Division of Pharmaceutical Chemistry
Faculty of Pharmacy
University of Helsinki

Understanding Human Drug Conjugating Enzymes;

Regio- and Stereoselectivity in Sulfotransferase 1A3 and the UDP-Glucuronosyltransferases

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium XII (Unioninkatu 34), on January 28th, 2011, at 12 noon.

Helsinki 2011

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ISBN 978-952-10-6756-3 (pbk.)
ISBN 978-952-10-6757-0 (PDF)
ISSN 1795-7079
http://ethesis.helsinki.fi

Helsinki University Print
Helsinki 2010

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Preface

This study was carried out during the years 2003-2010. Until 2009, I worked as an assistant teacher at the Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Helsinki, and the last year was funded by Helsinki University Pharmacy.

At the beginning of my postgraduate studies, I was fortunate to have Professor Jyrki Taskinen as my supervisor and I am grateful to him for introducing me to the world of enzyme kinetics and sulfotransferases. In 2003, I was allowed to visit Professor Michael Coughtrie's laboratory in Dundee, Scotland for three months. I am grateful to him as well as Vicky Butler and Lesley Wilson from his group for teaching me how to express human sulfotransferases in *E. coli* and to purify them.

I am deeply grateful to Docent Moshe Finel and Professor Risto Kostiainen, who were encouraging, enthusiastic and patient supervisors of my work after Jyrki retired. With Moshe's help, I finally started to make some progress with my research. Sanna Sistonen and Johanna Mosorin, who gave me their invaluable technical assistance with UGTs, are warmly acknowledged. I also thank Docent Liisa Laakkonen and my other co-authors for their contributions. Professor Hansruedi Glatt and Associate Professor Olivier Barbier are gratefully acknowledged for carefully reviewing the manuscript and providing valuable comments.

I started working at the Division of Pharmaceutical Chemistry in 2002 when Docent Hannele Salomies asked me to join the teaching staff during my undergraduate studies. I ended up working as an assistant teacher for more than six years and I really value that experience. I want to thank all the co-workers at the Division of Pharmaceutical Chemistry for creating such a cheerful working atmosphere there. Especially during the first years, when it was hard to believe that I would ever get my thesis done, the only thing that got me to Viikki every morning was the coffee break at 9 a.m. In addition to the people mentioned above, my special thanks go to Anna, Inkku, Jari, Kata, Kirsi, Leena, Mika, Mikko, Nina N., Nina S., Piia, Päivi U., and Tiina S.

There are also other friends that I want to thank for the fun times outside the work. Anna, Anu, Ellu, Hinni, Kati, Konna, Minna, and Piia are my oldest friends from Kokkola, where I started my academic journey as a child. We don't meet very often, but when we do, it is like we had never been apart. The same is true with Kaisa, Katja, Päivi, Salla, Tanja, and Tiina, who are my "pharmaceutical" friends. Marja-Liisa, Tilu, Diana, Kari, Mika, and Vilja have introduced me to the secrets of gourmet food, among other things. I feel very fortunate in having so many friends around me, who have helped me to loosen up every now and then.

I am sincerely grateful to my family and Tuukka's family for the support and encouragement. I value the fun and fruitful conversations that I have had with my siblings, Maaria and Tuomas, and their partners. Music has also been a connective hobby for us. Tuukka's parents, Sirpa and Sakari, have been for great help especially in childcare matters. My dear parents, Kaarina and Tapio, have given me the roots and the wings that every child needs. They have also taught me the art of discussion and argumentation, which I probably did not appreciate enough at the time. Without them, I definitely would not be here.

Finally, I owe my deepest gratitude to Tuukka for being such a loving and understanding partner during these years. He has taken care of my nutrition and social life, too. I still think that giving birth to our children is the most important accomplishment of my life although, I have to admit, giving birth to this book was sometimes harder and more painful, yet rewarding. Elli and Hilma: you are the sunshine of my life!

Helsinki, December 2010

Katriina Itäaho

Abstract

Sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs) are important detoxification enzymes and they contribute to the bioavailability and elimination of many drugs. They mainly catalyze sulfonation and glucuronidation of small lipophilic molecules that can be of endogenous or exogenous origin. Conjugation with a glucuronic acid or sulfonate moiety increases the water solubility of the substrates and their excretion through bile or urine.

SULT1A3 is an extra-hepatic enzyme responsible for sulfonation of phenols and catechols. The most important endogenous substrate is dopamine, which is often used as a probe substrate for SULT1A3. A new method for analyzing dopamine-3-*O*-sulfate and dopamine-4-*O*-sulfate by high-performance liquid chromatography (HPLC) with electrochemical detection was developed and the enzyme kinetic parameters for their formation were determined using purified recombinant human SULT1A3. The apparent K_m values for sulfonation at both hydroxyl groups were similar, but the maximal reaction rate was approximately six times higher for the formation of the 3-*O*-sulfate than the 4-*O*-sulfate. The results show that SULT1A3 strongly favors the 3-hydroxy group of dopamine over the 4-hydroxy group, which indicates that it may be the major enzyme responsible for the difference between the circulating levels of dopamine sulfates in human blood.

UGT1A10 is an important enzyme in the inactivation of drugs, among other compounds, and it is mainly expressed in the gastrointestinal tract. In this study, it emerged as the only UGT isoform capable of dopamine glucuronidation at a substantial level. All 19 known human UGTs were expressed as recombinant enzymes in baculovirus infected insect cells and their activity toward dopamine was screened using a sensitive liquid chromatography-tandem mass spectrometry method. Much lower or no dopamine glucuronidation activity was found for all other UGTs tested at 1 mM dopamine. The results obtained with recombinant enzymes were supported by studies with human intestinal and liver microsomes, because the affinities were similar for intestinal microsomes and UGT1A10. The affinity of dopamine to UGT1A10 was low and the K_A value was 1000 times higher than the K_m of SULT1A3, indicating that UGT1A10 is not an important enzyme in dopamine metabolism *in vivo*. Despite the low affinity, dopamine is a potential new probe substrate for UGT1A10 due to its selectivity.

Dopamine was used to study the importance of phenylalanines 90 and 93 in UGT1A10. The results revealed distinct effects that are dependent on differences in the size of the side chain as much as on differences in their positions within the protein. Examination of twelve UGT1A10 mutants that had the phenylalanines replaced by six amino acids of different sizes revealed lower dopamine glucuronidation in all of them. Enzyme kinetic studies of four mutants, F90A, F90L, F93A, and F93L, showed that their substrate affinities were similar to that of UGT1A10, suggesting that F90 and F93 are not directly involved in dopamine binding in the active site.

The glucuronidation of β-estradiol and epiestradiol (α-estradiol) 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. The results show that, although many UGTs can catalyze estradiol glucuronidation, there are marked differences in their kinetics, regioselectivity,

and stereoselectivity. The most active isoforms were UGT1A10 and UGT2B7, which demonstrated opposite regioselectivities. UGT1A10 favored the 3-OH of both estradiol diastereomers, as did UGT1A1, UGT1A3, UGT1A7, and UGT1A8. UGT2B7, like UGT2B4 and UGT2B17, favored the 17-OH. The stereoselectivities of UGT2B enzymes were more complex than those of UGT1As. UGT2B4 was specific for epiestradiol; UGT2B7 glucuronidated both diastereomers, with a high affinity for epiestradiol, whereas UGT2B17 only glucuronidated β-estradiol. In accordance with the results with recombinant enzymes, the main products of human liver and intestinal microsomes were epiestradiol-17-glucuronide and β-estradiol-3-glucuronide, respectively.

The amino acid sequences of the human UGTs 1A9 and 1A10 are 93% identical, yet there are large differences in their activities and substrate selectivities. UGT1A9 demonstrated much lower activity toward dopamine and estradiol than UGT1A10. However, inhibition studies revealed that β-estradiol had similar affinities to UGT1A9 and UGT1A10. β-Estradiol and epiestradiol, together with R- and S-propranolol and dobutamine, were used to further elucidate the differences in regio- and stereoselectivities of UGT1A9 and UGT1A10. Moreover, the kinetics of 1-naphthol glucuronidation was studied. To identify the residues responsible for the activity differences, several chimeras, in which segments of UGT1A9 were individually replaced by the corresponding segments from UGT1A10, were constructed and their activities were studied. In addition, the effects of various point mutations in UGT1A9 were studied. The results revealed that the residues between Leu86 and Tyr176 of UGT1A9 determine the substrate selectivity differences between UGT1A9 and UGT1A10. Within this region, residues at positions 115, 116, 117, 152, and 169 had significant impacts on the catalytic properties that were studied here. It appears that Phe117 of UGT1A9 participates in 1-naphthol binding and the residues at positions 152 and 169 contribute to the higher glucuronidation rates of UGT1A10.

In summary, the results emphasize that the substrate selectivity, including regio- and stereoselectivity, of UGTs is complex and it is controlled in subtle ways by many amino acids rather than one critical residue. Moreover, the results highlight the importance of the intestinal enzymes, SULT1A3 and UGT1A10, in the metabolism of dopamine and other physiological compounds as well as drugs and other xenobiotics.

List of original publications

This thesis is based on the following publications:

I Itäaho K, Alakurtti S, Yli-Kauhaluoma J, Taskinen J, Coughtrie MWH, and Kostiainen R (2007) Regioselective sulfonation of dopamine by SULT1A3 in vitro provides a molecular explanation for the preponderance of dopamine-3-*O*-sulfate in human blood circulation. *Biochem Pharmacol* **74**:504-510.

II Itäaho K, Mackenzie PI, Ikushiro S-i, Miners PO, and Finel M (2008) The configuration of the 17-hydroxy group variably influences the glucuronidation of β-estradiol and epiestradiol by human UDP-glucuronosyltransferases. *Drug Metab Dispos* **36**:2307-2315.

III Itäaho K, Court M, Uutela P, Kostiainen R, Radominska-Pandya A, and Finel M (2009) Dopamine is a low affinity and high specificity substrate for the human UDP-glucuronosyltransferase 1A10. *Drug Metab Dispos* **37**:768-775.

IV Itäaho K, Laakkonen L, and Finel M (2010) How many and which amino acids are responsible for the large activity differences between the highly homologous UDP-glucuronosyltransferases (UGT) 1A9 and UGT1A10? *Drug Metab Dispos* **38**:687-696.

The publications are referred to in the text by their roman numerals.

Abbreviations

ADME	absorption, distribution, metabolism, and excretion
cDNA	complementary deoxyribonucleic acid
Cl_{int}	intrinsic clearance
CYP	cytochrome P-450
DMSO	dimethyl sulfoxide
EC	enzyme classification
ER	endoplasmic reticulum
GI	gastrointestinal
GT-B	glycosyltransferase protein topology where two domains are linked flexibly
HEK293	a human embryonic kidney cell line
His-tag	polyhistidine tail
HPLC	high-performance liquid chromatography
K_A	substrate concentration at 0.5 ×V_{max} in Hill equation
k_{cat}	turnover number
K_i	dissociation constant of the inhibitory enzyme-substrate complex
K_m	the Michaelis-Menten constant
K_s	the substrate concentration at 0.5 ×V_{max} in the substrate inhibition equation
mRNA	messenger ribonucleic acid
n	the Hill coefficient
NSAID	non-steroidal anti-inflammatory drug
PAP	3'-phosphoadenosine 5'-phosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PCR	polymerase chain reaction
*p*NP	*p*-nitrophenol
PST	phenolsulfotransferase
RSD	relative standard deviation
RT	retention time
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
SL	saccharolactone
S_N2	bimolecular nucleophilic substitution
SNP	single-nucleotide polymorphism
SULT	sulfotransferase
SULT	a gene encoding SULT
UDPGA	uridine-5'-diphosphoglucuronic acid
[^{14}C]UDPGA	radiolabeled UDPGA
UDP-GlcNAc	uridine diphosphate N-acetylglucosamine
UGT	uridine diphosphoglucuronosyl transferase
UGT	a gene encoding UGT
V_{max}	limiting rate of an enzymatic reaction, "maximum velocity"

Amino acids	3-Letter	1-Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1 Introduction

The main goal of the drug development process is to produce safe and effective drugs within a reasonable time and for a reasonable cost. When a drug is administered to a patient, it is supposed to influence body functions, but that is not the only issue. The body also acts on the drug molecules, which affects both the safety and efficacy of the medication. First, the drug has to be absorbed. If the drug is taken orally, it usually goes from the intestine to the portal vein and liver before reaching the systemic circulation. Enzyme-catalyzed chemical transformation can occur in the intestine and liver during absorption, which is called first-pass metabolism. After reaching the systemic circulation, the drug is distributed to the tissues, including the target tissue, where it binds to its targets, e.g. receptors that mediate the drug's action. After doing its job, the drug has to be excreted from the body through bile or urine, and most drugs undergo some metabolic modifications before that. The liver is the major metabolizing organ in the body, but metabolizing enzymes are also expressed in other tissues, such as the intestine, kidney, lung, and skin. With evolution, the human body has gained a huge variety of enzymes to take care of drug metabolism and to protect the body from harmful xenobiotics. In addition to drugs and other xenobiotics, many endogenous compounds go through metabolic reactions before excretion, and they compete for metabolizing enzymes.

Drug metabolism can be divided into two phases that are catalyzed by different enzyme families. In phase I reactions, functional groups are added to or uncovered in the drug molecule. These reactions include oxidation, reduction, hydrolysis, hydration, and isomerization (Gibson and Skett, 2001). Cytochrome P450 (CYP) is the major enzyme family in phase I metabolism. In phase II reactions, the substrate molecules are conjugated to other molecules. Conjugation can occur on the drug molecule itself, or its metabolite from phase I reactions, and it usually renders the molecule much more hydrophilic and readily excreted, as well as less toxic. UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT) are the major phase II enzyme families. In addition to glucuronidation and sulfonation catalyzed by these enzymes, phase II reactions include glycosidation, methylation, acetylation, amino acid conjugation, glutathione conjugation, fatty acid conjugation, and condensation, all of which are catalyzed by a variety of different enzymes (Gibson and Skett, 2001).

When a new compound is discovered and evaluated for use as a drug, its metabolism is studied at an early stage in order to avoid unwanted outcomes. For example, bio-availability of a drug can be decreased if extensive first-pass metabolism prevents the molecule from getting to its target. In addition, some metabolites can be toxic or more active than the parent molecule. For example, morphine-6-*O*-glucuronide is an active metabolite of morphine (Lötsch and Geisslinger, 2001), minoxidil sulfate is the active form of minoxidil (McCall *et al.*, 1983; Buhl *et al.*, 1990), and many phase I metabolites (e.g. epoxides) as well as acyl glucuronides (Spahn-Langguth and Benet, 1992) may be toxic. Also, drug interactions may occur if two or more drugs are administered to a patient simultaneously or within a short period. If the drug molecules compete for the same metabolizing enzymes, or if one inhibits the function of a metabolizing enzyme, the concentration of the other can rise to a harmful level. Many chemicals can also induce the

expression of metabolizing enzymes, leading to more extensive metabolism and a lower blood concentration of the substrates. CYP-mediated interactions are quite common (Zhou, 2008), whereas clinical interactions mediated by UGT or SULT rarely occur, although many potential interactions have been found *in vitro* (Kiang *et al.*, 2005). Metabolism also affects the elimination half-lives of most drugs. For example, if we think about a prescription drug that is taken daily for years, it becomes clear that the elimination half-life should be short enough so that the drug does not accumulate in the body. On the other hand, it should be long enough so that the drug can be administered only once a day. This increases compliance by the patient. If the elimination half-life is too short, the drug must be administered very frequently. Another important feature of drug metabolism is polymorphism of the metabolizing enzymes, which may cause unexpected variation in metabolism. Drug concentration in the blood can rise to a detrimental level if the patient carries a mutation in the metabolizing enzyme. For instance, catechol-*O*-methyltransferase inhibitors may cause hepatotoxicity in people carrying mutations in UGT1A9 (Martignoni *et al.*, 2005). During the drug discovery and development process, the metabolism is normally studied from the CYP point of view, because most of the drugs that are currently in use are substrates of one or more CYPs. To avoid drug interactions, a new lead-compound may be discarded, if it is a substrate or inhibitor of CYPs. This has slightly increased the importance of the phase II enzymes in drug development because the compounds that are accepted for the next round in the development process may still be substrates or inhibitors of UGTs, SULTs, and other conjugating enzymes.

The increased ability to synthesize large numbers of potential drug candidates in a short time has made pharmacokinetic studies a bottleneck in the drug discovery process (Eddershaw and Dickins, 1999). There is a need for prediction tools that could be used *in silico* to assess potential problems in absorption, distribution, metabolism, or excretion (ADME) properties of a drug candidate in an early stage of the process. In order to be able to create predictive models for metabolism reactions, a lot of experimental data on the metabolizing enzymes and their structures and substrates must be generated. The lack of information on three-dimensional structures of the aglycone binding sites of UGTs has hindered the full understanding of the molecular basis of substrate recognition in these enzymes, and one way of getting more information about substrate recognition is to study their substrate specificities and the effects of site directed mutations on the proteins. For SULTs there are several crystal structures available (Kakuta *et al.*, 1997; Bidwell *et al.*, 1999; Dajani *et al.*, 1999b; Pedersen *et al.*, 2000; Lee *et al.*, 2003), which makes the case somewhat different. In this study, regio- and stereoselectivity of UGTs and SULT1A3 was examined using endogenous and exogenous substrates in concert with wild-type and mutated recombinant enzymes in order to get more detailed information on the molecular basis of their substrate recognition.

2 Review of the Literature

The first two chapters of the literature review are focused on the enzyme families that were studied in this work, UDP-glucuronosyltransferases and sulfotransferases. This review covers the reaction types that are catalyzed by these two enzyme families, the gene localization, the nomenclature of the genes and enzymes, their expression in different tissues, and what is known about their structure and function to date. The third chapter of the review is focused on enzyme kinetics and the equations used in this study.

2.1 UDP-glucuronosyltransferases (UGTs)

2.1.1 Glucuronidation

UDP-glucuronosyltransferases (EC 2.4.1.17; UGTs) are important conjugation enzymes that catalyze the glucuronidation of a large number of xenobiotic and physiological compounds. They are members of the UDP glycosyltransferase superfamily together with other enzymes that covalently attach glycosyl groups to other molecules.

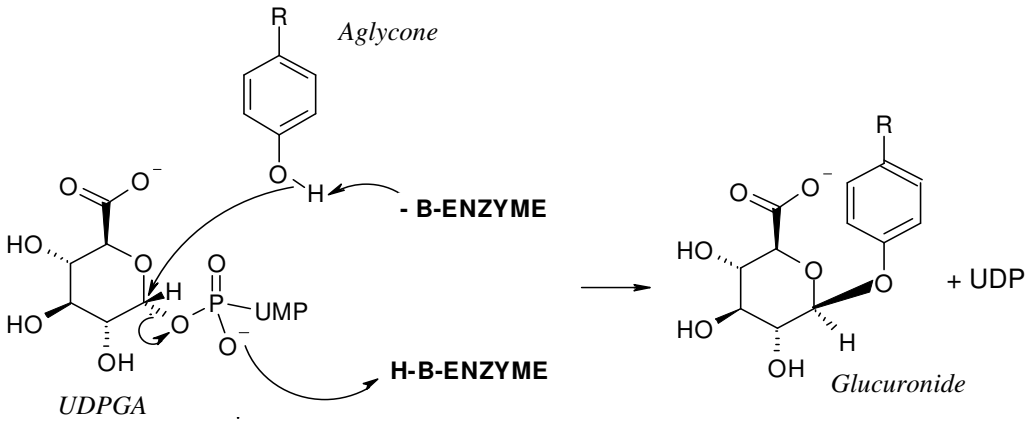


Figure 1. *O-Glucuronidation catalyzed by UGTs. The catalytic base (B, histidine) in UGT accepts a proton from the aglycone substrate (phenol in this case). Negatively charged oxygen attacks the C_1 atom of UDP-glucuronic acid (UDPGA). UDP is cleaved from UDPGA and it takes the extra proton from the enzyme* (Yin *et al.*, 1994).

In the glucuronidation reaction, the glucuronic acid moiety is transferred from UDP-glucuronic acid (UDPGA) to an aglycone substrate (Fig. 1). Aglycone substrates are mainly small lipophilic molecules and conjugation with glucuronic acid increases their water solubility and their excretion through bile or urine (Tukey and Strassburg, 2000; King *et al.*, 2000; Wells *et al.*, 2004). Glucuronides are substrates for transport proteins

that facilitate their passage through cell membranes. Multidrug resistance-associated proteins 2, 3, and 4, as well as the breast cancer resistance protein play a major role in the hepatic excretion of glucuronides (reviewed by Zamek-Gliszczynski *et al.*, 2006).

Glucuronidation occurs as an S_N2 reaction where the nucleophilic heteroatom of the aglycone attacks the C_1 atom of glucuronic acid (Fig. 1; Yin *et al.*, 1994; Johnson and Fenselau, 1978). In addition to the most common heteroatom, oxygen, the glucuronic acid moiety can also be attached to nitrogen, sulfur, or carbon atoms. The reaction follows a compulsory-order ternary-complex mechanism, which means that UDPGA and the aglycone always bind to the enzyme in the same order: UDPGA is bound first and the aglycone is bound after that (Luukkanen *et al.*, 2005). The reaction occurs when both substrates are present in the active site as a ternary complex with the enzyme and both products, the glucuronide and UDP, are released subsequently. The reaction is reversible in principle but in an intact cell the reaction products are rapidly removed and hence the reverse reaction is not likely to occur (Bock and Köhle, 2009).

2.1.2 UGT gene family

UGTs are divided into two families, UGT1 and UGT2, based on their sequence similarity and chromosomal localization (Mackenzie *et al.*, 2005; Fig. 2). The families are designated by a numeral following the abbreviation "UGT" and subfamilies are identified by letters. The isoforms are identified by numerals following the letter (e.g. UGT1A10).

The *UGT1* complex gene locus is located on chromosome 2 at position q37 and it encodes the nine functional members of the UGT1A subfamily, UGT1A1 and UGTs 1A3 through 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. However, there are also high levels of similarity among the N-terminal domains of many UGT1As, particularly among UGTs 1A3-1A5 and UGTs 1A7-1A10 (Guillemette, 2003).

The human *UGT2* genes are located on chromosome 4 at position q13 and they are divided into two subfamilies (Monaghan *et al.*, 1994; Jedlitschky *et al.*, 1999; Mackenzie *et al.*, 2005). In the UGT2A subfamily there are three members, UGTs 2A1-2A3, and there is exon sharing between UGT2A1 and UGT2A2 that leads to identical C-terminal halves, as in the UGT1A subfamily (Sneitz *et al.*, 2009). UGT2A3 and the UGT2Bs, on the other hand, are encoded by separate genes that contain all the exons (Mackenzie *et al.*, 2005). The human UGT2B subfamily contains at least 7 members: UGTs 2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28 (Turgeon *et al.*, 2001; Levesque *et al.*, 2001). In the UGT2B subfamily, a sequential numbering system, based on the chronological order of discovery of the genes, has been used, because orthologs across species are difficult to identify (Mackenzie *et al.*, 1997; 2005).

Recently, it was discovered that the diversity at the *UGT1* gene locus is amplified by alternative splicing of exon 5 (Girard *et al.*, 2007). This alternative splicing at the 3' end leads to nine additional UGT1A proteins (UGT1A1_i2 and UGT1A3_i2-UGT1A10_i2)

that are not capable of glucuronidation. Subsequently, even more alternative exons were found for UGTs (Guillemette *et al.*, 2010). Currently, the importance of this finding for glucuronidation in physiological conditions is not fully known, but it has been hypothesized that they may act as regulators of the glucuronidation activity of other UGTs. They also complicate the determination of the expression levels of active UGTs in tissues.

