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**Glucuronide Isomers: Synthesis, Structural
Characterization, and
UDP-glucuronosyltransferase Screening**

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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 lecture hall 3, Viikki B-building (Latokartanonkaari 7), on 5 June 2009, at 12 noon.

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Preface

This study was carried out in the Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Helsinki, during the years 2001–2009. I acknowledge the Graduate School of Pharmaceutical Research and the Finnish Cultural Foundation for funding the work.

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Helsinki, May 2009

Anna Alonen

Abstract

Drug metabolism reactions are divided into two phases. Glucuronidation is an important phase II metabolism reaction that is catalyzed by UDP-glucuronosyltransferases (UGTs). Due to glucuronic acid conjugation, bioactive compounds become inactive, and lipophilic substances become more hydrophilic, which enhances their excretion to urine and bile. Aglycones can be endogenous or exogenous compounds, such as hormones and drugs.

Synthetic glucuronide metabolites are needed as reference substances in drug research. Glucuronide conjugates have been synthesized by both chemical and enzymatic methods. The aim of this work was to study glucuronidation of aglycones that have multiple glucuronidation sites. Glucuronide isomers of dobutamine, losartan, candesartan, and zolarsartan were synthesized by enzyme-assisted methods whereas clenbuterol glucuronides were synthesized chemically using the Koenigs-Knorr reaction. The structures and the glucuronidation sites of the synthesized compounds were determined by tandem mass spectrometry and nuclear magnetic resonance spectroscopy. From dobutamine, the glucuronide synthesis produced three *O*-glucuronide regioisomers. Both *N*- and *O*-glucuronides, including two acyl glucuronides, of losartan, candesartan, and zolarsartan were obtained. The Koenigs-Knorr reaction produced *O*-glucuronide stereoisomers of clenbuterol. The yields varied between 0.7 and 34.6% (0.5–6.8 mg).

It is important to identify active UGT isoforms in order to understand drug-drug interactions, to clarify catalytic mechanisms, to assess the structure-function relationships, and to find isoform-selective probes. In this study, the glucuronidation activity of recombinant human UGTs was assessed using the synthesized glucuronides as reference compounds. In addition, species differences in glucuronidation were studied using liver microsomes from various animals. The formation of glucuronide isomers of dobutamine, losartan, candesartan, and zolarsartan brought a new perspective to evaluation of the structure-function relationships of UGTs and the results provided new data on their substrate specificities. UGT1A10 showed versatile substrate specificity whereas UGT1A3 was highly selective towards N2 in the tetrazole ring. The glucuronidation activity of liver microsomes was different between species. Compared to animal liver microsomes, human liver microsomes glucuronidated the studied aglycones inefficiently. There were no distinct correlations between the glucuronidation properties of human liver microsomes and recombinant hepatic UGTs, so it is difficult to predict the glucuronidation in human liver microsomes from the data obtained with recombinant UGTs.

List of original publications

This thesis is based on the following publications:

- I Alonen, A., Aitio, O., Hakala, K., Luukkanen, L., Finel, M., and Kostainen, R., Biosynthesis of dobutamine monoglucuronides and glucuronidation of dobutamine by recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* 33 (2005) 657-663.
- II Alonen, A., Jansson, J., Kallonen, S., Kiriazis, A., Aitio, O., Finel, M., and Kostainen, R., Enzyme-assisted synthesis and structure characterization of glucuronic acid conjugates of losartan, candesartan, and zolarsartan. *Bioorg Chem* 36 (2008) 148-155.
- III Alonen, A., Finel, M., and Kostainen, R., The human UDP-glucuronosyltransferase UGT1A3 is highly selective towards N2 in the tetrazole ring of losartan, candesartan, and zolarsartan. *Biochem Pharmacol* 76 (2008) 763-772.
- IV Alonen, A., Gartman M., Aitio O., Finel M., Yli-Kauhaluoma J., and Kostainen R., Synthesis, structure characterization, and enzyme screening of clenbuterol glucuronides. *Eur J Pharm Sci* (2009) accepted for publication.

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

Abbreviations

CYP	cytochrome P450
Da	dalton
DQFCOSY	double quantum filtered correlated spectroscopy
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
K_m	Michaelis constant
K_{si}	substrate inhibition constant
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
ROESY	rotating Overhauser effect spectroscopy
S_N2	bimolecular nucleophilic substitution
SPE	solid-phase extraction
UDP	uridine 5'-diphosphate
UDPGlcA	UDP-glucuronic acid
UGT	UDP-glucuronosyltransferase
<i>UGT</i>	UGT gene
UV	ultraviolet
V_{max}	maximal velocity

1 Introduction

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) are a family of enzymes that catalyze glucuronidation reactions. Glucuronidation is the major phase II metabolism reaction, where the substrate is conjugated to glucuronic acid. Both endogenous and exogenous compounds can serve as aglycones, and drugs are one important class of substrates of UGTs.