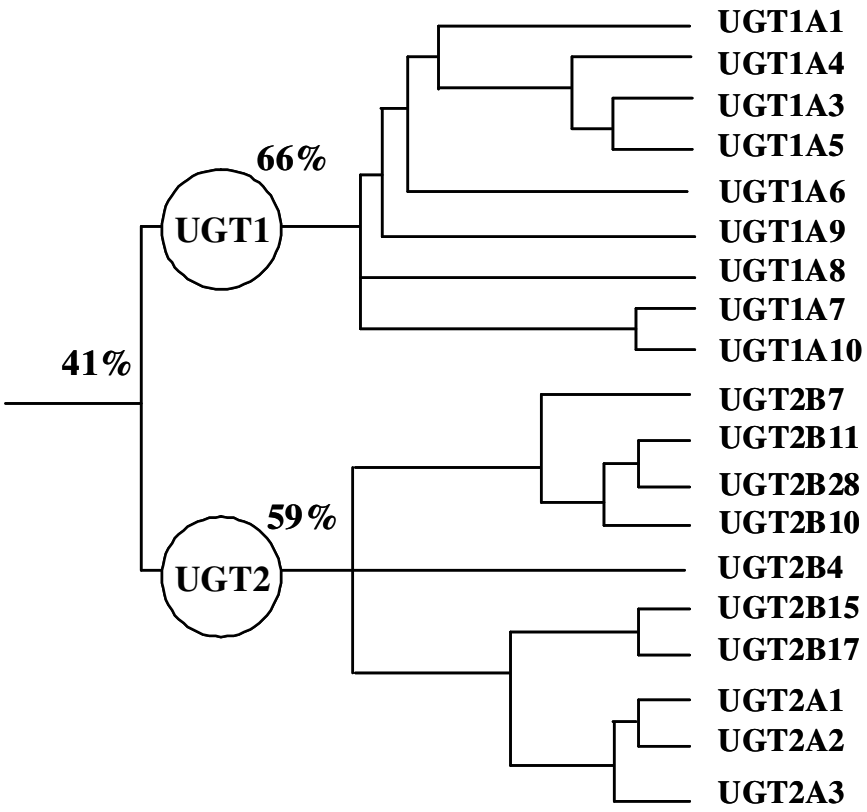


Figure 2. *A phylogenetic tree of the human UGTs showing percentage identity of the UGT families* (Guillemette, 2003; Guillemette *et al.*, 2010).

2.1.3 Diverse expression of UGTs

The UGTs are expressed mainly in the liver but also in many other tissues, such as the gastrointestinal tract and respiratory organs, both of which are important routes of drug administration (Table 1). The expression of *UGT* genes is regulated by many transcription factors like the hepatocyte nuclear factor 1α, octamer transcription factor 1, and caudal related homeodomain protein (Mackenzie *et al.*, 2003; Gregory *et al.*, 2004). In addition, many hormones and xenobiotic chemicals such as dihydrotestosterone, rifampicin, and phenobarbital can induce expression through nuclear receptors such as the pregnane X receptor and constitutive androstane receptor (Mackenzie *et al.*, 2003; Zhou *et al.*, 2005), which can cause interindividual variation in the metabolism of drugs that are substrates for UGTs.

Table 1. *Tissue-specific mRNA expression of UDP-glucuronosyltransferases.*

UGT	Tissues	References
1A1	Liver	Ritter et al., 1991
	Hepatic and biliary tissue	Strassburg et al., 1997
	Gastric tissue	Strassburg et al., 1998a
	Colon	Strassburg et al., 1998b
	Liver, small intestine, kidney, colon, trachea	Nishimura and Naito, 2006
	Liver and GI tract	Ohno and Nakajin, 2009
1A3	Liver and colon	Mojarrabi et al., 1996
	Hepatic, gastric and biliary tissue	Strassburg et al., 1997
	Liver, kidney, colon, prostate, small intestine	Mojarrabi and Mackenzie, 1998
	Liver and small intestine	Ohno and Nakajin, 2009
1A4	Liver	Ritter et al., 1991; Ohno and Nakajin, 2009
	Hepatic and biliary tissue	Strassburg et al., 1997
	Colon	Strassburg et al., 1998a
1A5	Low mRNA in liver and GI tract	Finel et al., 2005
	GI tract, kidney, esophagus	Ohno and Nakajin, 2009
1A6	Liver	Harding et al., 1988
	Hepatic, gastric and biliary tissue	Strassburg et al., 1997
	Colon	Strassburg et al., 1998a
	Liver, kidney and brain (cerebellum)	King et al., 1999
	Larynx	Zheng et al., 2002
	Liver, kidney, stomach, trachea, small intestine, adrenal, but not in brain	Nishimura and Naito, 2006; Ohno and Nakajin, 2009
1A7	Gastric tissue	Strassburg et al., 1997
	Esophagus	Strassburg et al., 1999
	Aerodigestive tract*	Zheng et al., 2002
	Esophagus, trachea, cervix, kidney, GI tract	Ohno and Nakajin, 2009
1A8	Small intestine, colon	Cheng et al., 1998
	Colon	Strassburg et al., 1998a; Mojarrabi and Mackenzie, 1998
	Esophagus	Strassburg et al., 1999
	Larynx	Zheng et al., 2002
	Hepatocytes	Li et al., 2007b
	Colon, small intestine, adrenal	Ohno and Nakajin, 2009
1A9	Liver	Wooster et al., 1991
	Colon	Strassburg et al., 1998a
	Esophagus	Strassburg et al., 1999
	Kidney, liver (low mRNA in adrenal, colon, small intestine, stomach and trachea)	Nishimura and Naito, 2006; Ohno and Nakajin, 2009

Table 1. *Continued.*

UGT	Tissues	References
1A10	Biliary and gastric tissue	Strassburg et al., 1997
	Colon	Strassburg et al., 1998a
	Colon and small intestine	Mojarrabi and Mackenzie, 1998; Cheng et al., 1999
	Esophagus	Strassburg et al., 1999
	Aerodigestive tract*	Zheng et al., 2002
	Hepatocytes	Li et al., 2007b
	Breast	Starlard-Davenport et al., 2008
	GI tract, esophagus, trachea, adrenal	Ohno and Nakajin, 2009
2A1	Olfactory tissue and brain	Jedlitschky et al., 1999
	Low mRNA in trachea and lung	Nishimura and Naito, 2006
2A2	Nasal mucosa	Sneitz et al., 2009
2A3	Small intestine, liver, colon and adipose tissue (low in pancreas, kidney, stomach, and testis)	Court et al., 2008
2B4	Liver, kidney, testis, mammary gland, placenta, adipose tissue, skin, prostate, adrenal, lung, heart, trachea, esophagus, thymus	Levesque et al., 1999; Ohno and Nakajin, 2009
	Tongue and floor of mouth	Zheng et al., 2002
2B7	Liver, kidney, pancreas and brain (cerebellum)	King et al., 1999
	Esophagus, liver	Strassburg et al., 1999
	Liver, kidney, mammary gland and intestine	Turgeon et al., 2001
	Liver, small intestine	Nishimura and Naito, 2006
	Kidney, liver, colon, small intestine	Ohno and Nakajin, 2009
2B10	Esophagus, liver	Strassburg et al., 1999
	Liver, kidney, mammary gland, intestine, lung, spleen	Turgeon et al., 2001
	Liver	Nishimura and Naito, 2006; Ohno and Nakajin, 2009
2B11	Mammary gland, adipose, skin, lung, adrenal, liver, kidney, prostate	Beaulieu et al., 1998
2B15	Liver, prostate, testes	Chen et al., 1993
	Liver (low mRNA in stomach and pancreas)	Nishimura and Naito, 2006
	Liver, GI tract, breast, prostate, trachea, testes	Ohno and Nakajin, 2009
2B17	Liver, testes, mammary gland, lung (low in prostate, uterus, kidney)	Beaulieu et al., 1996
	Tonsil and larynx	Zheng et al., 2002
	GI tract, liver (low mRNA in various other tissues)	Nishimura and Naito, 2006; Ohno and Nakajin, 2009
2B28	Liver and mammary gland	Levesque et al., 2001
	Bladder	Nakamura et al., 2008

*Aerodigestive tract tissues include tongue, tonsil, larynx, esophagus, and floor of mouth.

In addition to enzyme inducers (e.g. medication, smoking, alcohol), interindividual variation in drug metabolism is caused by genetic polymorphism (meaning the differences in DNA sequence among individuals). Polymorphisms have been described for almost all human UGTs [for a comprehensive list, see the UGT allele nomenclature homepage www.ugtalleles.ulaval.ca (accessed 9.7.2010)]. Some are single nucleotide polymorphisms (SNP) that do not result in an amino acid change in a polypeptide (i.e. silent mutation). In contrast, many types of mutations also lead to changes in the amino acid sequence. Missense mutations lead to amino acid changes in the protein, and nonsense mutations add a stop codon to the mRNA, leading to pre-mature truncation of the protein. Insertion and deletion (indel) mutations insert or delete nucleotides in the DNA sequence, leading to frameshifts and non-functional proteins. Deletion or amplification of a longer fragment of DNA causes copynumber variation. In addition, mutations in promoter regions and in splice acceptor or donor sites of the introns have been reported and they are listed on the UGT allele nomenclature homepage.

UGT1A10 is an important enzyme in the inactivation of dietary carcinogens (Dellinger *et al.*, 2007) and it is mainly expressed in the gastrointestinal tract (Table 1). To date, six polymorphic amino acid changes in the N-terminal domain have been detected: M59I, T202I (Saeki *et al.*, 2002), E139K, T240M, L244I (Elahi *et al.*, 2003), and I211T (Martineau *et al.*, 2004). The polymorphism where Glu139 is replaced by lysine lowers the glucuronidation activity of UGT1A10 toward carcinogenic polycyclic aromatic compounds (Dellinger *et al.*, 2006; 2007). The polymorphic variant T202I has lower glucuronidation activity toward estradiol than the wild-type UGT1A10 (Jinno *et al.*, 2003), whereas mutation I211T abolishes all activity (Martineau *et al.*, 2004). However, not all missense mutations affect the enzymatic activity. The activity of the variant M59I, for example, is similar to that of the wild-type UGT1A10 (Jinno *et al.*, 2003).

Copynumber variation has been found in the *UGT2B17* gene (Murata *et al.*, 2003; Wilson III *et al.*, 2004). It has been noticed previously that the mRNA levels of UGT2B17 can vary more than 100 fold between individuals (Congiu *et al.*, 2002). Because UGT2B17 is an important enzyme in testosterone glucuronidation, this leads to significant interindividual differences in the metabolism of this endogenous androgen (Jakobsson *et al.*, 2006).

Polymorphisms in the *UGT1A1* gene are clinically the most important, because UGT1A1 is the only enzyme that significantly catalyses bilirubin glucuronidation in man (Bosma *et al.*, 1994). A complete lack of UGT1A1 activity results in Crigler–Najjar syndrome type I, where the concentration of the neurotoxic bilirubin can rise to a fatal level if the patient is left untreated (Crigler and Najjar, 1952; Ritter *et al.*, 1991; Bosma *et al.*, 1994). There are at least 113 different alleles of UGT1A1 [www.ugtalleles.ulaval.ca (accessed 9.7.2010)]. Loss of activity can be due to mutations in the promoter region or any exon or intron. Milder UGT1A1 deficiency leads to Crigler–Najjar syndrome type II and Gilbert's syndrome, which cause jaundice but are often harmless.

Neonatal jaundice is also very common in normal healthy babies. One of the reasons is that the UGT activity in general is lower in newborns than in adults (Strassburg *et al.*, 2002), which also affects their drug metabolism. This should be taken into account when prescribing medications to newborns. On the other hand, old age seems to have only a

minor impact on glucuronidation activity in human liver and the same holds true for sex differences (Court, 2010).

2.1.4 Structure and substrate selectivity of UGTs

The UGTs are membrane proteins that are located mostly in the endoplasmic reticulum (ER) and to a lesser degree in Golgi membranes and nuclear envelope, but not in the cytosol (Hauser *et al.*, 1984; Radominska-Pandya *et al.*, 2002; Dellinger *et al.*, 2007). They demonstrate type I topology, meaning that the C-terminus is located on the cytoplasmic side of the membrane and N-terminus on the lumenal side. They form functional homo- and heterodimers or even higher oligomers in membranes (Fig. 3; Meech and Mackenzie, 1997; Ghosh *et al.*, 2001; Kurkela *et al.*, 2003; 2004; 2007; Fujiwara *et al.*, 2007; Operana and Tukey, 2007; Finel and Kurkela, 2008).

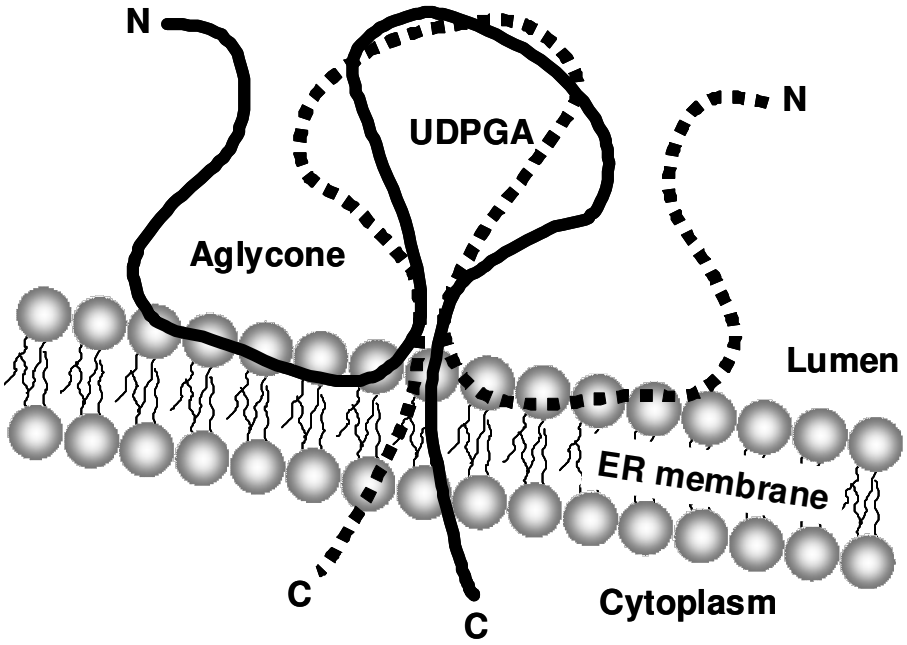


Figure 3. *A schematic model for the membrane topology of human UGTs* (Finel and Kurkela, 2008).

UGTs adopt a GT-B fold, which means that one monomer is mainly composed of two large domains, N-terminal and C-terminal, and the domains are connected by a flexible linker (Lairson *et al.*, 2008). There is a cytoplasmic tail of 20-25 residues in the C-terminal half and a 17-residue helix that spans the ER membrane. The rest of the protein is located in the lumen of the ER but there is most likely an additional membrane-binding hydrophobic region within the N-terminal domain (Ouzzine *et al.*, 1999). The active site is on the lumenal side of the membrane (Shepherd *et al.*, 1989), which means that the aglycone substrates and UDPGA must pass through the ER membrane in order to reach

the active site, and the glucuronides must be transported out from the lumen in order to be excreted from the cell and finally from the body. The aglycone substrates are usually hydrophobic small molecules, thus they may pass through the membranes passively. However, their access to the active site may be facilitated by the N-terminal membrane-bound domain (Ouzzine *et al.*, 1999). For UDPGA and glucuronides there are probably active transport mechanisms in the ER membrane (Bossuyt and Blanckaert, 1994; Muraoka *et al.*, 2001; Battaglia and Gollan, 2001; Csala *et al.*, 2004).

UGTs are difficult to purify and crystallize and hence no X-ray crystal structure of a whole UGT is available to date. Miley *et al.* (2007) were able to resolve the crystal structure of the C-terminal UDPGA binding domain of UGT2B7, which is the first crystal structure of any region of a mammalian UGT. At the core of this domain, there is a six-stranded β-sheet surrounded by seven α-helices (Miley *et al.*, 2007). The UDPGA binding pocket is a shallow cavity on the surface of the domain. In a native protein, the N-terminal domain sits on top of this cavity, forming a catalytic cleft between the domains. The aglycone binding site is located in the N-terminal domain (Mackenzie, 1990; Lewis *et al.*, 2007; Patana *et al.*, 2008).

In the absence of detailed structural data on the aglycone binding site, other approaches have been used in attempts to determine the amino acids that are essential for the substrate specificity and catalytic activity of the enzymes. These approaches include enzyme kinetic studies, structure-activity studies, construction of pharmacophore models, molecular modeling, use of amino acid-modifying reagents, and site-directed mutagenesis of individual UGTs (Dubois *et al.*, 1999; Smith *et al.*, 2003a; 2003b; Barre *et al.*, 2007; Kubota *et al.*, 2007; Lewis *et al.*, 2007; Nishiyama *et al.*, 2008; Sorich *et al.*, 2008; Laakkonen and Finel, 2010). Along with these methods, considerable *in vitro* glucuronidation data with diverse chemicals have been generated so far, but more data are still needed for better understanding of UGT structure and function. The amino acids that have been found to be important for the substrate selectivity or enzymatic activity of UGTs are given in Table 2. A recent review on this topic was written by Magdalou *et al.* (2010).

In the C-terminal domain, many amino acids have been identified that are involved in the binding of UDPGA. Consistent with the fact that, in the glucuronidation reaction, the bond between glucuronic acid and the phosphate group is broken, the amino acid residues closer to the glucuronic acid and phosphate group of UDPGA are more critical for the enzymatic function than those closer to the other end of the UDPGA binding site (Miley *et al.*, 2007; Patana *et al.*, 2007).

In the N-terminal domain, two amino acids have been identified as catalytic residues in UGTs (Table 2). A His residue is found at position 35-40 in all other UGTs except UGT1A4 and UGT2B10, and it functions as a catalytic base in *O*-glucuronidation (Kubota *et al.*, 2007; Li *et al.*, 2007a; Miley *et al.*, 2007; Patana *et al.*, 2008). An Asp at position 150 or 151 is the catalytic acid that stabilizes the protonated His during catalysis (Li *et al.*, 2007a; Miley *et al.*, 2007).

Table 2. *Some amino acids found to be important for enzymatic activity and substrate selectivity of UGTs. The amino acid residues are numbered according to the original publications.*

Amino acids (UGT)	Functions	References
N-terminal domain		
His^{40}/Pro^{40} (1A3/1A4) His^{35} (2B7) His^{37} (1A9)	Catalytic His is critical in O-glucuronidation but not in N-glucuronidation	Miley *et al.*, 2007; Kubota *et al.*, 2007; Li *et al.*, 2007a; Patana *et al.*, 2008
Asp^{150} (1A6) Asp^{151} (2B7)	Catalytic	Li *et al.*, 2007a Miley *et al.*, 2007
Aromatic residue at position 33 (2B4 and 2B7)	Important for substrate specificity and activity	Barre *et al.*, 2007
Arg^{52} and His^{54} (1A6)	Required for optimal function and structural integrity	Senay *et al.*, 1997
Phe^{90} and Phe^{93} (1A10)	Substrate binding	Xiong *et al.*, 2006; Starlard-Davenport *et al.*, 2007
Ser^{121} (2B17)	Required for activity toward the 3α-position of C_{19} steroids	Dubois *et al.*, 1999
Cys^{126} (1A6)	Maintaining the integrity of the substrate binding site	Senay *et al.*, 2002
Residues 61-194 (2B7 and 2B15)	Substrate binding and selectivity	Lewis *et al.*, 2007
Residues 69-132 (1A9)	Important in C-glucuronidation of phenylbutazone	Nishiyama *et al.*, 2008
Residues 96-101 (2B7)	Binding of opioids	Coffman *et al.*, 2003
Cys^{186} (1A1)	Affinity for bilirubin	Ghosh *et al.*, 2005
Cys^{127}, Cys^{156}, Cys^{177}, Cys^{223}, Cys^{280} (1A1)	Mutations abolish activity	Ghosh *et al.*, 2005
Ile^{211} (1A10)	Essential for activity	Martineau *et al.*, 2004
C-terminal domain		
Residues 308, 338, 356, 359, and 382 (2B7)	Interact with the nucleotide end of the UDPGA	Miley *et al.*, 2007
His^{371} and Glu^{379} (1A6)	Binding of UDPGA	Patana *et al.*, 2007
Residues 373, 374, 378, and 379 (2B7)	Interact with phosphate	Miley *et al.*, 2007
Residues 378, 398, and 399 (2B7)	Interact with the glucuronic acid moiety of UDPGA	Miley *et al.*, 2007
Cys^{383} (1A1)	Mutations abolish activity	Ghosh *et al.*, 2005
Asp^{393} (1A10)	Binding of UDPGA	Xiong *et al.*, 2008
Lys^{314} and Lys^{404} (1A10)	Binding of UDPGA	Banerjee *et al.*, 2008
Cytoplasmic tail		
Cys^{509}, Cys^{510}, Cys^{517} (1A1)	Stimulation of the enzyme activity by UDP-GlcNAc	Ghosh *et al.*, 2005

Table 3. *Typical substrates of UGTs.*

UGT	Characteristic substrates	References
1A1	Bilirubin	Bosma *et al.*, 1994; Senafi *et al.*, 1994
1A3	Small phenolic compounds, scopoletin, carboxylic acid drugs	Green *et al.*, 1998; Vashishtha *et al.*, 2000
1A4	Tertiary amines, imidazoles	Green *et al.*, 1995; Vashishtha *et al.*, 2001
1A5	1-Hydroxypyrene	Finel *et al.*, 2005
1A6	Small planar phenols, serotonin	King *et al.*, 1999; Ebner and Burchell, 1993; Krishnaswamy *et al.*, 2003
1A7	Phenolic compounds	Strassburg *et al.*, 1998a
1A8	Steroids, fatty acids, bile acids, flavonoids, anthraquinones, and other phenolic compounds	Mojarrabi and Mackenzie, 1998; Cheng *et al.*, 1999
1A9	Bulky phenols, propofol, entacapone	Ebner and Burchell, 1993 Lautala *et al.*, 2000
1A10	Steroids and other phenolic compounds; Polycyclic aromatic hydrocarbons	Strassburg *et al.*, 1998a; Mojarrabi and Mackenzie, 1998; Cheng *et al.*, 1999 Dellinger *et al.*, 2006
2A1	Phenolic compounds	Jedlitschky *et al.*, 1999; Sneitz *et al.*, 2009
2A2	Hyodeoxycholic acid, 3- and 4-phenylphenol	Sneitz *et al.*, 2009
2A3	Hyodeoxycholic acid and other bile acids	Court *et al.*, 2008
2B4	Hyodeoxycholic acid, catechol estrogens, phenols	Levesque *et al.*, 1999; Fournel-Gigleux *et al.*, 1991; Jin *et al.*, 1993
2B7	NSAIDs, steroids, bile acids, opioids	Jin *et al.*, 1993; Jin *et al.*, 1997; Coffman *et al.*, 1997; 1998; Gall *et al.*, 1999
2B10	Nicotine, medetomidine	Kaivosaari *et al.*, 2007; 2008
2B11	Not known	Beaulieu *et al.*, 1998
2B15	5α-Androstane 3α, 17β-diol and other steroids and phenols	Chen *et al.*, 1993; Green *et al.*, 1994
2B17	Dihydrotestosterone and other steroids and phenols	Beaulieu *et al.*, 1996
2B28	5β-Androstane 3α,17β-diol and other steroids and phenols	Levesque *et al.*, 2001

Additionally, in the N-terminal domain, some other amino acids have been found to be important for glucuronidation activity. In the absence of a three-dimensional structure of the N-terminal domain, it is not always easy to tell whether they participate in substrate binding. Many of the crucial amino acids may, on the other hand, be important for the

integrity of the protein structure. Differences in substrate selectivity among the UGTs are mainly due to the variable region within the N-terminal domain, roughly residues 60-200.

Substrate selectivity of UGTs is very complex since most of them can glucuronidate several different compounds that vary significantly in their chemical structure. There is often a partial overlap in the substrate specificity of human UGTs, reflecting the high sequence homology among them. On the other hand, there are some distinct differences in activity even between highly similar isoforms such as UGTs 1A3 and 1A4 (Kubota *et al.*, 2007), 1A9 and 1A10 (Sten *et al.*, 2006), and 2B15 and 2B17 (Dubois *et al.*, 1999). Some typical substrates of UGTs are summarized in Table 3. Some of them are more selective than others. Due to the lack of absolutely specific substrates or inhibitors for most UGTs, substrate selectivity is usually studied with individually expressed recombinant enzymes. One of the major challenges in current UGT research is to better understand the factors that determine the substrate specificity of UGTs in order to be able to predict the glucuronidation of new compounds. There is a clear need for additional detailed information on the preferences of these enzymes with respect to different substrates and regio- and stereoselective conjugation.

2.1.5 Substrate selectivity of UGT1A10

Although all the human UGTs were included in this work, the substrate selectivity of UGT1A10 deserved extra attention during the course of these studies. UGT1A10 belongs to the UGT1A subfamily where the percent identity among the isoforms in general is 66% or more (Fig. 2). UGTs 1A7, 1A8, 1A9, and 1A10 form a subgroup within the UGT1A subfamily since they are more than 80% identical in amino acid sequence. There are only 16 unique amino acid residues in mature UGT1A10 that are not identical to those from UGT1A7, 1A8, or 1A9: E67, N87, A102, Q103, S114, L117, T152, T169, H176, N192, D193, W208, V212, D216, L223, and R225 (Fig. 4). Yet it has somewhat different substrate selectivity from the other three (Kuuranne *et al.*, 2003; Alonen *et al.*, 2005; Sten *et al.*, 2006). Some of the differences will be further discussed under Results and Discussion.

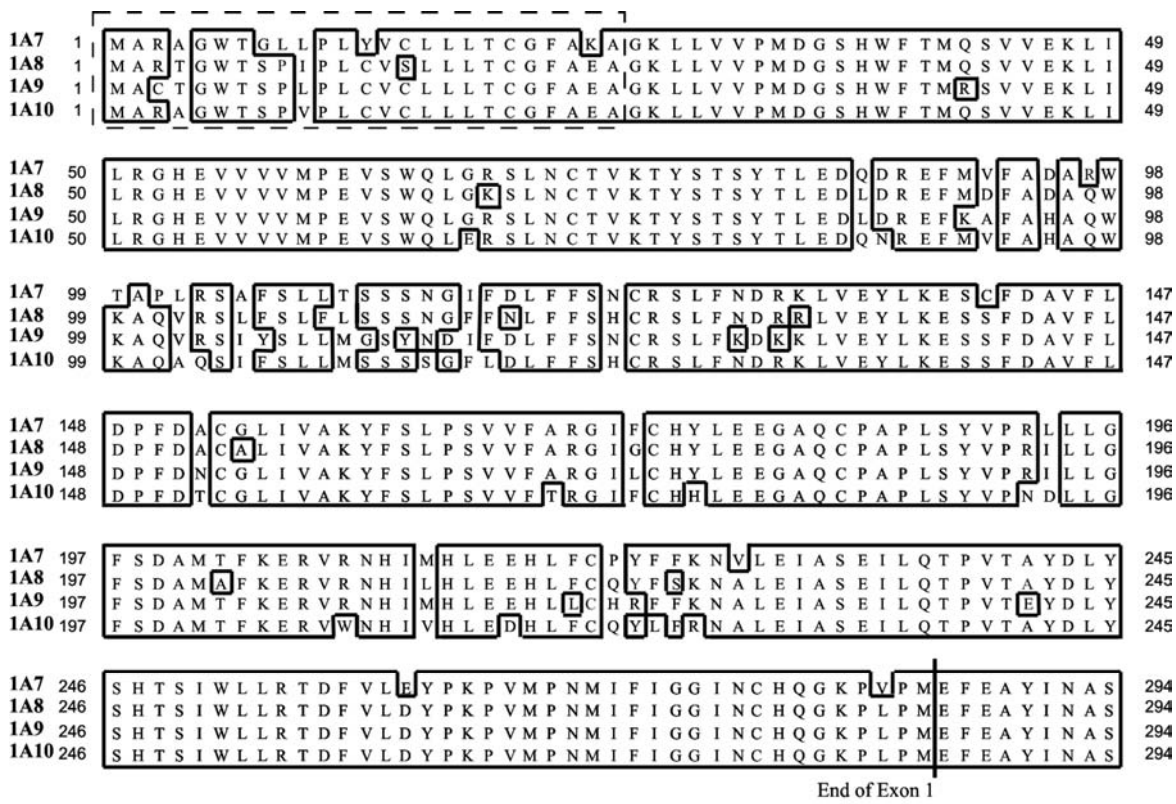


Figure 4. *Sequence alignment of the amino acid sequences encoded by the first exons of UGT 1A7, 1A8, 1A9, and 1A10. The signal sequence, which is marked with a dashed line, is cleaved from the protein after transport to the endoplasmic reticulum.*

2.2 Sulfotransferases (SULTs)

2.2.1 Sulfonation

Sulfotransferases (EC 2.8.2., SULTs) are generally considered to be cytosolic conjugation enzymes, but at least one of them (SULT2B1_v2) has also been found in the nucleus (He *et al.*, 2004; Dumas *et al.*, 2008). SULTs catalyze the transfer of a sulfonate group ($-SO_3^-$) from the donor substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to acceptor substrates that can be either xenobiotic or small endogenous compounds (reviews: Falany, 1997; Coughtrie, 1998; 2002; Glatt *et al.*, 2001; Allali-Hassani *et al.*, 2007). The most common acceptor groups are aromatic or aliphatic hydroxyls whose conjugation results in a sulfate ($R\text{-}OSO_3^-$) moiety (Fig. 5). A widely used term for this reaction, sulfation, was derived from early studies that identified phenolic sulfate esters in the urine of humans treated with phenols. However, it would be more appropriate to use the term sulfonation, because it is a sulfonate group that is actually transferred in the reaction. SULTs are also capable of conjugating sulfonate to groups other than hydroxyls, including primary amines, whose conjugation does not result in a sulfate but in a sulfamate group.

Sulfonation greatly increases the water-solubility of the acceptor substrate since the resulting group is negatively charged in the physiological pH range.

The mechanism of cytosolic sulfotransferases is a sequential Bi Bi kinetic mechanism that proceeds via a ternary complex between the enzyme and the two substrates, PAPS and the acceptor substrate (Duffel and Jakoby, 1981; Varin and Ibrahim, 1992; Zhang *et al.*, 1998). Both random ordered (Zhang *et al.*, 1998) and compulsory ordered (Whittemore *et al.*, 1985; Tyapochkin *et al.*, 2008) binding of substrates have been suggested. Sulfonation occurs as a dissociative substitution with a loose transition state and little nucleophilic involvement (Kakuta *et al.*, 1998; Bartolotti *et al.*, 1999; Hoff *et al.*, 2006). SULTs can also catalyze the reverse reaction, namely the transfer of the sulfonate moiety from a phenol substrate to PAP and other nucleotides (Duffel and Jakoby, 1981; Lin and Yang, 2000). Sulfonate group transfer between two phenols and PAP-independent hydrolysis of phenyl sulfate has also been demonstrated *in vitro* (Duffel and Jakoby, 1981). Divalent cations activate some sulfotransferases, although they are not necessarily required for the catalysis, unlike many other enzymatic transfer reactions that involve nucleotides (Zhang *et al.*, 1998).

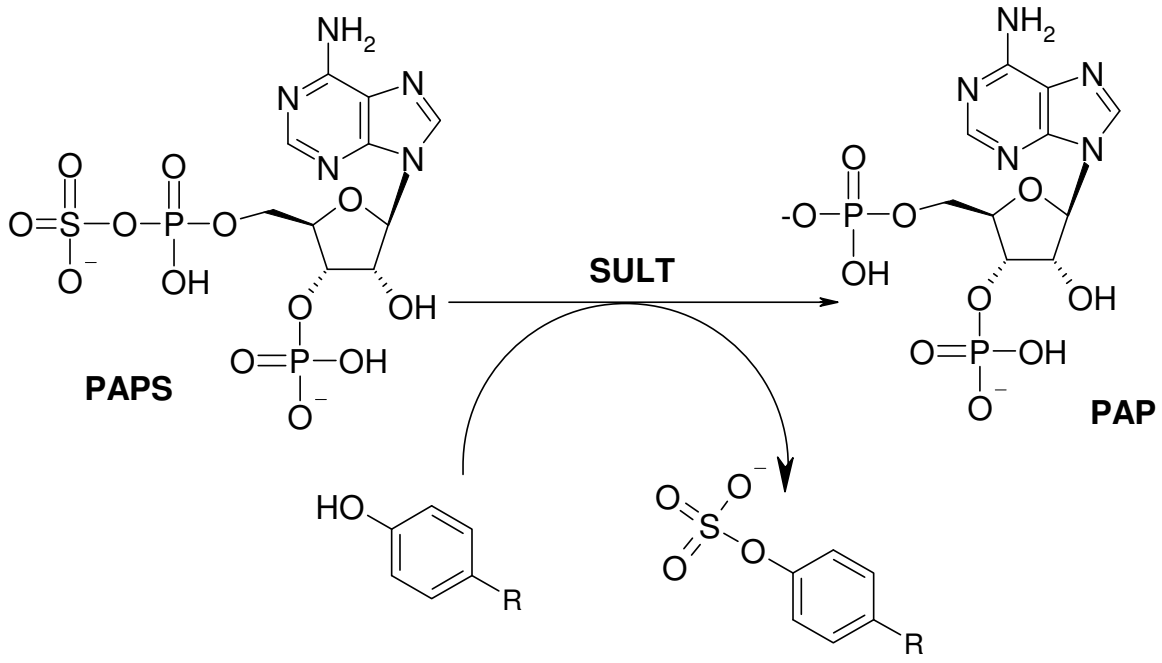


Figure 5. *Sulfonation of a phenolic compound catalyzed by SULT.*

SULTs are often referred to as “high affinity and low capacity enzymes” meaning that they are most effective at low substrate concentrations. They have a fast initial turnover rate, but the reaction velocity decreases as the concentration of available cofactor is rapidly depleted. The concentration of PAPS in the liver and other tissues is much lower than that of UDPGA, for example (Cappiello *et al.*, 1989; Cappiello *et al.*, 1991). The reason for this might be that the synthesis of PAPS requires two molecules of ATP, thus it consumes a lot of energy in the cell. Another important factor is the availability of

inorganic sulfate. *In vivo*, the sulfonation pathway is additionally affected by sulfatases and transport proteins that hydrolyze sulfates or pump them out of the cell, respectively (reviewed by Coughtrie *et al.*, 1998).

Although sulfonation is usually considered to be a detoxicating process for endogenous and xenobiotic compounds, it actually activates some drugs and promutagens (Buhl *et al.*, 1990; Garay *et al.*, 1990; Garay *et al.*, 1995). Many promutagens that are activated by sulfonation form covalent bonds to DNA and other macromolecules (Glatt, 1997). Most SULTs are expressed in the intestine (see below) and it has been suggested that sulfonation may have an impact on the initiation of intestinal cancer (Coughtrie *et al.*, 1998). A vegetable-rich diet may be useful in preventing such cancer because many SULTs are inhibited by the flavonoids and isoflavonoids that are found in fruits and vegetables (Harris and Waring, 2008).

2.2.2 SULT nomenclature

Blanchard *et al.* proposed nomenclature guidelines for cytosolic SULTs in 2004 (Blanchard *et al.*, 2004). Prior to that, naming of the sulfotransferases was very confusing as many research groups had their own names for them and sometimes it was difficult to know if they were speaking about the same isoform or not. The proposed system is parallel to the UGT nomenclature system: SULTs sharing at least 45% amino acid sequence identity are considered members of the same family and those sharing at least 60% identity are members of the same subfamily.

There are at least 12 human SULT isoforms divided into four families that are further divided into 8 subfamilies altogether (Coughtrie, 2002). In the SULT1 family there are at least 7 enzymes functionally expressed in humans belonging to four subfamilies: SULT1A, SULT1B, SULT1C and SULT1E (Blanchard *et al.*, 2004). Enzymes previously called SULT1C1 and SULT1C2 were re-named SULT1C2 and SULT1C4, respectively, in the proposed nomenclature system (Blanchard *et al.*, 2004), and this may cause some confusion as some scientists have not adopted the new names. In the SULT2 family, there are three isoforms belonging to two subfamilies: SULT2A1, SULT2B1_v1 and SULT2B1_v2. In the SULT4 family, there is only one member: SULT4A1. The newest finding is SULT6B1, which is expressed at least in human testes, as well as in other primates (Freimuth *et al.*, 2004), but there is very little data available about this isoform to date and it will not be discussed further in this review.

2.2.3 Tissue specific expression of SULTs

Like UGTs, many SULTs are highly expressed in the liver but also (and some exclusively) in other tissues like intestine, brain, endometrium, and kidney (Table 4). In liver, SULT1A1 is the most abundant isoform followed by SULT2A1 (Fig. 6; Riches *et al.*, 2009). In the small intestine, SULT1B1 and SULT1A3 are the most abundant forms and the total SULT content is higher than in the liver. Interindividual variation in both tissues

is very high. Some SULTs are expressed in different tissues in the fetus than in the adult: e.g. SULT1A3 has been found in fetal liver but not in adult liver (Richard *et al.*, 2001). The physiological implications of this are currently unknown.

Table 4. *Tissue-specific protein expression of human sulfotransferases.*

SULT	Tissue	References
1A1	Liver, adrenal gland, placenta, platelets	Heroux *et al.*, 1989
	Endometrium	Falany *et al.*, 1998
	Liver, gastrointestinal (GI) tract, brain, prostate, adrenal, bladder, cervix, uterus, ovary, skin, esophagus, and pancreas	Nowell *et al.*, 2005
1A2	Not detected at the protein level	Nowell *et al.*, 2005
	Liver and cecum	Teubner *et al.*, 2007
1A3	Brain	Whittemore *et al.*, 1985
	Platelets	Heroux and Roth, 1988
	Jejunal mucosa	Sundaram *et al.*, 1989
	Placenta, platelets	Heroux *et al.*, 1989
	Fetal liver	Richard *et al.*, 2001
1B1	Liver, small intestine, colon, blood leukocytes	Wang *et al.*, 1998
1C2	Stomach, kidney, thyroid, fetal kidney, and fetal liver (mRNA)	Her *et al.*, 1997
1C4	Fetal kidney, fetal lung (mRNA)	Sakakibara *et al.*, 1998b
1E1	Liver	Forbes-Bamforth and Coughtrie, 1994
	Liver, small intestine, adrenal gland, fetal lung, fetal liver, and fetal kidney	Her *et al.*, 1996
	Endometrium	Falany *et al.*, 1998
2A1	Liver and adrenal	Comer and Falany, 1992
	Liver and small intestine	Her *et al.*, 1996
2B1	Prostate, placenta, and trachea (mRNA)	Her *et al.*, 1998
	Skin (2B1_v2)	Higashi *et al.*, 2004
	Breast (2B1_v2)	Dumas *et al.*, 2008
4A1	Brain (mRNA)	Falany *et al.*, 2000
	Cerebral cortex, cerebellum, and brainstem	Liyou *et al.*, 2003

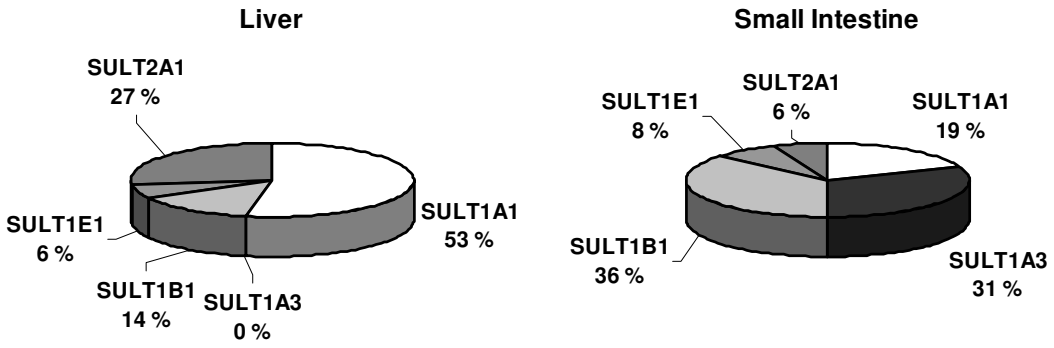


Figure 6. *Relative expression levels of five major sulfotransferases in liver and small intestine (Riches et al., 2009).*

2.2.4 Chromosomal localization and polymorphism of SULTs

SULT genes are located on various chromosomes. *SULT1A* genes are located on the short arm of chromosome 16 at 16p11.2-12.1 and they have probably evolved from a common ancient gene by duplication (Dooley *et al.*, 1994; Dooley and Huang, 1996). Two genes, *SULT1A3* and *SULT1A4*, encode the same enzyme, SULT1A3 (Hildebrandt *et al.*, 2004). *SULT1B1* and *SULT1E1* are located on the chromosome 4 (as the *UGT2* genes) at 4q13.1 (Glatt *et al.*, 2001; Her *et al.*, 1995), whereas *SULT1C* genes are located on chromosome 2 at 2q11.1-11.2 (Freimuth *et al.*, 2000). *SULT2* genes are located on chromosome 19 at 19q13.3 (Freimuth *et al.*, 2004; Her *et al.*, 1998), where *SULT2B1* gene encodes two protein products, SULT2B1_v1 and SULT2B1_v2, that have different first exons (Her *et al.*, 1998), like the products of the *UGT1* gene locus (see 2.1.2). *SULT4A1* is located alone on the long arm of chromosome 22 at 22q13, which suggests that it has developed individually without undergoing duplication (Freimuth *et al.*, 2004; Minchin *et al.*, 2008 and refs. therein).

Genetic polymorphisms have been found for many isoforms. At least SULT1A1 (Raftogianis *et al.*, 1997), SULT1A2 (Raftogianis *et al.*, 1999), SULT1A3 (Thomae *et al.*, 2003), SULT1C2 (Freimuth *et al.*, 2001), SULT1E1 (Adjei *et al.*, 2003), and SULT2A1 (Thomae *et al.*, 2002) are polymorphic. The physiological and pathological significance of these polymorphisms is still unclear, but it seems that there is a relationship between SULT1A1 polymorphism and various cancers (reviewed by Hildebrandt *et al.*, 2009).

2.2.5 Structure and substrate selectivity of SULTs

SULTs are soluble proteins, which makes them easier to purify and crystallize than the UGTs. There are therefore many crystal structures available for SULTs. The X-ray crystal structure of mouse estrogen sulfotransferase SULT1E1 was the first to be solved (Kakuta *et al.*, 1997). The first structural determination of human SULT was done for SULT1A3

(Bidwell *et al.*, 1999; Dajani *et al.*, 1999b; Lu *et al.*, 2005) followed by SULT2A1 (Pedersen *et al.*, 2000), SULT1E1 (Pedersen *et al.*, 2002), SULT1A1 (Gamage *et al.*, 2003), SULT1B1 and SULT1C2 (Dombrovski *et al.*, 2006), and most recently SULT1C4 and SULT4A1 (Allali-Hassani *et al.*, 2007). The overall structure consists of a five-stranded β-sheet and surrounding α-helices. A comprehensive review of the structure and function of SULTs has been done by Negishi *et al.* (2001).

The crystal structures and amino acid sequences reveal many conserved motifs in SULTs. One of them is the peptide sequence RKGxxGDWKNxFT in the C-terminal domain that is thought to take part in PAPS binding (Komatsu *et al.*, 1994). In the mouse SULT1E1 structure, residues 257-259 (Arg, Lys, and Gly, corresponding to the first three residues in the conserved motif) together with Arg130 and Ser138 are involved in 3'phosphate binding, and residues 48-51 (Lys, Ser, Gly and Thr) are involved in 5'phosphate binding of PAP (Kakuta *et al.*, 1997). The recognition of PAP was found to be similar in human SULT1A3 (Dajani *et al.*, 1999b). When PAPS is bound in the active site of human SULT1E1, the conformation of Lys47, corresponding to Lys48 in the mouse SULT1E1 structure, is changed so that it interacts with the adjacent Ser137 (Pedersen *et al.*, 2002). Ser137 corresponds to Ser138 in the mouse sulfotransferase structure and it forms a hydrogen bond with the 3'phosphate of PAPS. When the sulfonate moiety is cleaved from PAPS, the conformation of the lysine is changed so that it can form an ionic bond with the 5'phosphate, thus stabilizing the transition state of the reaction (Kakuta *et al.*, 1998). On the other hand, it has been postulated that in SULT1A1, Glu83 and Asp134 are important for activity and binding of PAPS (Chen *et al.*, 2000), but this is not evident from crystal structures of the other isoforms.

Human sulfotransferases are generally present as dimers in solution. They are capable of forming both homodimers and heterodimers (Kiehlbauch *et al.*, 1995). A conserved dimerization motif of cytosolic sulfotransferases (KxxxTVxxxE) forms a zipper-like structure between the two monomers of SULT and it is located near the C-terminus (Petrotchenko *et al.*, 2001). The monomers sit antiparallel with respect to each other and they are bound together by complementary hydrophobic interactions and backbone hydrogen bonds. In addition, lysine and glutamate form ion pairs at each end of the motif.

X-ray structures and site-directed mutagenesis of mouse estrogen sulfotransferase have revealed that His108 functions as a catalytic base in the reaction and Lys106 is involved in the binding of estradiol (Kakuta *et al.*, 1998). The histidine is conserved throughout the cytosolic sulfotransferase family. However, it is still mostly unclear which amino acids define the substrate specificities of individual SULT isoforms. When the structure of human SULT2A1 was compared to that of mouse SULT1E1, it was found that the amino acid differences in their binding pockets increased gradually from the bottom to the opening (Pedersen *et al.*, 2000). Residues from three loops form the majority of the substrate binding site in SULT2A1: Pro14-Ser20, Glu79-Ile82, and Asn136-Lys144. There is also a C-terminal loop, Tyr231-Gln244, that covers the binding pocket. In SULT1E1 Tyr81 was found to be important for its selectivity toward estradiol (Petrotchenko *et al.*, 1999). Tyr81 and Phe142 form a gate that regulates the binding affinity of SULT1E1 for estradiol.

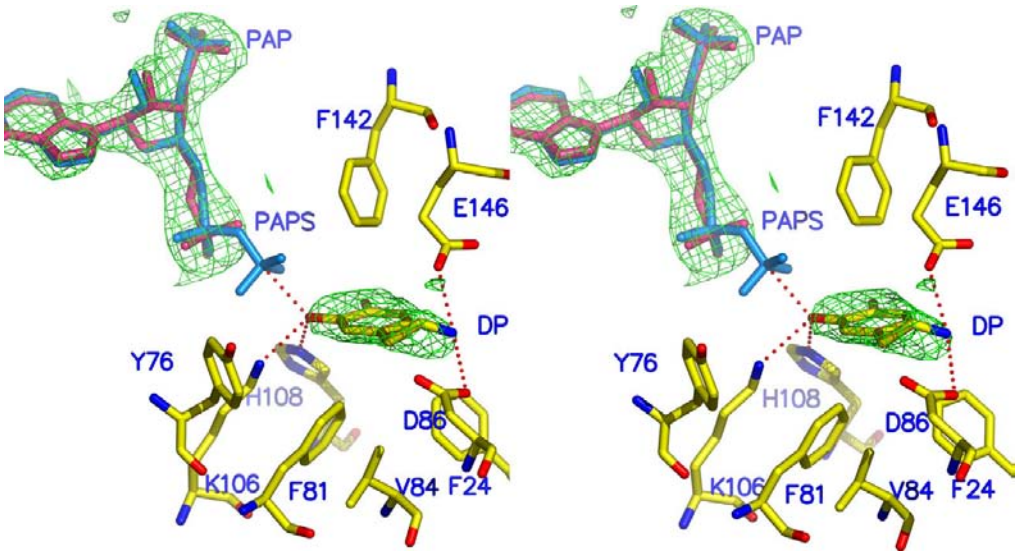


Figure 7. *Stereo view of the binding of PAP and dopamine (DP) to SULT1A3 (*Lu *et al.*, 2005; *Reprinted with permission from Elsevier).*

In the SULT1A3 structure, phenylalanines 24, 81, and 142 together with Glu146 and His149 form the substrate binding site (Dajani *et al.*, 1999b). For the most part, these are the same residues as those suggested by Lu *et al.* (2005; Fig. 7). Phe142 is highly conserved in cytosolic SULTs but all the other residues that are suggested to contribute to substrate binding are variable between SULT families. On the other hand, within the subfamilies they are more conserved. All the other residues, except Glu146, suggested to take part in substrate binding of SULT1A3 are identical to those of SULT1A1. Glu146 was identified as the single amino acid that defines the selectivity of SULT1A3 toward dopamine and other catecholamines by ion pairing with their amino group (Dajani *et al.*, 1998).