This study deals with syntheses of glucuronides and UGTs. The glucuronidation activities of recombinant human UGTs and liver microsomes from various species towards selected drugs were screened. The studied drugs — dobutamine, losartan, candesartan, zolarsartan, and clenbuterol — can be metabolized to different glucuronide regioisomers or stereoisomers. To characterize the structures of the glucuronides, they were first synthesized in milligram scale and the glucuronidation sites were determined by two-dimensional nuclear magnetic resonance spectroscopy (NMR).

Why is it important to identify the active UGT isoforms? Although glucuronidation is one of the most important phase II metabolism reactions, compared to cytochrome P450 (CYP) catalyzed reactions that play central role in drug metabolism, its implication for drug therapy is still quite unclear. On the basis of enzyme activity assays it may be possible to understand UGT mediated drug-drug interactions. No crystal structure of any full-length UGT has been determined and the catalytic mechanisms are still under investigation. Therefore UGT screening assays can provide important information for evaluating the structure-function relationships of UGTs. The activities of individual UGTs are generally overlapping, which has caused a lack of isoform-selective probe drugs and inhibitors. Recombinant enzyme screening assays can reveal possible probe compounds or structures that could be modified to serve as selective probes. In the last decades it has been found that many UGTs are polymorphic and this highlights the importance of pinpointing the isoforms responsible for the glucuronidation of a given drug.

1.1 Glucuronidation and UDP-glucuronosyltransferases

Drug metabolism changes the chemical structure of lipophilic compounds so that they are more easily excreted from the body, usually by increasing the hydrophilicity of the molecule [1]. Simultaneously bioactive compounds are often inactivated. Metabolic reactions are classified into two phases. Oxidation, reduction, and hydrolysis — the main phase I reactions — are functionalization reactions: as consequence a functional group is added to the parent drug molecule. Phase II reactions form conjugates, since an acetyl, amino acid, sulphate, glucuronic acid, glutathione, or methyl group is conjugated to the drug or to the metabolite formed in the phase I reaction. The conjugation reactions are usually catalyzed by specific transferases.

Glucuronidation is considered to be the most important phase II metabolic reaction, and it is involved in the metabolism of large number of endobiotics and xenobiotics. The human UGTs that use UDP-glucuronic acid (UDPGlcA) as the co-substrate are classified into three subfamilies, UGT1A, UGT2A, and UGT2B, according to their amino acid sequence (Fig. 1) and gene structure [2]. The substrate specificities of individual UGTs are often very wide and partly overlapping, reducing the risk of drug interactions caused by metabolism via glucuronidation. Human *UGT* genes and their regulatory elements are polymorphic and this has become an important area of research, because of the possible risk of drug-induced adverse reactions [3-7]. Further knowledge of polymorphism can also help to predict susceptibility to cancer [3, 4].