Substrate inhibition is a very common enzyme kinetic phenomenon for SULTs. Gamage *et al.* (2003) found that SULT1A1 accommodates two *p*-nitrophenol (*p*NP) molecules in the active site. They proposed that the substrate inhibition is due to binding of substrate to one of the binding sites for *p*NP and only the other binding site is catalytically active. The structural basis of the phenomenon was also studied by Lu *et al.* (2008), who found that two amino acids, Tyr238 and Met137, are critical for substrate inhibition in SULT2A1. They proposed that the substrate inhibition can occur from binding of the substrate in either orientation.

Overall, the substrate binding pockets of SULTs are flexible and can adopt their structures to accommodate many kinds of substrates. This makes their substrate specificity broad and overlapping with other SULTs. However, some characteristic substrates for most isoforms have been found and they are listed in Table 5. For instance, SULT1A1 sulfonates *p*-NP even at very low substrate concentrations, and SULT1A3 is selective for catecholamines, although they are 93% identical in their amino acid sequences. SULT4A1 is the isoform discovered most recently and it is expressed only in the brain. No substrate

has been found for this isoform and it seems that it is not capable of binding PAPS either (Allali-Hassani *et al.*, 2007). However, it binds some other compounds such as adrenaline and thyroid hormones, suggesting that it may contribute to their homeostasis in brain by some other mechanism than sulfonation.

Table 5. *Some typical substrates for human SULTs*

SULT	Characteristic substrates	References
1A1	*p*-Nitrophenol, *p*-nitrocatechol, *p*-cresol, 1-naphthol and other phenols, 17α-ethinyl-estradiol, 3,3'-diiodo-thyronine	Falany, 1997; Veronese *et al.*, 1994; Kester *et al.*, 1999; Brix *et al.*, 1999
1A2	*p*-Nitrophenol Ethanol	Raftogianis *et al.*, 1999 Schneider and Glatt, 2004
1A3	Dopamine, tyramine, dopa	Veronese *et al.*, 1994; Falany, 1997; Sakakibara *et al.*, 1998a; Brix *et al.*, 1999
1B1	Thyroid hormones Ethanol	Wang *et al.*, 1998 Schneider and Glatt, 2004
1C2	Thyroid hormones	Li *et al.*, 2000
1C4	Ethanol	Schneider and Glatt, 2004
1E1	Estradiol, estrone, 17α-ethinyl-estradiol and other steroids	Forbes-Bamforth and Coughtrie, 1994; Falany *et al.*, 1995; Falany, 1997
2A1	Dehydroepiandrosterone and other steroids	Falany, 1997; Pedersen *et al.*, 2000
2B1	Dehydroepiandrosterone, hydroxysteroids, pregnenolone (2B1_v1), cholesterol (2B1_v2)	Her *et al.*, 1998; Fuda *et al.*, 2002
4A1	Not known	Allali-Hassani *et al.*, 2007

2.2.6 The dopamine sulfonating isoform SULT1A3

SULT1A3 is the major isoform responsible for dopamine sulfonation in humans and it is encoded by two genes: *SULT1A3* and *SULT1A4* (Hildebrandt *et al.*, 2004). Sulfotransferase corresponding to human SULT1A3 is not expressed in rodents (Eisenhofer *et al.*, 1999; Honma *et al.*, 2001) and that is probably the reason why glucuronidation is a more important metabolic pathway for dopamine in rat than in human (Wang *et al.*, 1983). At least one non-synonymous coding single-nucleotide polymorphism has been found in *SULT1A3* or *SULT1A4* which leads to decreased levels of enzyme activity and protein levels in recombinant transient expression models (Thomae *et al.*, 2003).

Previously, SULT1A3 was called thermolabile phenolsulfotransferase (TL-PST) or monoamine-preferring PST (M-PST) to distinguish it from another phenolsulfotransferase, SULT1A1, previously called thermostable PST (TS-PST) or phenol-preferring PST (P-

PST). Glu146 is an important amino acid residue determining the substrate specificity of SULT1A3 for catecholamines (Dajani *et al.*, 1998). According to X–ray crystallographic studies, SULT1A3 is the only SULT having a carboxylate side chain in the active site capable of forming an ion pair with the protonated amino ethyl side chain of dopamine or other catecholamines (Dajani *et al.*, 1998; 1999b; Bidwell *et al.*, 1999; Lu *et al.*, 2005). Dopa and tyrosine are also good substrates for SULT1A3 although they both have a negatively charged carboxyl group next to the amino group (Sakakibara *et al.*, 1998a).

The crystal structure of SULT1A3 was resolved in complex with PAP (Dajani *et al.*, 1999b), with lithium sulfate (Bidwell *et al.*, 1999), and with PAP and dopamine (Lu *et al.*, 2005). In the latter structure (Fig. 7), the 3-hydroxy group of dopamine is aligned to form hydrogen bonds with residues His108 and Lys106 within the active site, whereas the 4-hydroxy group cannot easily form these bonds (Lu *et al.*, 2005). Only the 3-hydroxy group is also in line with the sulfate group of PAPS. In some studies, dopamine has been modeled in the active site so that the 4-OH is pointing to the catalytic His108 (Dajani *et al.*, 1999b; Brix *et al.*, 1999). It has been suggested that dopamine fits into the active site in such a way that it enables the sulfonation of either hydroxyl group because the flexible loops around the active site may undergo conformational change upon ligand binding (Bidwell *et al.*, 1999; Dajani *et al.*, 1999b; Barnett *et al.*, 2004). However, these findings have not been supported by an enzyme kinetic analysis of dopamine sulfonation *in vitro* because the most widely used assays measure only the sum of the 3-*O*- and 4-*O*-sulfates and are unable to resolve the two isomers (e.g. Foldes and Meek, 1973). One of the aims of this study was to characterize the regioselective enzyme kinetics of dopamine sulfonation by SULT1A3.

2.3 Enzyme kinetic equations

Enzyme kinetics is a discipline that focuses on the reaction rates of enzymatically catalyzed chemical reactions. In this section, the enzyme kinetic equations used in this study are briefly introduced. Originally, these enzyme kinetic equations were developed for single-substrate reactions, but they apply to two-substrate reactions as well, provided that the concentration of one substrate is kept at a constant saturating level while determining the enzyme kinetic parameters for the other.

The Michaelis-Menten equation, published in 1913, is the most commonly-used enzyme kinetic equation (Eq. 1 in Table 6.). In a usual enzyme-catalyzed reaction, the substrate is first bound to the enzyme. Then it is transformed into a product and released, or it is released back without transformation (Fig. 8A). The Michaelis-Menten constant (K_m) describes the substrate concentration at which the reaction velocity is half of the V_{max}, the limiting (or maximal) rate of the enzymatic reaction (Fig. 8B). The K_m is associated with the affinity of the enzyme for the given substrate but it does not directly define the affinity. V_{max} is mathematically the asymptote of the hyperbola that the Michaelis-Menten equation defines and the reaction velocity will never reach it at a finite substrate concentration (Cornish-Bowden, 1995).

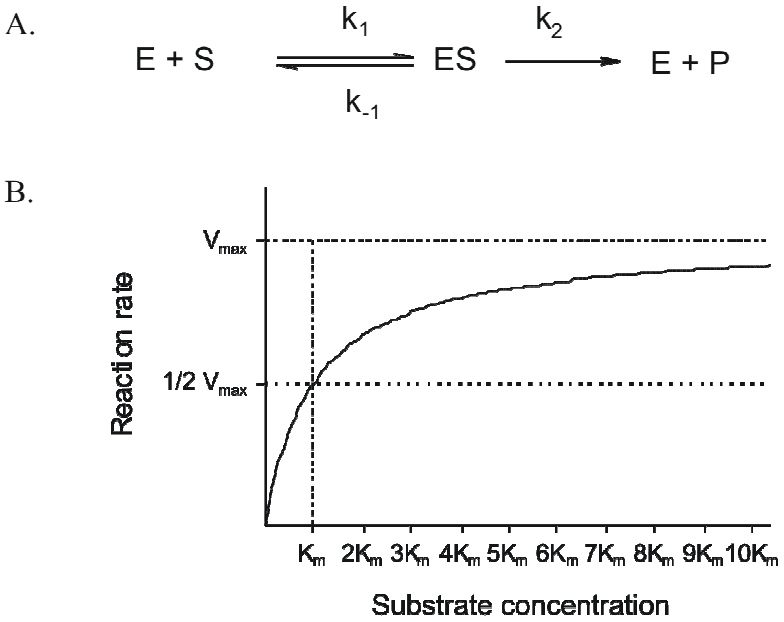


Figure 8. *The reaction scheme of a generalized enzyme-catalyzed reaction (A) and the curve defined by the Michaelis-Menten equation (B). E, S, and P represent the enzyme, substrate, and product, respectively.*

If enzyme is purified and quantified, one can determine the k_{cat} value, i.e. turnover number, of the enzyme (Cornish-Bowden, 1995; Copeland, 2000). The units of k_{cat} are reciprocal time and the value defines the maximal velocity at which an enzymatic reaction can proceed at infinite substrate concentration. It describes the velocity of multiple chemical steps that occur inside the enzyme after substrate binding, in other words, it tells how many substrate molecules the enzyme is able to transform into product molecules in a unit of time. Unlike UGTs, sulfotransferases are relatively easy to purify since they are cytosolic and not membrane bound proteins and hence the k_{cat} can be measured for them. When the actual amount of active enzyme is not known, the k_{cat} value can not be determined but one has to settle for determination of V_{max}. V_{max} values are reported in units of molecules of metabolite produced per incubation time units per amount of protein added to the reaction mixture (including proteins other than the enzyme studied). These values cannot be compared between different enzymes or enzyme preparations unless the expression levels of the enzymes in the preparations are somehow determined and the V_{max} values are normalized to those levels.

Table 6. *The enzyme kinetic equations used in this study.*

Name	No.	Equation	Definitions	Publi-cations
Michaelis-Menten	(1)	$v = \frac{V_{max} \times [S]}{K_m + [S]}$	S is the substrate concentration v is the reaction velocity V_{max} is the limiting rate of the enzymatic reaction K_m is the Michaelis-Menten constant $K_m = (k_{-1} + k_2)/k_1$ (Fig. 8A)	I-IV
Substrate inhibition	(2)	$v = \frac{V_{max} \times [S]}{K_S + [S](1 + [S]/K_i)}$	K_s is the substrate concentration at 0.5 $\times V_{max}$ K_i is the dissociation constant of the inhibitory SES complex (Fig. 9A)	II,III
Substrate inhibition (two sites)	(3)	$v = \frac{V_\infty \times [S]([K_1] + [S])}{K_2 K_3 + [S]K_3 + [S]^2}$	$V_\infty = k_2[E]_{total}$ $K_1 = k_1 K_{s1s2}/k_2$ $K_2 = 1/(1/K_{s1} + 1/K_{s2})$ $K_3 = K_{s1}K_{s1s2}(1/K_{s1} + 1/K_{s2})$ (Fig. 10)	I
Biphasic kinetics	(4)	$v = \frac{V_{max1} \times [S]}{K_{m1} + [S]} + \frac{V_{max2} \times [S]}{K_{m2} + [S]}$		IV
Hill	(5)	$v = \frac{V_{max} \times [S]^n}{K_A{}^n + [S]^n}$	K_A is the substrate concentration at 0.5 $\times V_{max}$ n is the Hill coefficient	II,III,IV
Competi-tive inhibition	(6)	$v = \frac{V_{max} \times [S]}{K_m(1 + [I]/K_i) + [S]}$	I is the inhibitor concentration K_i is the inhibition constant	II

Substrate inhibition (Eq. 2) is common in the enzyme kinetic studies of UGTs (e.g. Luukkanen *et al.*, 2005) and SULTs *in vitro* but it is not normally seen at physiological concentrations of the substrates (Cleland, 1983). In a simple single-substrate reaction, another substrate molecule binds to the ES complex and inhibits its function (Fig. 9A). Prior to the formation of an inactive SES complex, an ES complex must be formed, thus substrate inhibition usually occurs only at high substrate concentrations (Copeland, 2000).

At infinite substrate concentration, the reaction rate approaches zero (Fig. 9B) rather than the V_{max}. The V_{max} in the substrate inhibition equation describes the theoretical maximal rate of the reaction without substrate inhibition.

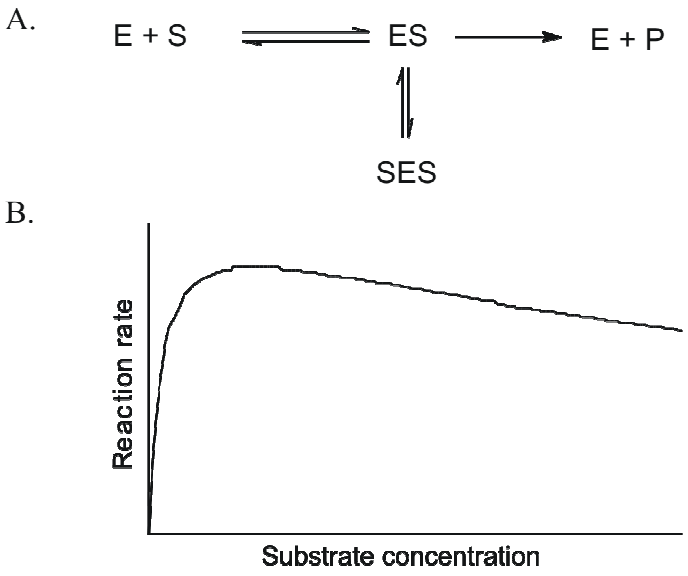


Figure 9. *The reaction scheme for substrate inhibition (A) and the curve defined by the substrate inhibition equation (B). E, S, and P represent the enzyme, substrate, and product, respectively.*

Sulfotransferases sometimes exhibit exceptional substrate inhibition kinetics, as described by Gamage *et al.* (2003; Eq. 3). SULT1A1 has been crystallized with PAP and two *p*-nitrophenol (*p*NP) molecules in the active site and this has been proposed to explain the substrate inhibition. Gamage *et al.* (2003) also studied the substrate inhibition with enzyme kinetic methods. They suggested that the enzyme can bind *p*NP at site 1 (the active site) or site 2 (an additional binding site) and that occupancy of site 1 does not affect the binding of *p*NP at site 2, although *p*NP cannot be bound to site 1 if site 2 is occupied (Fig. 10A). The product *p*NP-sulfate cannot be released if site 2 is occupied but occupancy of site 2 does not completely abolish the catalytic activity of site 1. At a high substrate concentration both sites are occupied but still some residual activity is observed, and the reaction rate does not approach zero with infinite substrate concentration as it does in the case of ordinary substrate inhibition. The major disadvantage of this model is that the constants obtained from equation 3 cannot be intuitively interpreted. The K values are combinations of different rate constants (Table 6 and Fig. 10A) and the V_{∞} is the only constant that is easily interpreted. It defines the limiting reaction velocity at infinite substrate concentration (Fig. 10B). A similar substrate inhibition pattern has been found for SULT1E1 (Zhang *et al.*, 1998). The equation used to fit the data was different in that study but the resulting plot was similar to that presented in Figure 10B.

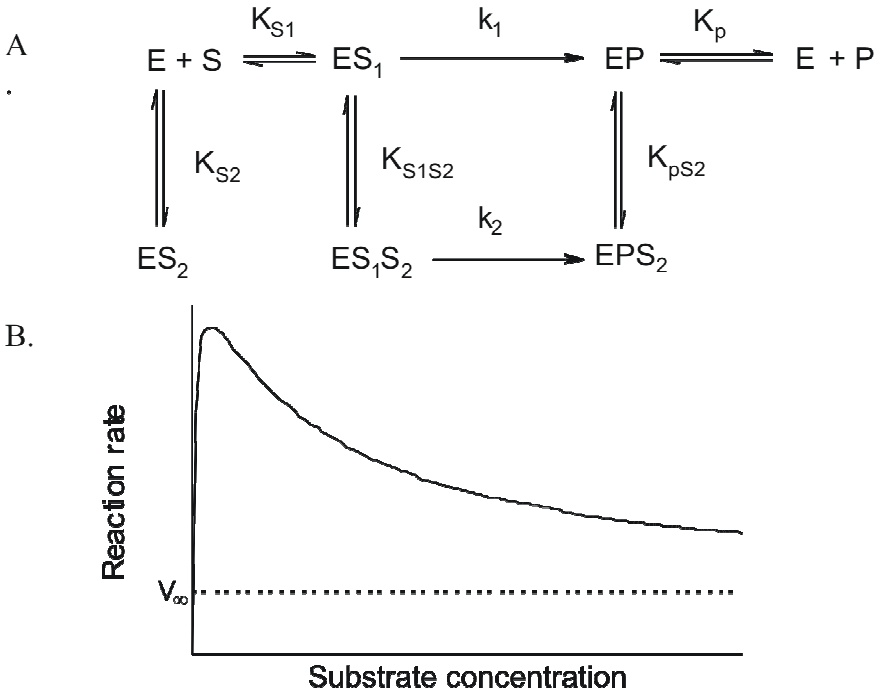


Figure 10. *The two sites model of substrate inhibition kinetics (A) and the curve defined by the equation of Gamage et al. (2003) (B). Site 1 is productive whereas Site 2 is unproductive. Binding of substrate at Site 2 prevents another substrate molecule from binding to Site 1. Binding to Site 1 does not affect the binding to Site 2. E, S, and P symbolize the enzyme, substrate, and product, respectively. The V_∞ defines the limiting reaction velocity at infinite substrate concentration*

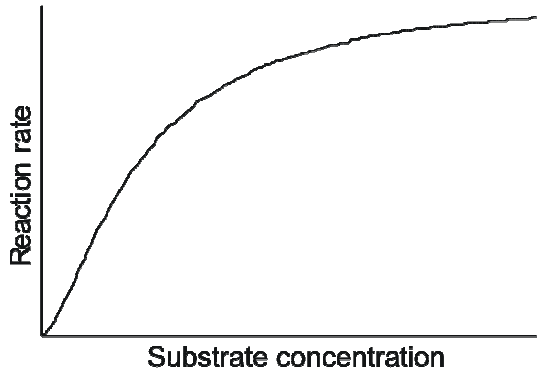


Figure 11. *The curve defined by the Hill equation.*

A biphasic equation is used to describe enzyme kinetics when there are multiple enzymes in the reaction mixture acting on the same substrate or one enzyme having multiple binding sites with different affinities and reaction rates. The resulting kinetic curve is a sum of two or more Michaelis-Menten curves (Eq. 4). This kind of kinetics has been seen e.g. with UGT2A isoforms (Sneitz *et al.*, 2009).

The Hill equation (Hill, 1910; Eq. 5) describes cooperative binding or autoactivation. The curve defined by the Hill equation is sigmoidal (Fig. 11) and the Hill coefficient (n) describes the degree of sigmoidicity (Eq. 5). If the binding sites are similar to each other (e.g. in homo-oligomeric enzymes) and connected so that they affect eachother's affinity for the substrate, they are said to exhibit cooperativity. With UGTs, sigmoidal curves are often produced (e.g. Soars *et al.*, 2003).

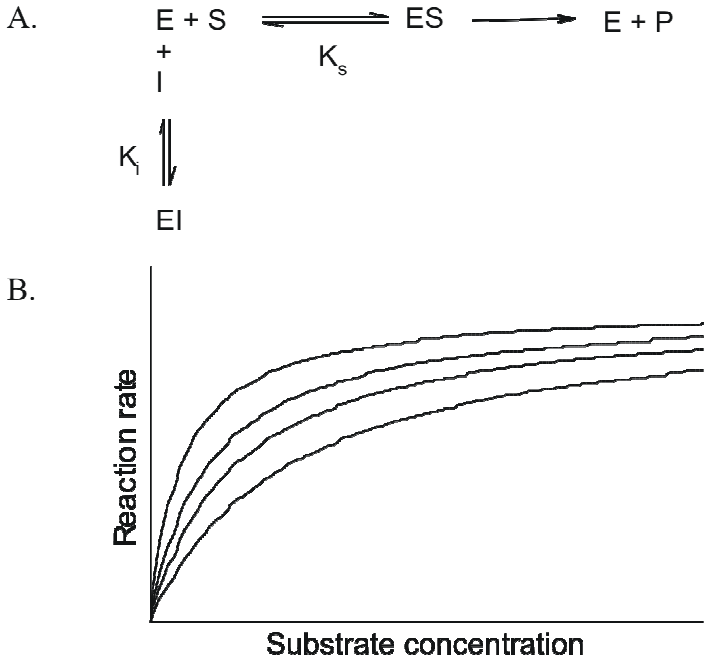


Figure 12. *The reaction scheme of the reversible competitive inhibition of an enzymatic reaction (A). E, S, I, and P represent the enzyme, substrate, inhibitor, and product, respectively. The curves are defined by the Michaelis-Menten equation in the absence and presence of a competitive inhibitor (B). The highest curve has been obtained without an inhibitor and the others have been obtained in the presence of three different concentrations of the inhibitor. The lowest curve has been obtained with the highest inhibitor concentration.*

Competitive inhibition is a mode of enzyme inhibition where two compounds, the substrate and the inhibitor, compete for the same form of the enzyme (Fig. 12). The enzyme molecules that bind an inhibitor instead of the substrate are temporarily unavailable to the substrate, but those enzyme molecules that bind substrate instead of the inhibitor have the same reaction rate as they would have in the absence of the inhibitor. When the substrate concentration is high compared to the inhibitor concentration the observed reaction rate is similar to the maximum velocity of the reaction without the inhibitor. Thus, the V_{max} is not affected by a competitive inhibitor. Instead, the inhibitor increases the substrate concentration that is needed to reach the half-maximal velocity and hence the apparent K_m.

3 Aims of the study

The aim of the study in publication I was to determine the regioselectivity of dopamine sulfonation by SULT1A3. Regioselectivity arose as an issue from molecular modeling studies with this enzyme at the Division of Pharmaceutical Chemistry when it was not obvious in which orientation the dopamine molecule should be placed in the model. The sulfonation kinetics of dopamine was determined using purified recombinant SULT1A3 and the samples were analyzed by HPLC. Another aim of the study was to develop a rapid analytical method to separate the regioisomers of dopamine sulfate.

After the first study, the focus was shifted from SULTs to UGTs. The aim of the study in publication II was to assess regio- and stereoselectivity of all known human UGT isoforms and 3 rat isoforms using β-estradiol and its diastereoisomer epiestradiol as substrates. Interspecies differences were also examined with human, rat, rabbit, pig, bovine, and moose liver microsomes.

Publication III aimed to find out which UGT isoforms are responsible for dopamine glucuronidation in humans. Regioselectivity of the reaction was also of interest. After finding that UGT1A10 was the only UGT capable of dopamine glucuronidation at a significant level, twelve mutants of UGT1A10 were studied in order to determine the role of the amino acid residues at positions 90 and 93 in dopamine glucuronidation. To find out if the mutations affect the affinity as well as the activity, four of the mutants were also subjected to enzyme kinetic studies.

During the course of studies II and III, it became obvious that UGT1A10 is a very highly active isoform in glucuronidation of estradiol and dopamine. In contrast, UGT1A9, which is very similar to UGT1A10 at the amino acid level, was less active and had opposite regio- and stereoselectivity. The aim of publication IV was to find out which amino acids determine the substrate specificity differences between UGT1A9 and UGT1A10 by examining chimeras and point-mutated enzymes that had parts from both isoforms.

4 Materials and Methods

4.1 Chemicals

Commercial chemicals and materials used in this study are listed in Table 7. Structures of the substrates are shown in Figure 13. Substrates used in this study were of the highest quality available (at least 98% purity). Dopamine sulfates (I) and dopamine-4-*O*-glucuronide (II) were not commercially available and they were synthesized in our laboratory and used as standards. Synthesis of the dopamine-4-*O*-glucuronide was carried out as described by Uutela *et al.* (2009). Synthesis of the dopamine sulfates was based on previously published methods (Jain *et al.*, 1986; Strobel *et al.*, 1988) and the details are found in publication I.

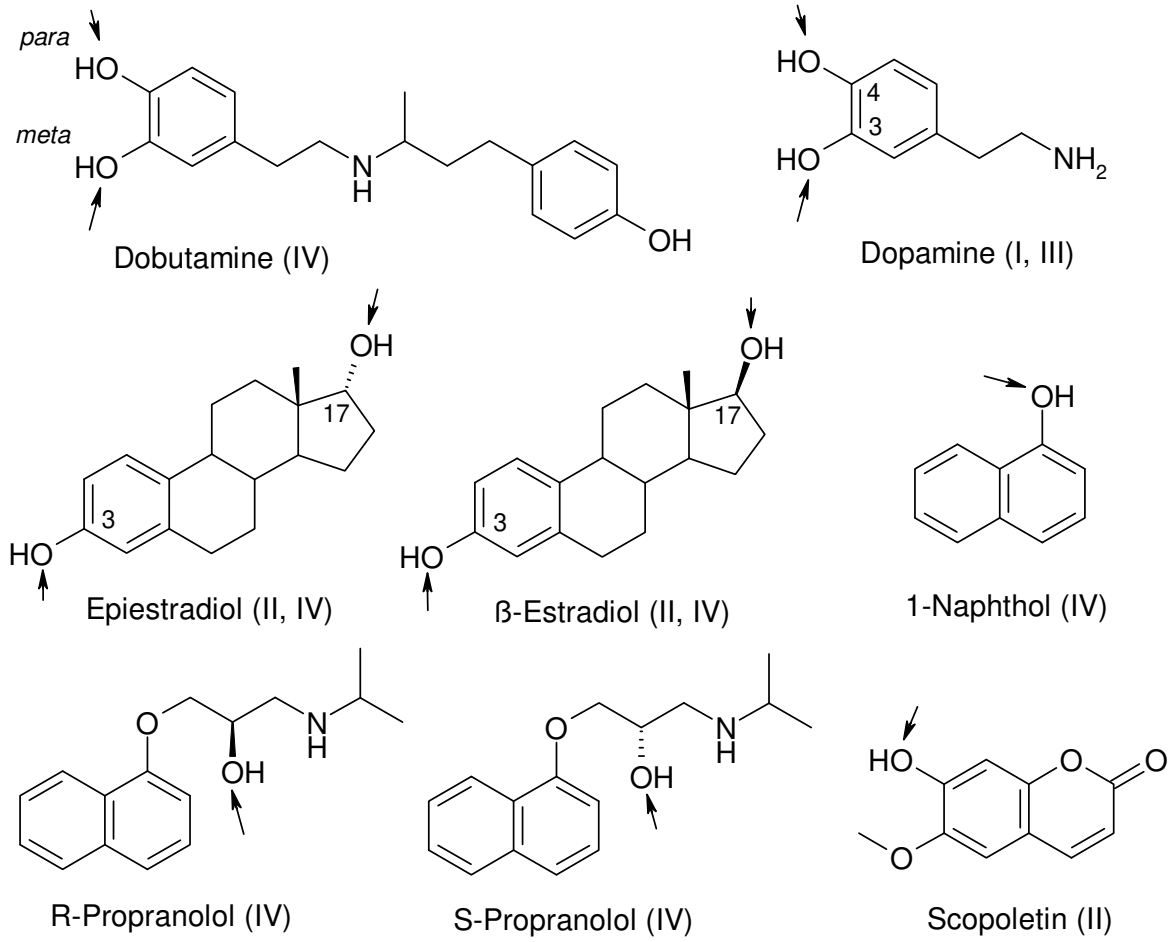


Figure 13. *Chemical structures of the substrates used in this study. The conjugation sites that were examined are marked with arrows.*

Table 7. *Chemicals used in the study.*

Chemical	Supplier	Use	Publi-cation
Acetic acid	Mallinckrodt Baker, Deventer, Holland	HPLC	IV
Acetonitrile	Rathburn Chemicals, Walkerburn, UK	HPLC	III, IV
Acryl amide	Flowgen, UK	SDS-PAGE	I
Ammonium acetate	Riedel-de Haën, Seelze, Germany	HPLC	IV
Ammonium persulfate	Bio-Rad Laboratories, Hercules, CA	SDS-PAGE	I
Ammonium sulfate	WVR International, UK	Enzyme purification	I
Ampicillin sodium salt	Sigma-Aldrich[a]	Enzyme purification	I
Barium acetate	WVR International, UK	Activity assay	I
$Ba(OH)_2$	Sigma-Aldrich[a]	Activity assay	I
Bradford reagent	Sigma-Aldrich[a]	Protein assay	I
Brij 58	WVR International, UK	Enzyme purification	I
Bovine serum albumin	Perbio Science, UK	Standard	I
Dimethylsulfoxide	Riedel-de Haën, Seelze, Germany	Activity assay	II-IV
Dipotassium hydrogen orthophosphate, anhydr.	WVR International, UK	Activity assay	I
Disodium hydrogen phosphate	Fluka, Germany	Activity assay	I-IV
Dobutamine hydrochloride	Eli Lilly & Co., Indianapolis, IN	Substrate	IV
Dopamine hydrochloride	Sigma-Aldrich[a]	Substrate	I, III
Dulton VII	Sigma-Aldrich[a]	SDS-PAGE	I
Emulsifier Safe	PerkinElmer Life and Analytical Sciences, Boston, MA	Activity assay	I
17α-Estradiol	Sigma-Aldrich[a]	Substrate	II, IV
17β-Estradiol	Sigma-Aldrich[a]	Substrate	II, IV
17β-Estradiol-β-D-glucuronides, sodium salt	Sigma-Aldrich[a]	Standard	II
Formic acid 98-100%	Riedel-deHaën, Seelze, Germany	HPLC	III
Glycine	WVR International, UK	SDS-PAGE	I
Hydrochloric acid 31.5-33%	WVR International, UK	SDS-PAGE	I
Idranal® III	Riedel-de Haën, Seelze, Germany	HPLC	I
Isopropyl β-D-Thiogalactopyranoside	Sigma-Aldrich[a]	Enzyme purification	I
Laemmli Sample Buffer	Sigma-Aldrich[a]	SDS-PAGE	I
Luria Agar	Gibco BRL Life technologies, UK	Enzyme purification	I
Luria Broth base	Invitrogen life technologies, UK	Enzyme purification	I

Table 7. *Continued.*

Chemical	Supplier	Use	Publi-cation
$MgCl_2$	Merck, Darmstadt, Germany	Activity assay	II-IV
$MgSO_4$	WVR International, UK	Enzyme purification	I
β-Mercapto-ethanol	WVR International, UK	Enzyme purification	I
Methanol	J.T. Baker, Deventer, Holland	Activity assay HPLC	I II, IV
1-Naphthol	Sigma-Aldrich[a]	Substrate	IV
1-Naphthyl glucuronide, sodium salt	Sigma-Aldrich[a]	Standard	IV
PAPS	Dr. H. Glatt, German Institute for Human Nutrition, Postdam, Germany	Co-substrate	I
PAP[^{35}S]	PerkinElmer Life and Analytical Sciences, Boston, MA	Co-substrate	I
Perchloric acid 70-72 %	Merck, Darmstadt, Germany	Activity assay	II-IV
Potassium dihydrogen orthophosphate	WVR International, UK	Activity assay	I
R-Propranolol	Sigma-Aldrich[a]	Substrate	IV
S-Propranolol	Sigma-Aldrich[a]	Substrate	IV
D-Saccharic acid 1,4-lactone	Sigma-Aldrich[a]	Activity assay	II, III
Scopoletin	Sigma-Aldrich[a]	Substrate	II
Sodium dihydrogen phosphate dihydrate	Fluka, Germany	Activity assay, HPLC	I, II, IV
Sodium dodecyl sulphate	WVR International, UK	SDS-PAGE	I
Tetramethylethylene-diamine	National diagnostics, UK	SDS-PAGE	I
Tris(hydroxymethyl)-methylamine	WVR International, UK	SDS-PAGE, Enzyme purification	I
UDP-glucuronic acid	Sigma-Aldrich[a]	Co-substrate	II-IV
[^{14}C]UDP-glucuronic acid	PerkinElmer Life and Analytical Sciences, Boston, MA	Co-substrate	II-IV
Water (Milli-Q Plus)	Millipore, Molsheim, France	HPLC	I-IV
$ZnSO_4$	Sigma-Aldrich[a]	Activity assay	I

[a]Sigma-Aldrich, St. Louis, MO or Steinheim, Germany

4.2 Enzyme sources

4.2.1 Expression and purification of SULT1A3 (I)

The human SULT1A3 cDNA was expressed in *E. coli* and the protein was purified as described previously (Dajani *et al.*, 1998; 1999a). Briefly, the SULT1A3 was purified from the *E. coli* cell-free extract by ammonium sulfate precipitation and two steps of chromatographic separation. First, the protein was applied to a HiTrap Q HP column (Amersham Biosciences) and thereafter to a 3',5'-adenosine diphosphate agarose affinity column. The fractions were collected and tested using SDS-PAGE and a dopamine sulfonation assay namely the barium precipitation method of Foldes and Meek (1973). The most active and pure protein fractions were pooled and dialyzed overnight between the purification steps and desalted using a PD-10 column (Amersham Biosciences) at the end of the procedure. The final composition of the buffer used to store the protein was 50 mM Tris/HCl buffer (pH 8) with 1 mM 2-mercaptoethanol. The protein concentration was estimated using bovine serum albumin as the standard (Bradford, 1976).

4.2.2 Microsomal and recombinant UGTs (II, III, IV)

The enzyme sources obtained commercially or according to previously published methods are listed in Table 8. For publication II, the cDNA of rat UGT2B3 was isolated by reverse transcription polymerase chain reaction 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-length DNA was subcloned as an 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 of the original stop codon by PCR. Thereafter virus preparation and protein production were performed as previously described (Kurkela *et al.*, 2003).

In publication III, the activity of UGT2B10 toward dopamine was studied using freshly harvested cells because of possible partial inactivation upon membrane/microsome preparation (Kaivosaari *et al.*, 2007).

Mutated recombinant UGTs 1A9 and 1A10 (910-mutants for publication IV) were produced in our laboratory as His-tagged proteins in baculovirus-infected insect cells (Kurkela *et al.*, 2003; Kuuranne *et al.*, 2003). Mutagenesis was done by polymerase chain reaction, and the correctness of the resulting mutants was confirmed by DNA sequencing of the entire fragment that had been amplified by polymerase chain reaction and subcloned into the previously sequenced vectors. The constructed chimeric and point mutants were expressed in baculovirus-infected insect cells as described previously (Kurkela *et al.*, 2003; Kuuranne *et al.*, 2003). Protein concentrations were determined by the BCA method (Pierce Biotechnology Inc., Rockford, IL). The relative expression levels of the recombinant UGTs were determined using a monoclonal antibody, tetra-His (QIAGEN,

Hilden, Germany), directed to the C-terminal His-tag that they carry, as described in detail previously (Kurkela *et al.*, 2007).

Table 8. *Enzyme sources used in the study.*

Enzyme source	Supplier or reference	Publi-cation
New Zealand rabbit liver microsomes	In Vitro Technologies, Baltimore, MD	II
Pooled human liver microsomes	BD Gentest, Woburn, MA	II, III
Pooled human intestinal microsomes	BD Gentest, Woburn, MA	II, III
Recombinant human UGT2B15	BD Gentest, Woburn, MA	II, III
Rat liver microsomes	Luukkanen *et al.*, 1997	II
Pig, bovine and moose liver microsomes	Luukkanen *et al.*, 1997	II
Recombinant human UGTs 1A1, 1A3-1A10, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28	Kurkela *et al.*, 2003; 2007; Kuuranne *et al.*, 2003	II, III, IV
Recombinant human UGTs 2A1-3	Sneitz *et al.*, 2009	II, III
rat UGTs 2B1 and 2B2	Kurkela *et al.*, 2003; Mackenzie *et al.*, 1984; Mackenzie, 1986a; 1986b	II
1A10F90 and 1A10F93 mutants	Xiong *et al.*, 2006; Starlard-Davenport *et al.*, 2007	III

4.3 Enzyme assays

In enzyme kinetic studies, the initial rate of the enzyme-catalyzed reaction should be measured (Allison and Purich, 1983). The initial rate period is shorter for low substrate concentrations and longer for high substrate concentrations because the relative substrate concentration changes faster at low concentration. The linear period ends approximately when more than 10% of the substrate is consumed. (There is no true linear range, but the curve is virtually straight.) Enzyme stability is another factor influencing the duration of the initial rate period. The initial rate period can be estimated by continuous assays, e.g. following the reaction by spectrophotometer or coupling the reaction to another (Cornish-Bowden, 1995) or by assaying separate samples. In this study, preliminary tests were always conducted prior to enzyme kinetic studies in order to determine the linear range of the reaction with respect to incubation time and protein concentration.

When determining enzyme kinetic parameters, the substrate concentration range should be at least from 0.2 to 5 times K_m (Cornish-Bowden, 1995; Allison and Purich, 1983). To estimate this range, preliminary studies were usually conducted prior to the selection of concentration levels. In estradiol glucuronidation assays it was not always possible to use as wide a concentration range as recommended, because of the solubility

problems encountered with concentrations above 300 µM. In dopamine glucuronidation studies, dopamine concentrations as high as 10 mM were used, but that was only 3 to 4 times K_m in most cases.

Dimethyl sulphoxide (DMSO) was used in glucuronidation assays to enhance the solubility of the substrates. Of the substrates studied here, estradiols were especially poorly soluble in water, and a 5% DMSO concentration was used in the assays. Dehal *et al.* (2002) have shown in a poster that 5% DMSO did not inhibit the formation of estradiol-3-*O*-glucuronide by recombinant UGT1A1 or human liver microsomes. Uchaipichat *et al.* (2004) have investigated the effects of organic solvents on activities of recombinant UGTs in cell lysates of a human embryonic kidney cell line (HEK293). They concluded that some isoforms are more affected than others. UGT2B17 was especially sensitive to solvents. Kuuranne *et al.* (2003) noticed that DMSO was a better solvent for studying steroid glucuronidation than methanol or ethanol, and they used DMSO concentrations as high as 10%. Also, Dehal *et al.* (2002) have shown that DMSO is the least inhibitory solvent in UGT assays, although the effect is dependent on the isoform and the substrate. For SULTs, on the contrary, ethanol was recommended as a preferred solvent by Ma *et al.* (2003), but in the dopamine sulfonation study (I) no organic solvent was used.

4.3.1 SULT assays (I)

Dopamine sulfonation assays were carried out in 10 mM sodium phosphate buffer, pH 6.8, in a final volume of 160 μl, and the reactions were started with the addition of the enzyme. The reaction mixtures contained 0.5-1000 μM dopamine (12 different concentrations) and 10 μM PAPS and they were incubated at 37°C for 10 minutes. All samples were assayed in duplicate and the reactions were stopped with 160 μl of chilled methanol. Control samples were incubated in the absence of dopamine, PAPS or enzyme to verify that no peaks eluted in HPLC chromatograms at the same time as dopamine sulfates. To some control samples, the enzyme was added after the addition of methanol to verify that the enzyme was properly deactivated under these conditions. The reaction mixtures were then frozen at –70°C and lyophilized. The dried samples were dissolved in 50 μl of HPLC mobile phase and centrifuged for 5 min at 16,100*g*, after which 20 μl of supernatant was injected into the HPLC system. Four sets of samples were made and analyzed on different days.

4.3.2 UGT assays (II-IV)

The detailed descriptions of the assay conditions for activity screening and enzyme kinetic studies can be found in the original publications II-IV. All incubations were done at 37°C and pH 7.4, mimicking physiological conditions. All samples contained 50 mM phosphate buffer and 5 mM $MgCl_2$. The reactions were terminated by adding chilled 4 M perchloric acid (10% v/v of the total volume of the sample) and cooling the tubes in a cold block,

followed by centrifugation at 16,100*g* to remove the precipitated proteins. Screening assays were incubated in duplicate or triplicate and all enzyme kinetic samples were incubated in triplicate. Other conditions are summarized in Table 9.

In glucuronidation studies, saccharolactone was traditionally added to reaction mixtures as an inhibitor of endogenous β-glucuronidase that might be present in enzyme preparations. However, in 2008, it was shown by Oleson and Court that saccharolactone was not needed for optimal activity. On the contrary, saccharolactone slightly inhibited some glucuronidation reactions (Oleson and Court, 2008). Consequently, although saccharolactone was used in studies II and III, it was omitted from the samples of the last publication (IV).

Table 9. *Incubation conditions in glucuronidation assays.*

Substrate	S (μM)	Enzyme	Protein (mg/ml)	Time (min)	UDPGA (mM)	DMSO (%)	SL[d] (mM)
II							
β-Estradiol, Epiestradiol[a]	1-100	19 human and 3 rat recombinant UGTs	0.04-0.2	30	1	5	5
β-Estradiol, Epiestradiol[a]	100	HIM, HLM, rabbit, pig, elk, bovine and rat liver microsomes	0.4-3	60	1	5	5
β-Estradiol, Epiestradiol[b]	0.5-300	1A1, 1A3, 1A4, 1A7, 1A8, 1A10, 2A1, 2A2, 2B4, 2B7, 2B15, 2B17, r2B1, r2B3	0.008-0.2	10-45	1	5	5
Scopoletin[c] (β-Estradiol)	5-500 (5-20)	1A9	0.04	15	1	4	5
III							
Dopamine[a]	1000 (or 5000)	HLM, HIM, 19 human recombinant UGTs, F90 and F93 mutants	0.4-1.6	60	1	2	5
Dopamine[b]	100-10 000	1A10, F90 and F93 mutants, HLM, HIM	0.1-0.4	30-45	1 and 5	2	5
IV							
β-Estradiol, Epiestradiol[a]	100	1A9, 1A10, 910 mutants	0.5-4	60	2	5	-
R- and S-Propranolol[a]	500	1A9, 1A10, 910 mutants	0.5-4	60	2	2	-
Dobutamine[a]	1000	1A9, 1A10, 910 mutants	0.5-4	60	2	2	-
1-Naphthol[b]	0.1-250	1A9, 1A10, 910 mutants	0.05-0.5	10-30	2	2	-

[a]Screening assays; [b]Enzyme kinetic assays; [c]Inhibition of UGT1A9 by β-estradiol; [d]Saccharolactone

4.4 Liquid chromatography

HPLC was used to analyze the samples. The methods and equipment are listed in Table 10. The flow rate was 1 ml/min with two exceptions. In publication III, the flow rate was 0.9 ml/min and in publication IV, the flow was 1 ml/min up to 9.5 min and 2 ml/min from 10 to 19.5 min when estradiol-glucuronides were analyzed.

The methods for glucuronidation studies were validated by determining the linearity, quantitation limit and repeatability of the retention times when applicable. Screening assays in publication IV were done without quantitation and hence no standard curves or quantitation limits were determined. When authentic glucuronide standards were not available, radioactive glucuronides were produced by incubation and used as standards. More details can be found in the original publications II-IV. The method for the dopamine sulfonation assay was systematically validated and the validation data are presented under Results and Discussion.

4.5 Enzyme kinetic analyses

The enzyme kinetic parameters were determined by fitting the observed values of the reaction rates to different enzyme kinetic equations (Table 6) by a non-linear least squares regression method using SigmaPlot 9.0 with Enzyme Kinetics 1.1 (SPSS, Chicago, IL, US) in publication I or GraphPad Prism version 4.03 or 5 for Windows (GraphPad Software Inc., San Diego, CA) in publications II-IV. The best kinetic model was selected by considering the randomness of the residuals, the standard errors of the estimates, and the correlation coefficients.

4.6 Molecular modeling (IV) and mRNA quantitation (III)

A homology model for human UGT1A9 was constructed using Modeler 9v6 with a standard modeling scheme (Sali and Blundell, 1993). For the C-terminal domain, the human UGT2B7 was used as a template and for the N-terminal domain, UGT72B1 from *Arabidopsis thaliana*, GtfB from *Mycolatopsis orientalis*, and macrolide glycosyltransferase from *Streptomyces antibioticus* were used. The modeling of the segments of poor homology was aided by secondary structure predictions from PredictProtein (Rost *et al.*, 2004). Full details of the model construction are described elsewhere (Laakkonen and Finel, 2010). Eventually, 1-naphthol was docked manually to this resultant model of UGT1A9.

UGT1A9 and UGT1A10 mRNA quantitation in human tissues was done in the collabotarory laboratory as described in publication III.

Table 10. *HPLC methods and equipment used in the study.*

Compounds	RT (min)	Mobile phase	Column	Detector
Dobutamine glucuronides (IV)[a]	*meta*: 17.9 *para*: 14.9	A: 20 mM ammonium acetate (pH 4.5) and B: methanol 0-5 min: 10% B, 5-25 min: 10-35% B, 25-30 min: 35% B, 30-31 min: 35-10% B	Hypersil BDS-C18 (250×4 mm 5μm)	Fluorescence detector[a] Wavelengths: Ex 285nm, em 313nm
Dopamine-glucuronides (III)[a]	*meta*: 5.3 *para*: 4.7	A: 0.1 % aqueous formic acid and B: acetonitrile: 0-1 min: 5% B, 1-11 min: 5-20% B, 11-13 min: 5% B	Discovery® HS F5 (4×150mm) (Bellafonte, PA)	API3000 triple-quadrupole mass-spectrometer[b] with a turbo ion spray source
Dopamine-sulfates (I)[c]	*meta*: 2.65 *para*: 2.44	25 mM phosphate buffer (pH 3.0) and 0.1 mM EDTA in water	Synergi Polar-RP[d] (75×4.6 mm)	Electrochemical[e] Electrode potentials: 200 mV and 400 mV
Estradiol-glucuronides (II)[c]	β3: 2.2 β17: 3.0 Epi3: 2.4 Epi17: 4.2	50% 25 mM phosphate buffer (pH 3.0) and 50% methanol	Chromolith SpeedRod[f]	Fluorescence detector[c] Wavelengths: Ex 216nm, em 316nm
Estradiol-glucuronides (II)[a]		55% 25 mM phosphate buffer (pH 3.0) and 45% methanol	Chromolith SpeedRod	Radioactivity detector (Reeve Analytical, Glasgow, U.K)
Estradiol-glucuronides (IV)[a]	β3: 4.8 Epi3: 5.7	60% 25 mM phosphate buffer (pH 3.0) and 40% methanol	Chromolith SpeedRod	Fluorescence detector[a] Wavelengths: Ex 216nm, em 316nm
1-Naphthyl-glucuronide (IV)[a]	4.2	58% 50 mM phosphate buffer (pH 3.0) and 42% methanol	Hypersil BDS-C18 (150×4.6mm 5 μm)	Fluorescence detector[a] Wavelengths: Ex 285nm, em 335nm
Propranolol-glucuronides (IV)[a]	R: 5.5 S: 31.2	A: 50 mM phosphate buffer (pH 3.0) and B: methanol R-propra: 55%A/45%B S-propra: 70%A/30%B	Zorbax Eclipse Plus C18 (150×4.6mm 5 μm)[g]	Fluorescence detector[a] Wavelengths: Ex 230nm, em 342nm
Scopoletin-glucuronide (II)[a]	4.6	90% 50 mM phosphate buffer (pH 3.0) and 10% methanol	Chromolith SpeedRod	Fluorescence detector[a] Wavelengths: Ex 335nm, em 455nm

[a]Analyzed by Agilent 1100 HPLC, Agilent Technologies, Waldbronn, Germany; [b]Applied Biosystems/MDS Sciex, Concord, Canada; [c]Analyzed by Shimadzu HPLC, Kyoto, Japan; [d]Phenomenex; [e]Coulochem II® Multi-Electrode Detector with Model 5011A Analytical Cell, ESA Biosciences, MA, US; [f]Chromolith SpeedRod RP18e (50×4.6 mm) Merck, Darmstadt, Germany; [g]Agilent Technologies, Waldbronn, Germany

5 Results and Discussion

The main results of the study are presented and discussed in this chapter. More details are found in the original publications I-IV. In addition, some unpublished results are presented here.