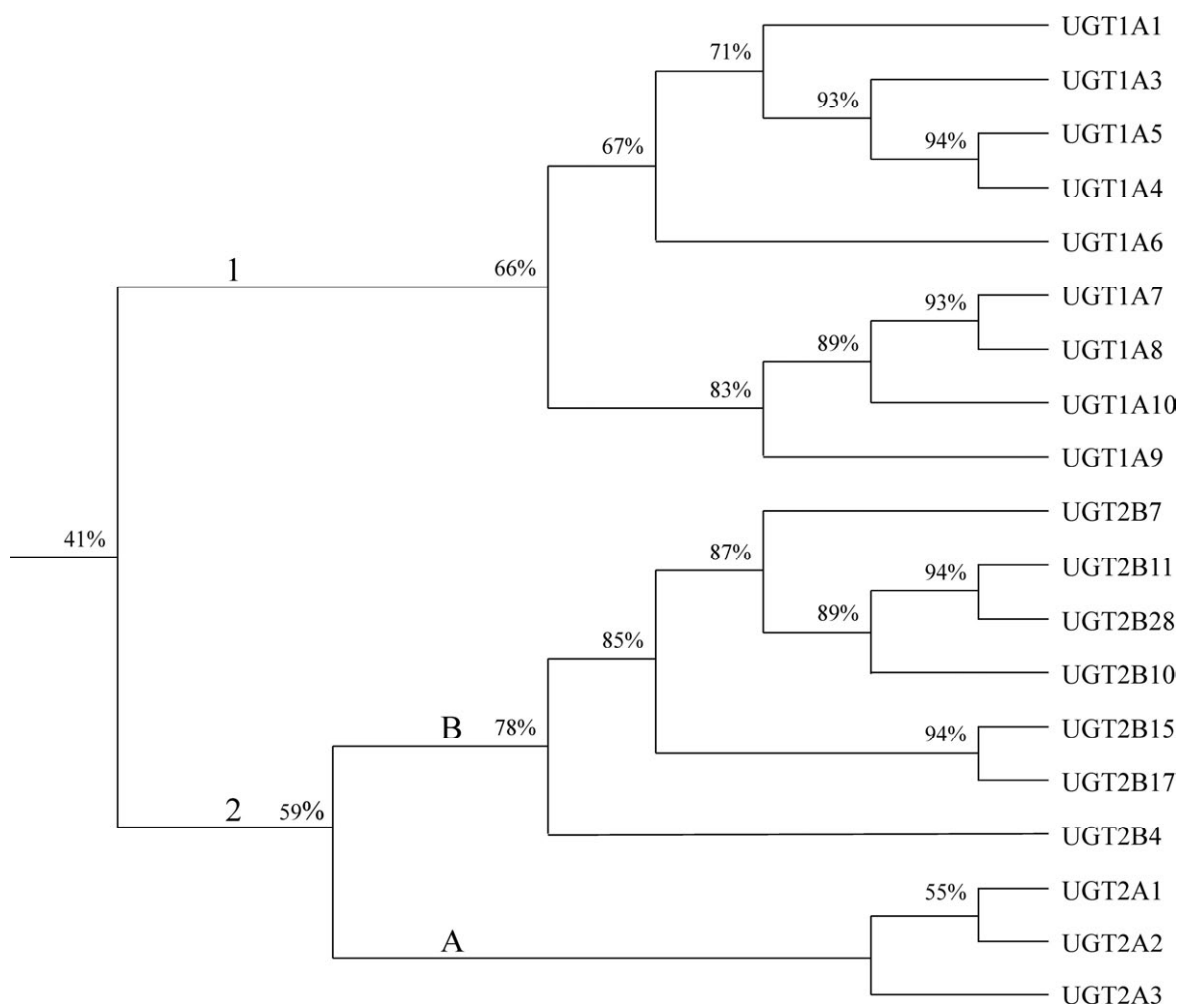


Figure 1 *Phylogenetic tree for UGTs. The homology of the enzymes at the amino acid level is presented [2, 3, 8].*

Metabolizing enzymes often appear in the tissues and organs that are prone to exposure to exogenic compounds, thus protecting the organism from foreign compounds. The liver is such an organ, as it is directly exposed to absorbed drugs and other xenobiotics from the intestine via the hepatic portal vein. The liver is the main organ where UGTs are expressed, although glucuronidation is also active in different parts of the gastrointestinal tract. UGTs are expressed in other extrahepatic tissues as well (Table 1). Polymorphic expression of UGTs produces inter-individual variation in the content of UGTs in tissues [9, 10].

Table 1. *Expression of UGT isoforms in human tissues and organs.*

UGT isoform	Tissues and organs	Refs (and Refs. therein)
1A1	Bile ducts, colon, liver, small intestine, stomach	[6, 11-13]
1A3	Bile ducts, colon, liver, small intestine, stomach	[6, 11-13]
1A4	Bile ducts, colon, liver, small intestine	[6, 11-13]
1A5	Colon, liver, small intestine, stomach	[14]
1A6	Bile ducts, brain, colon, liver, small intestine, stomach	[6, 11-13]
1A7	Esophagus, lung, small intestine, stomach	[6, 11-13]
1A8	Colon, esophagus, kidney, small intestine	[6, 11-13]
1A9	Colon, esophagus, kidney, liver	[6, 11-13]
1A10	Bile ducts, colon, esophagus, kidney, small intestine, stomach	[6, 11-13]
2A1	Brain, fetal lung, olfactory epithelium	[6, 11]
2A2	Liver, small intestine	[8]
2A3	Adipose, colon, kidney, liver, pancreas, small intestine, stomach, testis	[15]
2B4	Liver, small intestine	[6, 11-13]
2B7	Brain, colon, esophagus, kidney, liver, pancreas, small intestine	[6, 11-13]
2B10	Esophagus, liver, mammary gland, prostate, small intestine	[6, 11-13]
2B11	Adipose, adrenal, kidney, liver, lung, mammary gland, prostate, skin	[6, 11-13]
2B15	Esophagus, liver, prostate, small intestine	[6, 11-13]
2B17	Liver, prostate	[6, 11, 12]
2B28	Liver, mammary gland	[6]

UGTs are transmembrane proteins located in the endoplasmic reticulum [13, 16-18]. About 95% of the polypeptide chain is located on the luminal side, including the catalytic domain. The access of hydrophilic UDPGlcA from the cytosol into the endoplasmic reticulum lumen across the membrane is restricted. Therefore, in *in vitro* studies, the

glucuronidation activity can be increased with membrane-disrupting agents like phospholipase, detergents, sonication, and alamethicin, probably due to the greater access of UDPGlcA (and aglycone) to the enzyme active site [16, 17, 19]. It has been proposed that there are transporters to carry the co-substrate into the lumen of the endoplasmic reticulum, as well transporters to carry the glucuronide formed out to the cytosol [13, 17, 18]. Even the action of UGTs as transporters has been suggested [13, 17, 18]. In particular it has been speculated that two UGT monomers could form a dimer, which would then act as a channel carrying the UDPGlcA to the active site. Indeed, it strongly appears that UGTs act as dimers or oligomers, where two or more UGT polypeptide chains form homo- or heteromers [13, 17, 18, 20].

UGTs catalyze the conjugation of UDPGlcA to a nucleophilic functional group of the substrate that already exists in the aglycone or is formed in a phase I metabolic reaction (Fig. 2). Aryl, alkyl, enolic, and acyl hydroxy groups are glucuronidated to *O*-glucuronides (ether or ester linkage) whereas *N*-glucuronides are formed from amines and sulfonamides [16, 17]. *S*- and *C*-glucuronides, formed from thiols and enol α -carbon atoms, are rare. The enzymatic glucuronidation reaction is believed to be a bimolecular nucleophilic substitution (S_N2) reaction where the aglycone substrate attacks the UDPGlcA and an inversion of the configuration converts the glucuronic acid from α - to the β -anomer.

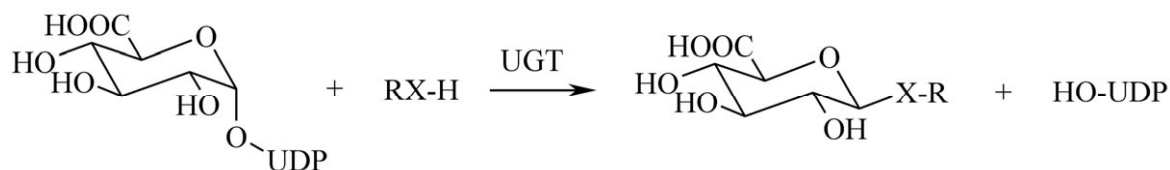


Figure 2 Glucuronidation of compound *RX-H*. The UGT-catalyzed reaction produces β -D-glucuronides from α -anomeric UDPGlcA and aglycone substrate. The *X* refers to *O*, *N*, or *S* atoms.

Glucuronidation is considered as a detoxification reaction: the compound usually loses its biological activity, although some exceptions exist. Probably the most well-known active glucuronide is morphine 6-*O*-glucuronide, which has even more analgesic action than morphine [21, 22]. Another example is α -hydroxymidazolam glucuronide which is active and may accumulate in patients with renal failure, causing prolonged sedation [23]. *N-O*-Glucuronides of hydroxamic acids, formed from the glucuronidation of an *N*-hydroxy group, are also considered reactive metabolites [21]. They can react with nucleophiles in cell, such as methionine, tryptophan, and guanosine residues in proteins and DNA. However, the formation of *N-O*-glucuronides is rare, and their reactivity has not been widely tested.

The most noteworthy group of reactive glucuronide metabolites is acyl glucuronides (**1**) (Fig. 3), which are formed from carboxylic acids. Electrophilicity of the ester group carbonyl carbon leads to instability of the acyl glucuronide [21, 24-27]. Susceptibility to