5.1 Validation of the HPLC method for analysis of dopamine sulfates (I)

The aim of publication I was to study regioselective sulfonation of dopamine by SULT1A3. A new analytical HPLC method was developed and validated by determining resolution, the limit of quantitation, linearity and the repeatability of retention times and peak areas (Table 11). The resolution (1.15) between the regioisomers was sufficient for accurate and reproducible separation of dopamine sulfates. Separation of dopamine-4-*O*-sulfate, dopamine-3-*O*-sulfate, and dopamine was achieved within three minutes (Fig. 14).

Table 11. *Validation parameters for the HPLC method.*

	Dopamine-3-*O*-sulfate	Dopamine-4-*O*-sulfate
Retention time	2.65 min	2.44 min
RSD of retention time		
Within a day	0.37%	0.24%
Between days	2.15%	2.02%
RSD of peak area (including sample preparation)	5.55%	14.6%
Linearity (R^2)	0.997	0.994
Limit of quantitation	33 nM	33 nM

The analytical method developed here has many advantages over previous methods. One widely-used method for sulfotransferase activity studies is that developed by Foldes and Meek (1973), which is based on the use of the radioactive co-substrate PAPS. The radioactivity is transferred to the acceptor substrate during incubation and after that the unreacted PAPS is precipitated with barium hydroxide and zinc sulfate. After centrifugation, the resulting supernatant is mixed with scintillation fluid and the radioactivity is measured by a scintillation counter. The advantage of this method is that it is fast and it can be used for different substrates. However, one of the disadvantages is the poor repeatability of the precipitation as it depends e.g. on the temperature of the laboratory and the purity of the precipitation reagents. Also, the recovery of the products varies depending on the substrate and the precipitation conditions (Toth *et al.*, 1987). In addition, if the substrate can be sulfonated at more than one position, like dopamine, the resulting regioisomers cannot be separated and quantitated with this method. The new HPLC method was developed to solve these problems.

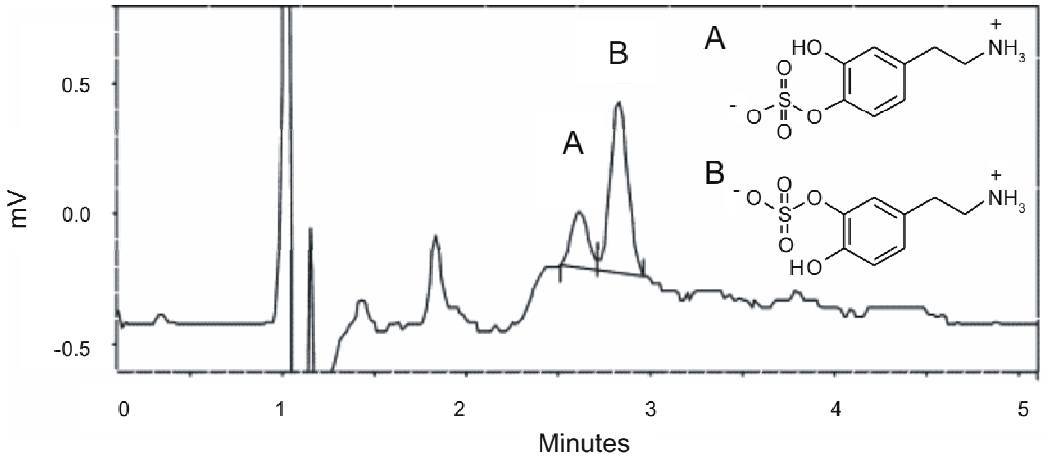


Figure 14. *The separation of dopamine sulfates by HPLC with electrochemical detection. A is dopamine-4-O-sulfate and B is dopamine-3-O-sulfate.The sample was incubated in the presence of the lowest dopamine concentration used, 0.5 µM.*

The new HPLC method is relatively fast for a conventional HPLC and much faster than previous methods (Elchisak and Carlson, 1982; Elchisak, 1983; Swann and Elchisak, 1986; Toth *et al.*, 1987; Kienzl and Eichinger, 1988). The fast separation of sulfates was achieved using a Synergi Polar-RP ether-linked phenyl column (75 mm × 4.6 mm, Phenomenex) that allows the use of an aqueous mobile phase without any organic solvent. It provides enhanced selectivity for polar and aromatic compounds and is therefore optimal for the separation of dopamine sulfates. Dopamine sulfates are not easily separated by ordinary reverse phase columns, and ion pairing agents have often been used in previous methods (Elchisak and Carlson, 1982; Elchisak, 1983; Swann and Elchisak, 1986; Kienzl and Eichinger, 1988). Disadvantages of these methods include a longer equilibration time for the system, longer retention times for analytes and shorter life-times for the columns.

In summary, the HPLC method developed here provides a fast, reliable and easy method for the separation and analysis of the regioisomers of dopamine sulfate and the use of electrochemical detection provides high selectivity and sensitivity.

5.2 Conjugation of dopamine by SULT and UGT

5.2.1 Sulfonation by SULT1A3 (I)

Enzyme kinetics and regioselectivity of dopamine sulfonation by SULT1A3 was studied and the samples were analyzed by the newly developed HPLC method. The reaction was regioselective: the V_{max} and Cl_{int} were, respectively, 7.5 and 6.4 times higher for dopamine-3-*O*-sulfate than for dopamine-4-*O*-sulfate (Table 12). The K_m, on the other

hand, was similar for both regioisomers. The reactions followed the Michaelis-Menten equation at dopamine concentrations below 60 µM (Fig 15). At higher concentrations the reaction rate decreased markedly as a result of substrate inhibition.

Our results from the enzyme kinetic analysis are in accordance with previous observations that SULT1A3 has selectivity for the 3-*O*-sulfonation of l-dopa (Suiko *et al.*, 1998) and that dopamine-3-*O*-sulfate is the form predominantly found in human blood. The K_m and V_{max} values obtained are in good agreement with those published previously using the barium precipitation assay, which showed a K_m of approximately 1 µM and a V_{max} of almost 200 nmol/min/mg, obviously representing the combined formation of dopamine-3-*O*-sulfate and dopamine-4-*O*-sulfate (Dajani *et al.*, 1999a).

As expected, between 35 and 85% substrate inhibition was observed at high dopamine concentrations. In the present study, variable substrate inhibition by dopamine was observed, and in some cases the data fitted well in the equation proposed by Gamage *et al.* (2003, Eq. 3 in Table 6) where the residual enzyme activity is taken into account. In other cases, however, the substrate inhibition seemed to behave differently, and the activity approached zero at high dopamine concentrations.

Table 12. *Enzyme kinetic parameters for dopamine sulfonation by SULT1A3 (n=4).*

	Dopamine-3-*O*-sulfate	Dopamine-4-*O*-sulfate
K_m (µM)	2.59 ± 1.06	2.21 ± 0.76
V_{max} (nmol/min/mg)	344 ± 139	45.4 ± 16.5
Cl_{int} (V_{max}/K_m) (ml/min/mg)	134 ± 39	20.8 ± 6.2
Substrate inhibition (%)	35–85	40–80

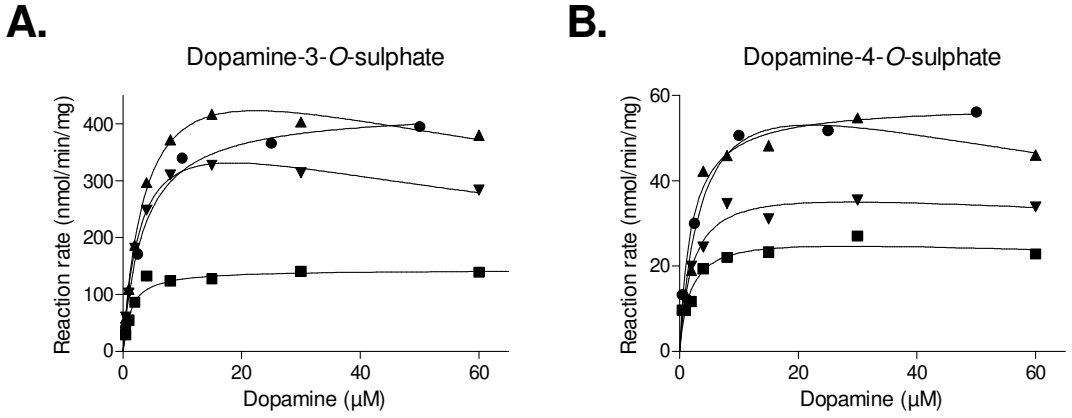


Figure 15. *The formation of dopamine-3-O-sulfate (A) and dopamine-4-O-sulfate (B) by SULT1A3. Four sample sets were analyzed on different days and the sets are marked with different symbols. The samples were incubated in duplicate and the mean values are shown.*

The variation in enzyme kinetic parameters was high between days (Table 12; Fig. 15). The reason for the different enzyme kinetics observed is not clear, but one explanation might be that the enzyme was not stable during processing and storage. Instability is more common with purified enzymes than microsomes, because the other proteins and lipids, that often protect enzymes, are not present (Allison and Purich, 1983). Also, adsorption to container walls and high dilution into the assay mixture may have caused problems. Ma *et al.* (2003) have recommended that incubations with expressed enzyme contain >12 µg/ml total protein, especially when organic solvents are used in the reaction mixture. The SULT1A3 assays contained only 0.16 µg/ml protein per sample but, then again, no organic solvent was used. Some enzymes require the addition of reducing agents, e.g. 2-mercaptoethanol or dithiothreitol, to the assay mixture in order to restore their activity. Reducing agents reduce critical thiol groups and prevent their cross-linking. Previously, it has been observed that oxidation and reduction affect the function of SULTs *in vitro* (Zhang *et al.*, 1998; Marshall *et al.*, 2000). In this study, the SULT1A3 enzyme was in the reduced form because 2-mercaptoethanol was present in the enzyme storage buffer, so the variation in the enzyme kinetics could not be due to different redox stages of the enzyme.

In summary, despite the high variation in enzyme kinetic parameters between days, the results presented here show clearly that SULT1A3 strongly favors the 3-hydroxy group of dopamine over the 4-hydroxy group and may indeed be primarily responsible for the difference between the circulating levels of the regioisomers of dopamine sulfate in human blood.

5.2.2 Glucuronidation by human UGTs (III)

To find out which UGTs are responsible for glucuronidation of dopamine in human, 19 human UGTs were expressed as recombinant proteins in insect cells and screened for dopamine glucuronidation activity. The main finding of this study was that UGT1A10 was the only isoform that glucuronidated dopamine at a significant rate. Very low activity was detected with UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 2A1, 2A3, 2B7, 2B11, 2B15, and 2B17, but, for example, the normalized activity of UGT1A6, the second-best UGT after UGT1A10, was less than 1.3% of the activity of UGT1A10.

The enzyme kinetic studies showed that the glucuronidation of dopamine by UGT1A10 followed slightly sigmoidal kinetics (Fig. 16) and the data were fitted to the Hill equation (Eq. 5 in Table 6). Dopamine glucuronidation by UGT1A10 was not regioselective and the K_A and V_{max} values were similar for both hydroxyls (Table 13).

Human intestinal microsomes (HIM) exhibited similar enzyme kinetics to those of recombinant UGT1A10: the reactions exhibited slightly sigmoidal kinetics and the K_A values were of the same order of magnitude (Fig. 16, Table 13). Human liver microsomes (HLM) glucuronidated dopamine at such a low rate and affinity that the enzyme kinetic parameters for the reaction could not be determined. The outcome of the studies with the human liver and intestinal microsomes, for its part, shows that the assumption that UGT1A10 is the only UGT that catalyses the reaction at a significant rate is correct, because the expression of UGT1A10 in the intestine is much higher than its expression in

the liver (III; Mojarrabi and Mackenzie, 1998; Cheng *et al.*, 1999). The slight difference between the K_A values of HIM and recombinant UGT1A10 may be explained by the fatty acids that are presumably more abundant in intestinal microsomes than in the membrane preparations of the insect cells. At least UGT1A9 and UGT2B7 are inhibited by fatty acids (Rowland *et al.*, 2008), but to date it is not known whether fatty acids inhibit UGT1A10 or not.

Table 13. *The enzyme kinetic parameters for dopamine glucuronidation by UGT1A10 and human intestinal microsomes.*

	Dopamine-3-*O*-glucuronide			Dopamine-4-*O*-glucuronide		
	K_A (μM)	V_{max} (pmol/min/mg)	n	K_A (μM)	V_{max} (pmol/min/mg)	n
UGT1A10	1950 ± 171	116 ± 4.89	1.52	2190 ± 205	140 ± 6.50	1.49
HIM	2870 ± 267	25.3 ± 1.13	1.26	3410 ± 398	28.1 ± 1.56	1.17

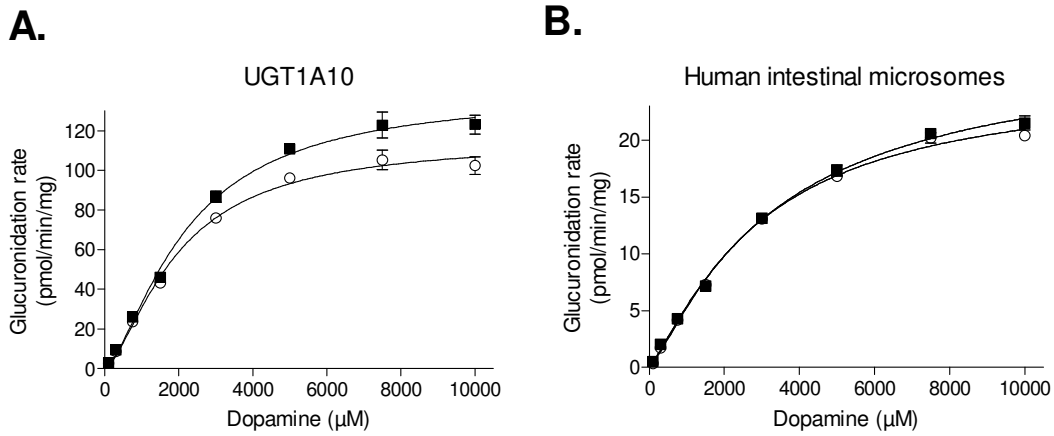


Figure 16. *Glucuronidation of dopamine by UGT1A10 (A) and human intestinal microsomes (B). The samples were incubated in triplicate and the mean values are shown with SEM. ○ Dopamine-3-O-glucuronide; ■ Dopamine-4-O-glucuronide*

UGT1A7, UGT1A8, and UGT1A9 are the isoforms that are the most homologous to UGT1A10. Although their activity was very low for the both hydroxyls of dopamine, these three UGTs strongly favored the 3-OH over the 4-OH, unlike UGT1A10. The regioselectivities of these UGTs in the case of dopamine were in agreement with previous findings for the two catecholic hydroxyls of dobutamine (Alonen *et al.*, 2005). It is interesting to note that there are only 16 amino acid residues in UGT1A10 that differ from the residues at the corresponding positions in UGT1A7, UGT1A8, or UGT1A9 (Fig. 4). Hence, during the later work for publication IV, dopamine was used in preliminary studies in an attempt to find the amino acids that are responsible for the differences in substrate selectivity between UGT1A9 and UGT1A10. Dopamine was also used to study the significance of phenylalanines 90 and 93 in UGT1A10 (III).

In conclusion, despite the low affinity, dopamine seems to be a useful probe substrate for UGT1A10, because it largely follows the two criteria for a good probe compound outlined by Court (2005): it is selective for one isoform and it exhibits similar affinity to the individual enzyme and to the microsomes from human intestine, where UGT1A10 is mainly expressed.

5.2.3 Glucuronidation by rat UGTs 2B1, 2B2, and 2B3 (unpublished results)

Dopamine glucuronide was recently found in rat brain (Uutela *et al.*, 2009). In rat, the glucuronidation is a more important metabolic pathway for dopamine than in human (Wang *et al.*, 1983), most likely because a sulfotransferase corresponding to human SULT1A3 does not exist in rodents (Eisenhofer *et al.*, 1999; Honma *et al.*, 2001). To date, it is not known which of the UGT isoforms are responsible for dopamine glucuronidation in rat. To begin, three rat UGTs, UGT2B1, UGT2B2, and UGT2B3, were expressed as recombinant proteins in insect cells and their activity toward dopamine was studied. The samples were incubated in duplicate for 60 minutes in the presence of 1 mM dopamine. The protein concentrations were 0.6-0.8 mg/ml. Under these conditions, UGT2B2 did not show any detectable activity, but the two other rat UGTs, UGT2B1 and UGT2B3, exhibited low activity toward dopamine (Fig. 17).

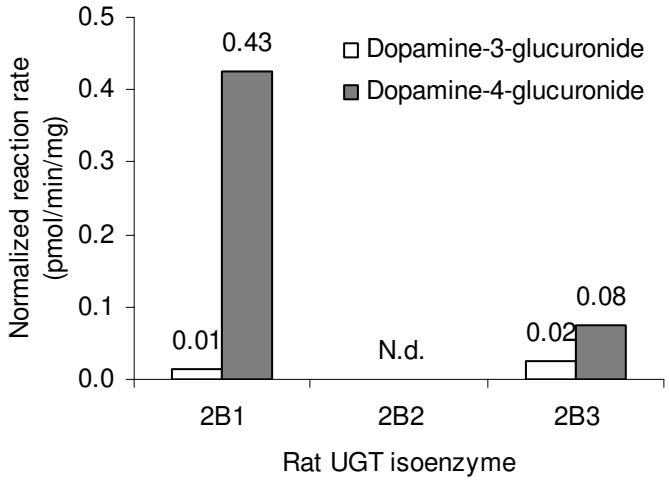


Figure 17. *Dopamine glucuronidation by three rat recombinant UGTs. The mean values for duplicate samples are shown. The samples were incubated in the presence of 1mM dopamine and UDPGA for 60 min. N.d. means that no activity was detected.*

Interestingly, in contrast to human UGT2Bs, the reaction was highly regioselective for the 4-OH of dopamine with both rat UGTs, especially with UGT2B1. This observation is in line with the previous study by Uutela *et al*. (2009), where dopamine-4-glucuronide was the major product in incubations with rat liver microsomes. Although UGT2B1, UGT2B2, and UGT2B3 are mainly expressed in the liver, low levels of mRNA were also found in

the rat brain (Shelby *et al.*, 2003), thus they may contribute to the glucuronide formation there as well. In addition to these three, there are many other UGTs expressed in the rat brain, UGT1A1 being the most abundant (Shelby *et al.*, 2003).

In the future, it will be interesting to examine the dopamine glucuronidation activity of the other rat UGTs in order to find out which isoforms are mainly responsible for dopamine glucuronidation in different tissues in rat. Rat is one of the most important laboratory animals in drug development, which makes it especially relevant to study the differences between rat and human UGTs.

5.3 The roles of phenylalanines 90 and 93 in dopamine glucuronidation by UGT1A10

Recent studies have shown that two phenylalanine residues in UGT1A10, Phe90 and Phe93, may be directly involved in substrate binding (Xiong *et al.*, 2006). The effect of mutating these Phe residues into Ala (Xiong *et al.*, 2006; Miller *et al.*, 2008) or Ala and Leu (Starlard-Davenport *et al.*, 2007) on several enzymatic activities have been examined previously. The results were quite complex, revealing effects on either the V_{max} or the K_m, depending on the substrate. In this work, the effect on dopamine glucuronidation was studied.

5.3.1 Activity screen of twelve F90 and F93 mutants (III and unpublished results)

Phe90 and Phe93 residues were separately replaced by 6 residues of varying sizes and hydrophobicities. The smallest residue was glycine, followed by alanine, valine, leucine, isoleucine, and tyrosine. The 12 resulting mutants were expressed in baculovirus-infected insect cells and their activities toward dopamine were determined using one protein concentration and 5 mM dopamine. The activities were normalized for the relative expression levels of the mutants and the results were compared to control UGT1A10.

The hypothesis was that Tyr would not affect the activity much because its structure is very similar to phenylalanine. They are both aromatic amino acids and their only difference is the phenolic hydroxyl found in tyrosine but not in phenylalanine. As expected, the most active mutant in this set was 1A10F93Y whose activity was 35 – 47% of wild-type UGT1A10 (Fig. 18). Less expectedly, 1A10F90Y turned out to be one of the least active enzymes in this study. All mutations lowered the activity toward both hydroxyls. In general, F90 mutations affected the normalized activity more than the F93 mutations, but mutant F90L had higher activity than most of the F93 mutants (Fig. 18).

Based on these results, it is not possible to tell whether the mutations affect the binding of dopamine to the active site. Enzyme kinetic studies were therefore conducted with four of the mutants.

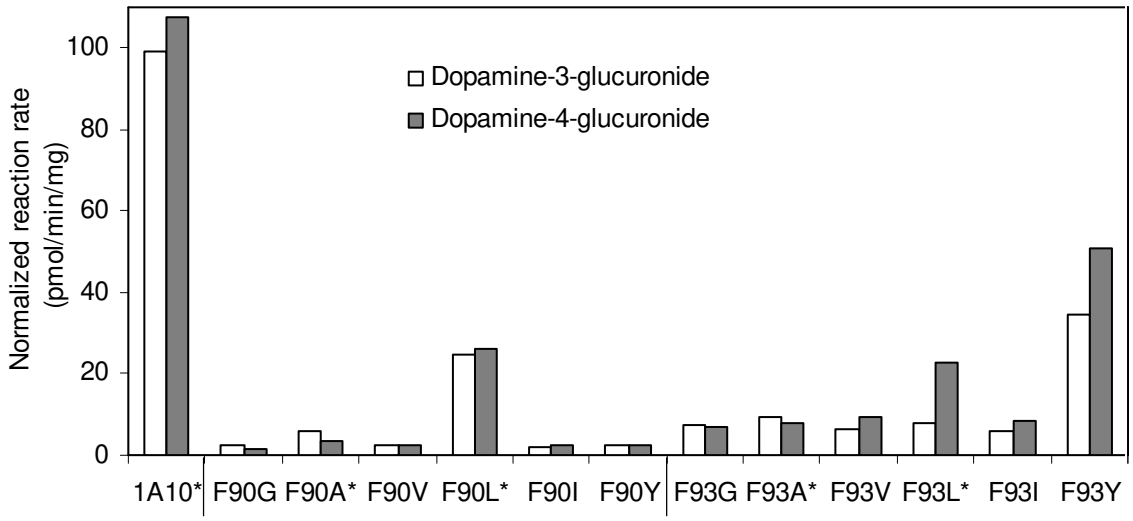


Figure 18. *The effect of the mutations in F90 and F93 on the glucuronidation activity of UGT1A10 toward dopamine. The results that have been published previously in publication III are marked with asterisks. The mean values of duplicate samples are shown.*

5.3.2 Enzyme kinetic studies of four F90 and F93 mutants (III)

The enzyme kinetics of dopamine glucuronidation by four previously described UGT1A10 mutants, 10F90A, 10F90L, 10F93A, and 10F93L (Xiong *et al.*, 2006; Starlard-Davenport *et al.*, 2007), were studied in order to find out if the lower activity observed in the screening assays was due to alterations in affinity or in the maximal reaction rate. Dopamine glucuronidation by UGT1A10 was best described by the Hill equation (Eq. 5 in Table 6) and the same type of sigmoidal kinetics was exhibited by the two mutants of phenylalanine 90, whereas mutants 10F93A and 10F93L exhibited Michaelis-Menten kinetics (Fig. 19). All the four mutations lowered the normalized V_{max} of the reaction without significantly affecting K_m or K_A values. Substitution of either Phe90 or Phe93 with alanine, as in 10F90A and 10F93A, lowered the activity toward the 4-OH of dopamine much more than replacement of either of these two phenylalanine residues with leucine. A possible explanation for this is that the latter mutations are more conservative when it comes to the hydrophobicity and size of the side chain.

The two F to L mutants, 10F90L and 10F93L, differed sharply from each other with respect to dopamine glucuronidation at the 3-OH. The regioselectivity of the 10F90L mutant was similar to that of the wild type (i.e. no selectivity), whereas mutant 10F93L exhibited very different regioselectivity, glucuronidating dopamine at the 4-OH approximately 3 times faster than at the 3-OH.

In conclusion, the results obtained in this study do not suggest that F90 or F93 are necessarily involved in direct interactions with dopamine in the active site of UGT1A10 because the mutations did not affect the K_m. There is an apparent disagreement between these results and those of previous studies that suggested that these phenylalanines are

located within the binding site of estrogens and other phenolic substrates (Xiong *et al.*, 2006; Starlard-Davenport *et al.*, 2007). However, the explanation may be that the phenylalanines are located too far away from the catalytic His for the dopamine to reach when it sits in the active site. Unlike estrogens, dopamine is a small molecule with a flexible ethylamine side chain.

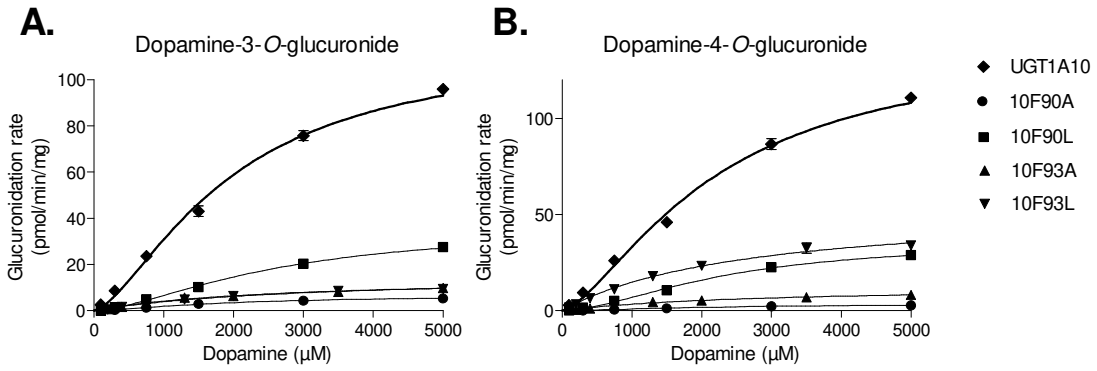


Figure 19. *Formation of dopamine-3-O-glucuronide (A) and dopamine-4-O-glucuronide (B) by UGT1A10 and its mutants 10F90A, 10F90L, 10F93A, and 10F93L. The reaction rates are normalized with respect to protein expression levels in each sample.*

5.4 Regio- and stereoselectivity in estradiol glucuronidation (II)

In publication II, the regio- and stereoselectivities of human UGTs were studied using β-estradiol and epiestradiol as substrates. The focus of this work was mainly on structure-activity relationships of the human UGTs, and these two substrates were selected for this study because both of them can be glucuronidated at two different positions, providing an interesting system for examining 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.

5.4.1 Estradiol glucuronidation by human recombinant UGTs

Initially, 19 human UGTs were screened for activity toward β-estradiol and epiestradiol. The UGTs that exhibited undetectable or barely detectable activity under the conditions used in this study were UGT1A5, UGT1A6, UGT1A9, UGT2A3, UGT2B10, UGT2B11, and UGT2B28. Next, the UGTs that catalyzed the glucuronidation of at least one of the estradiol diastereoisomers were subjected to kinetic analyses. Detailed enzyme kinetic data are presented in original publication II.

The general picture that emerges from this study is a distinct regioselectivity between the three subfamilies of the human UGTs. Regio- and stereoselectivity was more prominent in the UGT2B subfamily than in UGT1A or UGT2A subfamilies (Table 14). UGT1As were in general more active on 3-OH whereas UGT2Bs were mainly active on 17-OH. The human UGTs of subfamily UGT2A that exhibited estradiol glucuronidation activity, UGT2A1 and UGT2A2, seem to have more promiscuous binding sites because they can glucuronidate these steroids at both positions. These patterns of estradiol glucuronidation by members of the different UGT subfamilies are not absolute, however, and the two exceptions to the rule were UGT1A4 and UGT2B15. Unlike most UGTs of the UGT1A family, UGT1A4 glucuronidated the two diastereoisomers solely at the 17-OH position, but only at low rates. UGT2B15 was the only member of subfamily UGT2B that conjugated the two estradiols at the 3-OH rather than the 17-OH position (Table 14).

The most active isoforms in this study were UGT1A10 and UGT2B7. They had completely opposite regioselectivity, as UGT1A10 was more active on 3-OH and UGT2B7 was only active on 17-OH of both enantiomers. The activity of UGT1A10 toward epiestradiol was very high, as was its activity toward the 3-OH group of β-estradiol. In the latter case, a combination of low K_s and high V_{max} value led to an exceptionally high Cl_{int} value. UGT2B7, in turn, revealed exceptionally high affinity for epiestradiol.

Table 14. *Regio- and stereoselectivity of human UGTs in estradiol glucuronidation. A minus sign means that glucuronide formation, if any, was below the detection limit, whereas each additional "+" indicates an approximate 10-fold increase in Cl_{int}. UGTs 1A5, 1A6, 1A9, 2A3, 2B10, 2B11, and 2B28 had very low or no activity with both substrates and were not included in the enzyme kinetic studies.*

UGT	Epiestradiol-3-glucuronide	β-Estradiol-3-glucuronide	Epiestradiol-17-glucuronide	β-Estradiol-17-glucuronide
1A1	++	++	-	-
1A3	++	+	(+)	+
1A4	-	-	+	+
1A7	+	-	-	-
1A8	++	+	-	(+)
1A10	+++	++++	-	++
2A1	++	+	+	+
2A2	+	+	+	(+)
2B4	-	-	++	-
2B7	-	-	++++	+++
2B15	++	(+)	-	-
2B17	-	-	-	++

The rules of stereoselectivity among the different UGTs are even more complex than those of regioselectivity 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, and UGT2B7 exhibited a clear preference, but not strict specificity, for epiestradiol (Table 14).

When only glucuronidation at the 17-OH position is considered, the results are in line with the results of Sten *et al.* (2009) on the glucuronidation of testosterone and epitestosterone. In these steroids, 17-OH is the only hydroxyl group (Fig. 20). Therefore, consistent with the results obtained with estradiols, UGT1As were not highly active on testosterone or epitestosterone. The stereoselectivities of all UGTs toward testosterones were similar to those towards estradiols. UGT2B17 even had very similar K_m values for β-estradiol and testosterone. UGT2B7, on the other hand, had much lower activity for testosterone than for β-estradiol and its K_m for testosterone was not determined, but its K_m values for both epiestradiol and epitestosterone were very low. Thus, it can be concluded that the K_m values of UGT2B7 and UGT2B17 for these steroids are not affected by the configurations of the A and B rings.

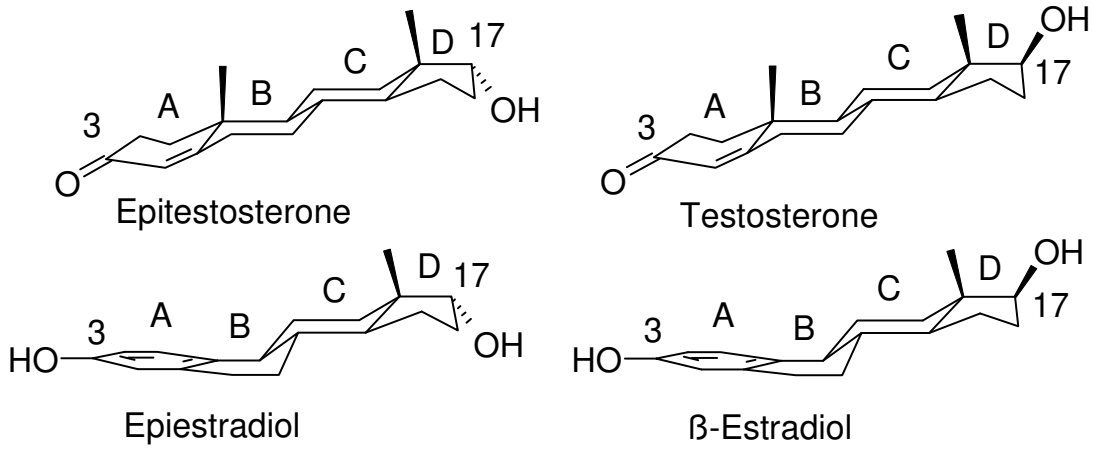


Figure 20. *The structures of estradiol and testosterone diastereomers.*

In study II, UGT1A10 was the most active human enzyme in glucuronidation of β-estradiol and epiestradiol at the 3-OH position. The estradiol glucuronidation activity of UGT1A10 was reported to be low in a previous study (Lepine *et al.*, 2004), perhaps because of the use of a low activity batch of recombinant UGT1A10. In another study (Cheng *et al.*, 1999), estradiol was not included, but the activity of UGT1A10 toward many other substrates including estrogens was studied and found to be lower than the activity of UGT1A8. For example, activity toward propranolol or 1-naphthol was not detected. On the other hand, Strassburg *et al.* (1998a) reported that UGT1A10 had higher activity than UGT1A8 toward a range of substrates including β-estradiol and 1-naphthol. Also, Basu *et al.* (2004) observed high activity toward various substrates including β-estradiol. Therefore, we took further steps to ensure that our findings were correct. Both UGT1A8 and UGT1A10 were expressed in human embryonic kidney cells (HEK293) without any C-terminal fusion peptide as described previously (Uchaipichat *et al.*, 2004) and their estradiol glucuronidation activities were examined in the collaboratory

laboratory. The results were broadly consistent with the results obtained with the "his-tagged" UGTs expressed in the insect cells. In particular, the normalized V_{max} value for UGT1A10 was 2 orders of magnitude higher than that of UGT1A8 (data not shown).

Some studies have suggested that UGT1A10 activity depends on phosphorylation (Basu *et al.*, 2004; 2005). Protein kinases that are involved in the phosphorylation may not be present or as active in insect cells as in human cells. However, this does not explain the differences in UGT1A10 activity toward β-estradiol in this study and the previous study of Lepine *et al.* (2004), because insect cells were used in both studies. On the other hand, Dellinger *et al.* (2007) have suggested that in HEK293 cells, the majority of the UGT1A10 is not localized in the microsomal fraction, which is most often used in glucuronidation studies. That might explain the low activity observed by Cheng *et al.* (1999). Incubation conditions, such as pH and use of organic solvents or detergents, may also have a great impact on the results, but clearly further studies are required to fully elucidate the reasons behind the large variability in the activity of UGT1A10 found by different research groups around the world.

There are large differences in estradiol glucuronidation activities between the human UGT1A7 through UGT1A10, despite the high degree of sequence similarity among them (Fig. 4). UGT1A10 was very active toward the 3-OH of epiestradiol and even more active toward the 3-OH of β-estradiol (Table 14). UGT1A7 and UGT1A8 showed higher activity toward 3-OH of epiestradiol than that of β-estradiol. In the case of UGT1A9, the activity toward the 3-OH of epiestradiol was barely detectable at 100 μM substrate concentration, but toward β-estradiol, it was below the detection limit (data not shown).

Taken together, our results show that many UGTs are capable of estradiol glucuronidation, but they catalyze these reactions with varying regioselectivities and stereoselectivities 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.

5.4.2 Competitive inhibition of human UGT1A9 by β-estradiol

To determine whether UGT1A9 binds either estradiol diastereomer, we examined the inhibition of scopoletin glucuronidation activity of UGT1A9 by the two estradiols. The results confirmed that β-estradiol can substantially inhibit the activity of UGT1A9 as reported previously (Mano *et al.*, 2004). In contrast, the inhibitory effect of epiestradiol on this activity was limited.

To further clarify the mode of inhibition by β-estradiol, the apparent K_m values for scopoletin glucuronidation in the absence and presence of three concentrations of the inhibitor were determined. To determine what type of inhibitor β-estradiol was toward UGT1A9, the method recommended by Copeland (2000) was used. Briefly, the untransformed data from incubations in the absence and presence of three concentrations of β-estradiol were fitted separately to the Michaelis-Menten equation, and the apparent K_m and V_{max} values for scopoletin were determined. These values were then substituted into the reciprocal transformation of the Michaelis-Menten equation and thus four straight

lines were obtained. In the case of competitive inhibition the intersection of these lines is located on the y-axis, as observed in this case (Fig. 6C in publication II). Other types of inhibition, noncompetitive and uncompetitive, would not produce a similar intersection, but in the case of noncompetitive inhibition the intersection would be on the left side of the y-axis, whereas in the case of uncompetitive inhibition the lines would not intersect at all. The K_i value for β-estradiol was determined by fitting the untransformed data to Eq. 6 (Table 6). The outcome of this experiment 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.

In summary, although UGT1A9 does not glucuronidate β-estradiol or epiestradiol, it binds β-estradiol with nearly the same affinity as UGT1A10. Hence, no activity does not always mean no binding. Because the dissimilarity between UGT1A9 and UGT1A10 was so obvious, β-estradiol and epiestradiol were used as model substrates when the selectivity differences between these two UGTs were studied in more detail in the later publication (IV).

5.4.3 Estradiol glucuronidation by human liver and intestinal microsomes

The results obtained with recombinant human UGTs were further verified by incubations with human liver and intestinal microsomes. Of those UGTs that were active toward estradiol, UGT1A10 and UGT1A8 are expressed in the intestine but not (or at very low level) in the liver, whereas UGT1A1, UGT1A3, UGT1A4, and UGT2Bs are mainly expressed in the liver (Table 1), UGT2B4, UGT2B7, UGT2B10, and UGT2B15 being the most abundant isoforms (Izukawa *et al.*, 2009; Ohno and Nakajin, 2009). Accordingly, β-estradiol-3-glucuronide, the preferred product of UGT1A10, was also the main product in human intestinal microsomes (Fig. 21). Epiestradiol-17-glucuronide, mainly produced by UGT2B7 and UGT2B4, was the most abundant product in liver microsomes (Fig. 21). These results suggest that the recombinant proteins, which are expressed with an additional polyhistidine tail (His-tag), are good tools for predicting glucuronidation activities also in the native enzymes.

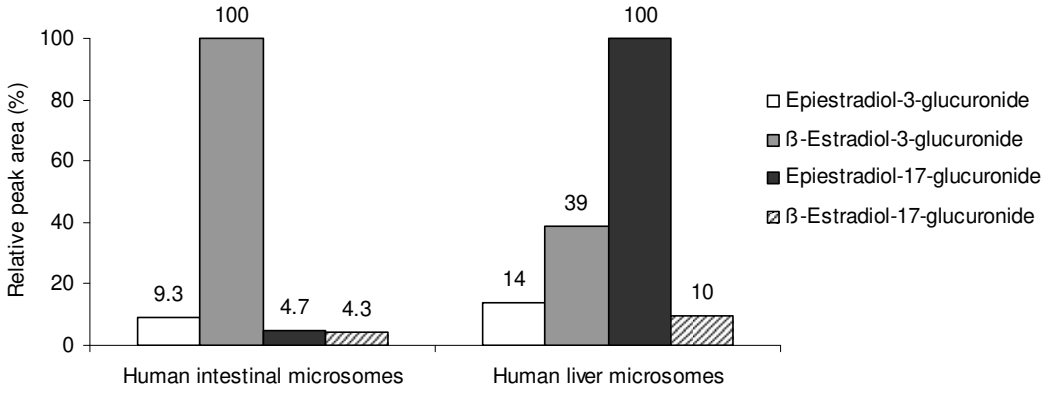


Figure 21. *Glucuronidation of estradiols by human liver and intestinal microsomes.*

5.4.4 Interspecies differences in estradiol glucuronidation

Interspecies differences in estradiol glucuronidation were studied with three recombinant rat UGTs and liver microsomes from various species. Glucuronidation of β-estradiol and epiestradiol by three rat UGTs from the UGT2B subfamily, UGT2B1, UGT2B2, and UGT2B3, were studied. The results showed that UGT2B2 was not active toward either estradiol diastereomer, UGT2B1 only glucuronidated β-estradiol, and UGT2B3 glucuronidated both diastereomers but only at the 17-OH position (Table 15). The regioselectivity and stereoselectivity of UGT2B1 were similar to those of the human UGT2B17, with activity nearly restricted to the 17-OH of β-estradiol. Interestingly, UGT2B3 was highly and similarly active toward the 17-OH of both diastereomers, a clear exception among all the tested UGTs.

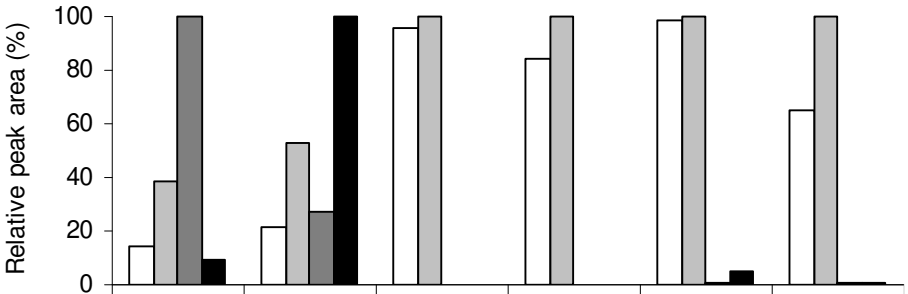
It is difficult to identify UGT2B orthologues across species, because most of the members in the mammalian UGT2B subfamily are at least 70% similar in sequence (Mackenzie *et al.*, 1997; 2005). Based on the results with estradiols and dopamine, it can be concluded that the rat UGTs 2B1 and 2B3 are unique and different from human UGT2Bs at least when it comes to regio- and stereoselectivity toward these substrates.

Table 15. *Regio- and stereoselectivity of rat UGTs in estradiol glucuronidation. A minus sign means that glucuronide formation, if any, was below the detection limit, whereas each additional "+" indicates an approximate 10-fold increase in* Cl_{int}.

UGT	Epiestradiol-3-glucuronide	β-Estradiol-3-glucuronide	Epiestradiol-17-glucuronide	β-Estradiol-17-glucuronide
2B1	-	(+)	-	+++
2B2	-	-	-	-
2B3	-	-	+++	+++

.

In addition, liver microsomes from several different animal species were examined. Rat liver microsomes were from animals that had been treated with Aroclor 1254 (a mixture of polychlorinated biphenyls) in order to induce the expression of UGTs. UGTs 1A1, 1A6, 1A7, 2B1, and 2B12 are more induced by polychlorinated biphenyls than the other UGTs in rat liver (Shelby and Klaassen, 2006). Both human and rat liver microsomes exhibited higher glucuronidation activity at the 17-OH position than at the 3-OH position (Fig. 22). Rat liver microsomes exhibited a clear preference for β-estradiol. However, the estradiol glucuronidation activity of microsomes from rabbit, pig, bovine, and moose was high, and glucuronidation occurred mainly at the 3-OH position. The results are in line with a previous report (Falany *et al.*, 1983) where only the β-estradiol glucuronidation activities of rat and rabbit liver microsomes were studied. To summarize, this study showed that the differences in glucuronidation between animal species are large and they should be taken into account when using animal models to predict drug metabolism in human.



	Human	Rat	Rabbit	Pig	Bovine	Moose
□ Epiestradiol-3-glucuronide	14	21	96	85	98	65
▒ ß-Estradiol-3-glucuronide	39	53	100	100	100	100
▒ Epiestradiol-17-glucuronide	100	27	0.16	0.34	0.67	1.1
■ ß-Estradiol-17-glucuronide	10	100	0	0.10	5.1	0.82

Figure 22. *Estradiol glucuronidation in human and animal liver microsomes.*

5.5 Key amino acid residues involved in functional differences between UGT1A9 and UGT1A10 (IV)

The aim of publication IV was to find the location of the residues that play a central role in determining the activity differences between UGT1A9 and UGT1A10. The activities of the different UGT1A9-UGT1A10 (910) chimeras and other mutants, alongside the parent enzymes, were examined using compounds whose glucuronidation varied considerably between UGT1A9 and UGT1A10. R- and S-propranolol, β-estradiol, epiestradiol, and dobutamine (Fig. 13) were selected for this large screen because earlier studies showed differences in their stereoselectivity or regioselectivity (II; Alonen *et al.*, 2005; Sten *et al.*, 2006). Dopamine was used in preliminary studies but it was replaced by dobutamine because the latter was a better substrate for both UGTs studied. 1-Naphthol was chosen as a substrate for the enzyme kinetic studies because it is a substrate for both UGT1A9 and UGT1A10 and there is a relatively large difference between their K_m values as well as two orders of magnitude difference in their V_{max} values.

In the beginning, a minimalist assumption was made that a single amino acid difference between UGT1A9 and UGT1A10 could lead to the activity differences between them. The chimera approach was adopted to eliminate most of the 33 differing residues in the first round of mutagenesis and then to focus on the central residues. Different mutations were designed to confer UGT1A10-like activity on UGT1A9, since it would have more evidential value than the opposite arrangement in which UGT1A10 would lose its properties as a result of mutation. Although this study focused on the residues that differ between UGT1A9 and UGT1A10, UGT1A7 and UGT1A8 were also taken into account when trying to locate the most important residues that affect the substrate selectivity.

5.5.1 UGT1A9-UGT1A10 chimeras

The N-terminal domain encoded by exon 1 was divided into 5 parts and they were named A, B, C, D, and E (Table 16). Five single-segment chimeras were constructed. In these chimeras, each of the five N-terminal segments of UGT1A9 was separately replaced by the corresponding segment from UGT1A10. The chimeric mutants generated in this way were named 910 (combination of UGT1A9 and UGT1A10) and a letter (A–E), denoting the segment originating from UGT1A10.

The activities of the chimeras toward estradiols, propranolols, and dobutamine were screened. None of the single-segment chimeras greatly resembled UGT1A10 in the activity studies (Table 17), strongly suggesting that the functional differences between UGT1A9 and UGT1A10 could not be explained by a single amino acid difference. The results implied that segments A and E and even segment D with 10 variable residues (Fig. 4, Table 16) do not contribute significantly to the substrate selectivity differences between the two UGTs. 910B and 910C, on the other hand, showed qualitative changes in regio- and stereoselectivity patterns toward UGT1A10, but the reaction rates were only a fraction of that exhibited by UGT1A10 so the observation was inconclusive.

Table 16. *Mutations in segments A-E in UGT1A9-UGT1A10 chimeras. In segment B, the five point-mutations that were separately studied after the chimeras are shown in bold text.*

Segment	Amino acids	Mutations
A	1-83	(C3R, T4A, and L10V in signal peptide), R42Q, G67E
B	84-147	L86Q, **D87N**, K91M, A92V, **V102A**, **R103Q**, Y106F, G111S, Y113S, **N114S**, D115G, I116F, **F117L**, N123H, K129N, K131R
C	148-181	N152T, A169T, L173F, Y176H
D	182-235	R192N, I193D, R208W, M212V, E216D, L219F, H221Q, R222Y, F223L, K225R
E	236-285	E241A

Four double-segment chimeras were then constructed and their activities were analyzed. In these chimeras, two of the five segments of UGT1A9 were replaced by the corresponding segments from UGT1A10 and they were named 910AB, 910BC, 910CD, and 910BD. Construction of these double-segment chimeras was undertaken because it was reasoned that if the minimal number of crucial residues for the differences between UGT1A9 and UGT1A10 is two and if they are located within different segments, then a double-segment chimera may contain the correct combination and will yield a UGT1A10-like activity. Segment E was not included in the double-segment chimeras because the single amino acid difference between UGT1A9 and UGT1A10 in that segment had no significant effect on the enzymatic activity. Of double-segment chimeras, 910BC and 910BD were the most similar to UGT1A10 when considering regio- and stereoselectivity and the results were quite promising, although their activities were not nearly as high as that of UGT1A10 (Table 17).

Table 17. *Regio- and stereoselectivity of UGT1A9, UGT1A10, and 910 chimeras.*

	S-Propranolol-glucuronide	R-Propranolol-glucuronide	β-Estradiol-3-glucuronide	Epi-estradiol-3-glucuronide	Dobutamine-*para*-glucuronide	Dobutamine-*meta*-glucuronide
	Peak area relative to protein concentration and UGT expression level*					
1A9	3300	470	10	76	69	440
1A10	2000	8900	86000	18000	16000	5300
910A	1300	190	-	31	26	190
910B	-	240	280	77	69	54
910C	-	170	480	190	510	1800
910D	870	180	36	120	15	56
910E	3000	480	51	81	51	480
910AB	-	88	73	-	36	28
910BC	280	810	3600	1200	840	510
910BD	460	1000	6500	280	300	110
910CD	150	270	320	1300	89	460

*The mean values of duplicate or triplicate samples are shown.
- No detectable activity

Table 18. *Enzyme kinetic parameters for the glucuronidation of 1-naphthol by UGT1A9, UGT1A10, and the 910 chimeras. The V_{max} values are normalized with respect to the relative expression level of the enzyme in each sample.*

Enzyme/Mutant and Kinetic Model	K_{m1} μM	V_{max1} pmol/min/mg	K_{m2} μM	V_{max2} pmol/min/mg	[S] Range μM
1A9[B]	0.04 ± 0.01	12.5 ± 0.4	15.7 ± 3.9	17.4 ± 1.6	0.1-25
1A10[MM]	4.9 ± 0.2	1970 ± 35	-	-	0.1–25
910A[B]	0.10 ± 0.02	13.0 ± 1.0	4.5 ± 3.2	4.8 ± 0.8	0.1-25
910B[MM]	6.3 ± 0.4	7.7 ± 0.1	-	-	1–250
910C[MM]	1.0 ± 0.05	914 ± 10	-	-	0.1–25
910D[MM]	11.8 ± 0.6	217 ± 5.4	-	-	0.1–25
910E[B]	0.13 ± 0.02	10.9 ± 0.6	27.0 ± 12.3	19.6 ± 4.1	0.1-25
910BC[MM]	7.1 ± 0.4	131 ± 3.0	-	-	0.1–25
910BD[MM]	7.1 ± 0.3	146 ± 1.8	-	-	0.5–100
910CD[MM]	17.9 ± 1.0	1950 ± 36	-	-	0.5–100

[MM]Michaelis-Menten; [B]Biphasic

Because the activity screenings were performed in the presence of a single concentration for each substrate and enzyme, enzyme kinetic studies were conducted to learn more about the substrate affinity and maximal turnover rate of each mutant. To keep the study a reasonable size, one substrate, 1-naphthol, was selected for these analyses.

The 1-naphthol glucuronidation analyses of the single- and double-segment chimeras revealed that the activity (low), affinity (high), and kinetic model (biphasic) of 910A and 910E were similar to those of the parent UGT1A9 (Table 18). On the other hand, 910D and 910CD exhibited much higher K_m values than UGT1A9, or even UGT1A10, whereas 910C had intermediate K_m and V_{max} values. The highest 1-naphthol glucuronidation rate of the chimeras was observed with 910CD and the lowest with 910B. The K_m most similar to that of UGT1A10 was observed with 910B, even though the V_{max} was very low (Table 18). In addition, two double-segment chimeras that contained segment B, 910BC and 910BD, exhibited K_m values close to that of UGT1A10, and their activity rates were higher than that of UGT1A9 (Table 18). The kinetic parameters for 910AB were not determined because of its very low activity toward 1-naphthol.

In summary, the results obtained with chimeras showed that those in which segment B was replaced by that from UGT1A10 were the most similar to UGT1A10 when considering regio- and stereoselectivity together with affinity for 1-naphthol (Tables 17 and 18). In addition, segment B includes the residues and regions that were previously reported to play a major role in substrate binding (Dubois *et al.*, 1999; Lewis *et al.*, 2007; Nishiyama *et al.*, 2008); hence, it was subjected to further studies.

5.5.2 Point mutations within residues 84-147 of UGT1A9

Assuming that the most important residues are in segment B, five point mutations were made in that segment and their effects on the glucuronidation activity were studied. Within segment B there are 16 residues that differ between UGT1A9 and UGT1A10, but only 5 of them are unique to UGT1A10 compared to UGT1A7, 1A8, and 1A9 (Fig. 4). We concentrated on these 5 unique residues, because UGT1A7 and UGT1A8 resembled UGT1A9 rather than UGT1A10 in dobutamine and dopamine glucuronidation (III; Alonen *et al.*, 2005) and to a large extent also in stereoselectivity in estradiol glucuronidation (II). The point mutants were named 9D87N, 9V102A, 9R103Q, 9N114S, and 9F117L.

Four of the mutations, namely D87N, V102A, R103Q, and N114S, did not change the regio- and stereoselectivity of UGT1A9 (Table 19). Their normalized activities were also very close to those of UGT1A9 with all three substrates and they did not affect either the affinity or the activity of UGT1A9 toward 1-naphthol (Table 20). The only substantial difference was in the V_{max1} of 9N114S, which was about twice that of UGT1A9.

The effect of the F117L mutation varied between the substrates (Table 19 and 20). With dobutamine, the regioselectivity was changed toward that of UGT1A10 but the activity was very low. With propranolol, the ratio of the reaction rates was not inverted, although the glucuronidation rate of S-propranolol was reduced more than that of R-propranolol when compared to rates with UGT1A9. With estradiol there was no change in stereoselectivity or activity. With 1-naphthol, on the other hand, 9F117L had a K_m very close to that of UGT1A10 and its V_{max} was also approximately ten times higher than that of UGT1A9.

Table 19. *Regio- and stereoselectivity of the point mutants and the triple mutant in segment B.*

	S-Propranolol-glucuronide	R-Propranolol-glucuronide	β-Estradiol-3-glucuronide	Epi-estradiol-3-glucuronide	Dobutamine-*para*-glucuronide	Dobutamine-*meta*-glucuronide
	Peak area relative to protein concentration and UGT expression level*					
1A9	3300	470	10	76	69	440
1A10	2000	8900	86000	18000	16000	5300
9D87N	6100	900	21	180	100	620
9V102A	3200	530	15	89	160	520
9R103Q	3500	450	11	75	62	470
9N114S	2900	430	28	170	120	430
9F117L	420	210	11	36	87	56
$910B_{(115-117)}$	18000	1100	100	280	1700	1700

*The mean values of duplicate or triplicate samples are shown.

Table 20. *Enzyme kinetic parameters for the glucuronidation of 1-naphthol by UGT1A9, UGT1A10, the point mutants, and the triple mutant in segment B.*

Mutant and Kinetic Model	K_{m1} or K_s μM	V_{max1} pmol/min/mg	K_{m2} μM	V_{max2} pmol/min/mg	K_i μM	[S] Range μM
$1A9^{B}$	0.04 ± 0.01	12.5 ± 0.4	15.7 ± 3.9	17.4 ± 1.6	-	0.1-25
$1A10^{MM}$	4.9 ± 0.2	1970 ± 35	-	-	-	0.1–25
$9D87N^{B}$	0.09 ± 0.01	12.3 ± 0.3	41.9 ± 9.4	38.4 ± 5.1	-	0.1-25
$9V102A^{B}$	0.11 ± 0.01	8.7 ± 0.2	26.7 ± 3.4	24.8 ± 1.5	-	0.1-25
$9R103Q^{B}$	0.15 ± 0.01	14.8 ± 0.5	32.4 ± 16	16.9 ± 4.3	-	0.1-25
$9N114S^{B}$	0.09 ± 0.02	25.5 ± 1.8	30.0 ± 3.2	11.5 ± 4.6	-	0.1-25
$9F117L^{SI}$	3.4 ± 0.2	107 ± 4.0	-	-	81.4 ± 14	0.1–25
$910B_{(115–117)}{}^{SI}$	2.3 ± 0.2	436 ± 10	-	-	167 ± 16	1–100

^{B}Biphasic; ^{MM}Michaelis-Menten; ^{SI}Substrate inhibition

Finally, we made one more construct, $910B_{(115-117)}$, with three mutated amino acids: D115G, I116F, and F117L. This mutant was more active than 9F117L with all substrates studied. Like 9F117L, it did not change the stereoselectivity with estradiol or propranolol but this mutant had different regioselectivity than UGT1A9 with dobutamine (Table 19). With dobutamine, the activity toward both catecholic hydroxyls was higher than that of UGT1A9 but the mutant did not show any preference for either one of them. With 1-naphthol, $910B_{(115-117)}$ showed even higher activity than 9F117L, although not as high as 910C or UGT1A10, and the K_m was close to that of UGT1A10. The effect of this triple mutant on the K_m for 1-naphthol (Table 20) was probably due to the F117L mutation alone, suggesting that Asp115 and Ile116 of UGT1A9 do not directly interact with the

substrate. In the molecular model of UGT1As (Laakkonen and Finel, 2010), this segment is predicted to be helical, which prevents three consecutive residues from pointing in the same direction.

Based on the results with the five point mutants and the triple-mutant, it was concluded that residue 117 in UGT1A9 and UGT1A10 affects the K_m of 1-naphthol and may be in direct contact with the substrate when it is bound in the active site.

5.5.3 Combinations of single-segment chimeras and point mutations

Next, 6 single-segment chimeras with additional point mutations in another segment of the protein were constructed. In four of these mutants (910B-N152T, 910B-A169T, 910B-L173F, and 910B-Y176H), segment B was similar to that of UGT1A10 and, additionally, one of the four dissimilar amino acids in segment C was mutated. Two other mutants (910C-F117L and 910D-F117L) had segment C or D from UGT1A10 in addition to the point mutation F117L in segment B.

Concerning regio- and stereoselectivity of these mutants, 910B-L173F and 910B-Y176H were similar to 910B and 910D-F117L was similar to 9F117L, suggesting that the mutations L173F and Y176H and segment D were not very important in substrate selectivity differences. Three other mutants, 910B-N152T, 910B-A169T, and 910C-F117L, exhibited similar regio- and stereoselectivity to UGT1A10, and 910B-N152T had the highest activity among them (Table 21).

All the six mutants had K_m values for 1-naphthol that were similar to, or higher than, those of UGT1A10 and 910B. 910D-F117L had the highest K_m of all the enzymes studied, and its V_{max} was also rather high, reaching 50% of that of UGT1A10. 910B-L173F and 910B-Y176H had very low activities, like 910B. 910B-A169T had somewhat higher activity but its K_m was also higher. 910B-N152T had the highest activity of all the mutants that had part B from UGT1A10 and an affinity similar to those of 910B and UGT1A10. It may thus be suggested that the residue at position 152 faces the active site. This would be in agreement with the previous findings of Fujiwara *et al.* (2009). However, 910C-F117L was the most similar to UGT1A10 when considering both affinity and activity.

Finally, assuming that both Phe117 and Asn152 in UGT1A9 affect substrate specificity and interact with the bound substrate, we prepared the double mutant 9F117L-N152T hoping that this combination would largely confer UGT1A10-like activity on UGT1A9. However, the results were disappointing because the activity of this mutant was very low in the glucuronidation of propranolols, estradiols, and dobutamine. The affinity for 1-naphthol was also very low, even lower than that of UGT1A10, although the V_{max} toward this substrate was high.

In summary, residues between Leu86 and Tyr176 of UGT1A9 appear to determine the substrate selectivity differences between UGT1A9 and UGT1A10. Within this region, residues at positions 115, 116, 117, 152, and 169 seem to contribute to the catalytic properties that were studied here. The results indicate that interactions between several amino acids, rather than the presence of a single residue in a “strategic” location within the active site, govern the complex substrate selectivity of the UGTs.

Table 21. *Regio- and stereoselectivity of the chimeras having an additional point mutation in another segment and the double mutant 9F117L-N152T.*

	S-Propranolol-glucuronide	R-Propranolol-glucuronide	β-Estradiol-3-glucuronide	Epi-estradiol-3-glucuronide	Dobutamine-*para*-glucuronide	Dobutamine-*meta*-glucuronide
	Peak area relative to protein concentration and UGT expression level*					
1A9	3300	470	10	76	69	440
1A10	2000	8900	86000	18000	16000	5300
910B-N152T	1300	3000	2500	590	800	800
910B-A169T	290	1900	880	72	160	100
910B-L173F	19	31	110	22	57	52
910B-Y176H	53	150	110	14	36	27
910C-F117L	400	620	650	390	410	140
910D-F117L	860	360	33	43	63	53
9F117L-N152T	720	500	96	28	110	140

*The mean values of duplicate or triplicate samples are shown.

Table 22. *Enzyme kinetic parameters for the glucuronidation of 1-naphthol by UGT1A9, UGT1A10, the chimeras having an additional point mutation in another segment, and the double mutant 9F117L-N152T.*

Mutant and Kinetic Model	$K_{m(1)}$ or K_s μM	$V_{max(1)}$ pmol/min/mg	K_{m2} μM	V_{max2} pmol/min/mg	K_i μM	[S] Range μM
1A9[B]	0.04 ± 0.01	12.5 ± 0.4	15.7 ± 3.9	17.4 ± 1.6	-	0.1-25
1A10[MM]	4.9 ± 0.2	1970 ± 35	-	-	-	0.1–25
910B-N152T[MM]	6.2 ± 0.4	112 ± 2.6	-	-	-	0.1–25
910B-A169T[SI]	12.8 ± 1.3	67.7 ± 3.6	-	-	240 ± 49	0.5–100
910B-L173F[SI]	2.4 ± 0.2	1.8 ± 0.06	-	-	287 ± 59	0.5–100
910B-Y176H[MM]	11.6 ± 0.5	6.6 ± 0.1	-	-	-	0.5–100
910C-F117L[MM]	2.8 ± 0.1	1090 ± 17	-	-	-	0.1–25
910D-F117L[MM]	121 ± 5.7	838 ± 19	-	-	-	1–250
9F117L-N152T[SI]	20.8 ± 1.8	1320 ± 74	-	-	111 ± 14	0.5–100

[B]Biphasic; [MM]Michaelis-Menten; [SI]Substrate inhibition

5.5.4 Molecular modeling of the active site of UGT1A9

During the course of the experimental work described above, a homology model for selected human UGTs was constructed (Laakkonen and Finel, 2010). In addition to the conserved structural elements of the N-terminal domain, there are two long and highly

variable interstrand loops, which are predicted to fold into several short helices. These variable loops correspond closely to segments B and D, as defined in this study. In accordance with the experimental results, the model showed that segments A and E are the farthest away from the active site, without any direct contacts with the bound substrate except the catalytic His37 within segment A (Miley *et al.*, 2007; Patana *et al.*, 2008). His37 of either UGT1A9 or UGT1A10 is expected to face the substrate binding site of the enzyme and be crucial for activity but not for the selectivity differences between these enzymes. In contrast, the results with the 910-mutants suggested that Phe117 may be responsible for the high affinity of UGT1A9 for 1-naphthol.

To test this hypothesis (and the model itself), 1-naphthol was docked between the side chain of His37 of UGT1A9 and the anomeric carbon of the glucuronic acid moiety of UDPGA. Because of the rigidity of the 1-naphthol structure and the presence of a single reactive group within this substrate, it may be assumed that it only possesses rotational freedom in the active site. Depending on the rotational orientation of the bound aglycone, segments B, C, and D come into close proximity with it. The two most extreme orientations for 1-naphthol point either to segments C and D (169–173 and 188–192) or to segment B (residues 116–119). In the latter position, the substrate comes in contact with Phe117, which is found close to the catalytic His and may stack with the aromatic ring of 1-naphthol, in full agreement with the activity data. It is tempting to speculate that the biphasic kinetic model observed with UGT1A9 may originate from the different orientations of 1-naphthol in the active site, the high-affinity and the low-affinity orientations, and when the F117 is removed by mutation, the high affinity is lost.

5.6 mRNA expression of UGT1A9 and UGT1A10 in human tissues (III)

Because UGT1A10 seemed to be the most active UGT in dopamine and estradiol glucuronidation, the expression of this isoform in human tissues was evaluated. Although studies with some tissues were done previously, there were inconsistencies in the results, particularly regarding whether this enzyme is expressed in liver (Strassburg *et al.*, 1997; 1998a; 1999; Mojarrabi and Mackenzie, 1998; Cheng *et al.*, 1999; Zheng *et al.*, 2002; Li *et al.*, 2007b; Nakamura *et al.*, 2008). The expression pattern of UGT1A9 was also analyzed in the same tissues. The results confirm that UGT1A10 is expressed mainly in the intestine followed by adipose tissue, trachea and stomach, but very low mRNA levels were also found in the liver, testis, and prostate. UGT1A9, in contrast to UGT1A10, is expressed mainly in the liver and in the kidneys. UGT1A9 is also expressed to considerable levels in the small intestine, colon, and adipose tissue, and to low levels in adrenal gland, placenta, prostate, stomach, testis, trachea, and thyroid, but neither UGT1A9 nor UGT1A10 was detected in the brain, ovary, or uterus, which are target tissues of dopamine and β-estradiol.

6 Summary and Conclusions

During the course of the studies for this doctoral thesis, many important new findings were made that provide better understanding of the drug metabolizing enzymes and their substrate selectivity. First, a new HPLC-method was developed and used for dopamine sulfonation studies (I). This was the first time that regioselectivity of dopamine sulfonation by sulfotransferase (SULT) 1A3 was studied by enzyme kinetic analysis. Previously, it has been suggested that specificity of transport proteins or arylsulfatases might be the factors influencing the circulating levels of dopamine-3-*O*-sulfate and dopamine-4-*O*-sulfate in the blood (Strobel *et al.*, 1990). The results presented here indicate that SULT1A3 strongly favors the 3-hydroxy group of dopamine over the 4-hydroxy group and may indeed be primarily responsible for the difference in the circulating levels of dopamine sulfates in human blood (I). These results are fully consistent with the increasing amount of crystallographic and other data appearing on the mechanism of the sulfonation reaction.

Glucuronidation of dopamine and estradiol by 19 human recombinant UDP-glucuronosyltransferases (UGTs) was studied (II, III). The results highlight the importance of the human UGT1A10, which has sometimes been considered a low activity isoform (Cheng *et al.*, 1999; Lepine *et al.*, 2004). On the other hand, high activity toward various substrates has been observed by other groups (Strassburg *et al.*, 1998a; Basu *et al.*, 2004). The reason for the varying results is not clear, although some speculations have been presented e.g. phosphorylation and extra-microsomal localization of UGT1A10 (Dellinger *et al.*, 2007; Basu *et al.*, 2004; Basu *et al.*, 2005). Further studies are required to fully elucidate the reasons behind the large variability observed in the activity of UGT1A10. However, our results with recombinant UGT1A10 were supported by the results obtained with microsomal preparations from human liver and intestine (II, III).

Both SULT1A3 and UGT1A10 are expressed mainly in the intestine. Although the liver is usually considered to be the major metabolizing organ, the surface area of the intestinal mucosa is enormous. Therefore, these two intestinal enzymes may play important roles in the first step of inactivation and detoxification of drugs and other xenobiotics *in vivo*. Moreover, almost half of the endogenous dopamine is produced in the mesenteric organs, where most of the dopamine sulfates are produced (Eisenhofer *et al.*, 1997). *In vivo*, UGT1A10 is probably much less important in dopamine metabolism than SULT1A3, whose affinity to dopamine is approximately 1000 times higher (I, III).

Despite the low affinity, dopamine was identified as a new possible probe substrate for UGT1A10 due to its selectivity, and it was used to study mutants of this enzyme (III). Phenylalanines 90 and 93 were replaced by six amino acids of different sizes. The results revealed distinct effects that are likely to be dependent on differences in the size of the side chain and in their positions within the protein. Four mutants were subjected to kinetic analyses and the amino acid changes were found to affect only the V_{max} of the dopamine glucuronidation but not the K_m, presumably reflecting the lack of direct contact of these residues with dopamine in the active site. Studies with more substrates are under way in our laboratory and the findings should facilitate better understanding of the substrate specificity of the human UGT1A10 and the factors that determine it.

Glucuronidation of β-estradiol and epiestradiol was studied with respect to regio- and stereoselectivity of UGTs (II). In general, UGT1As were more active on the 3-OH than on the 17-OH of both diastereomers, whereas the regioselectivity of UGT2Bs was the reverse. The stereoselectivity of UGT2B7 and UGT2B17 toward the 17-OH of β-estradiol and epiestradiol was similar to that toward testosterone and epitestosterone (Sten *et al.*, 2009). UGT2B7 and UGT1A10 were the most active enzymes in estradiol glucuronidation. UGT1A10 was more active on the 3-OH of β-estradiol than on the same hydroxyl of epiestradiol. UGT1A9 glucuronidated only epiestradiol, and only to a barely detectable level, but it also bound β-estradiol with an affinity similar to that of UGT1A10. In consequence, epiestradiol and β-estradiol were used in the latter study of the differences between UGT1A9 and UGT1A10 (IV).

The substrate selectivities of UGT1A9 and UGT1A10 partly overlap, but there is some dissimilarity in their regio- and stereoselectivities as well as in affinities and activities toward many substrates. In mature proteins, there are only 33 amino acids that differ between these two enzymes and modify their function in subtle ways. In study IV, seven amino acids were identified that were unimportant for the selectivity differences between UGT1A9 and UGT1A10: R42, G67, D87, V102, R103, N114, and E241. On the other hand, F117 was identified as an important residue in the binding of 1-naphthol. In addition, residues at positions 115, 116, 152, and 169 had significant impacts on the catalytic properties that were studied here, and the residues at position 152 and 169 especially affected activity. Because there is no crystal structure of the N-terminal domain of any UGT, determination of the important amino acids is a difficult task. Previously, some membrane proteins have been studied with atomic force microscopy *in situ* (Engel and Gaub, 2008) and perhaps that would be a useful tool in determining the structures of UGTs in the future.

In drug discovery, metabolism studies are part of the non-clinical phase. The glucuronidation studies with rat UGTs and liver microsomes from five species implied that animal models are not always reliable in predicting glucuronidation in humans. In addition, the studies with recombinant enzymes provide new information about the substrate selectivity of human UGTs and SULT1A3 that can even be of help in creating new predictive models for early ADME studies.

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Appendix: Original Publications I-IV