hydrolysis, which is more rapid at neutral and basic pH than with acidic conditions and can even be catalyzed by esterases and β -glucuronidase, can prolong the half-life of carboxylic-acid containing drugs due to enterohepatic recirculation. In addition, an intramolecular rearrangement called acyl migration can take place, and either the acyl glucuronide itself or the migration isomers may bind covalently to macromolecules, such as proteins or DNA, causing adverse effects. Acyl glucuronides can form protein adducts by two distinct types of reactions. In the transacylation reaction, glucuronic acid (**2**) is lost and aglycone is directly bound to the protein (**3**) (Fig. 3). The other reaction type is called glycation, where the 2-*O*-, 3-*O*-, or 4-*O*-acyl migration product (**4**, **5**, or **6**) undergoes transient ring opening to give an open-chain aldehyde (**7**) that reacts further with amino groups of biomolecules and forms an imine (Schiff base) (**8**). Amadori rearrangement of the imine (**8**) to ketone (**9**) is possible in the cases of 3-*O*- and 4-*O*-acyl migration products. The migration isomer can undergo anomerization by a ring closing reaction of the open-chain aldehyde (**7**) leading to an α -configuration of the isomer (**10**). Overall, the reactivities of acyl glucuronides differ enormously and depend on the electronic, steric, and other characteristics of the drug aglycone [26]. For example, diclofenac is a carboxylic acid-containing nonsteroidal anti-inflammatory agent, the therapeutic use of which leads sometimes to rare hepatotoxicity [28]. The toxicity is caused by reactive metabolites. Diclofenac is glucuronidated by UGT2B7 and the acyl glucuronide formed can bind covalently to proteins. However, diclofenac can also be bioactivated by oxidation. The biochemical mechanism of diclofenac hepatotoxicity is not fully understood, but acyl glucuronidation certainly plays a role.

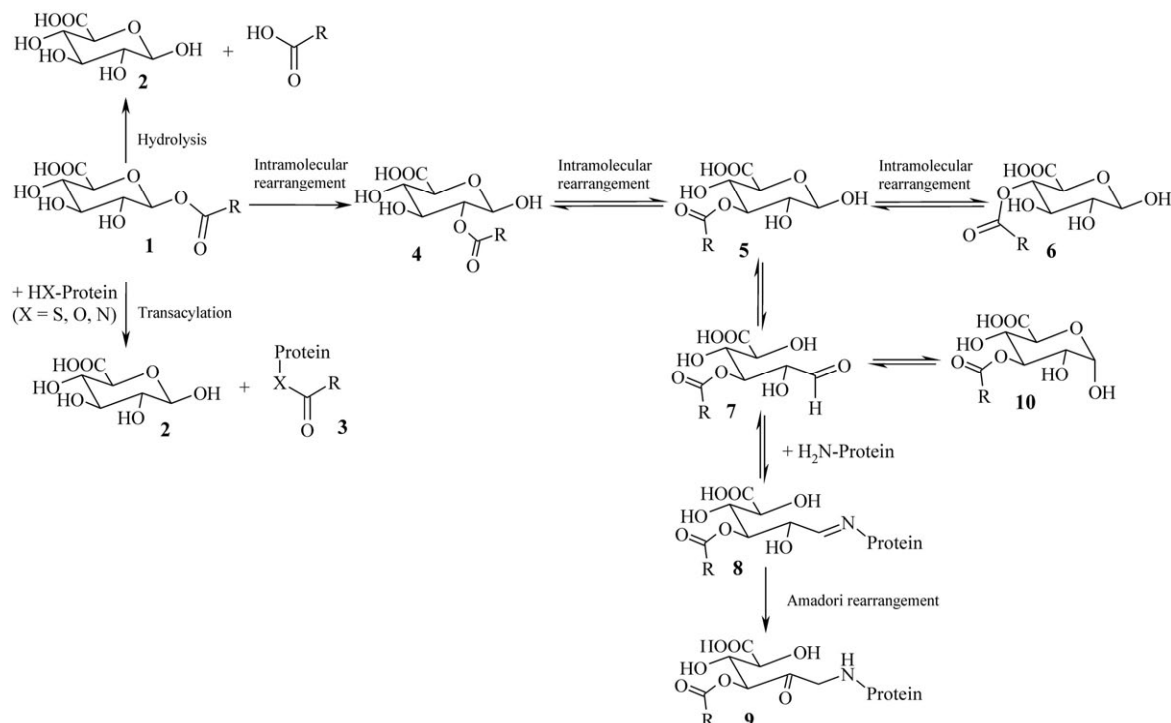


Figure 3 Reactivity of acyl glucuronide (**1**) [21, 24-27]. The figure shows glycation of a 3-*O*-isomer (**5**), but similar reactions take place with other isomers (**4** and **6**). (See text for details.)

1.2 Glucuronides as Standard Compounds

Drug metabolites, including glucuronides, are needed as reference substances for analytical studies in pharmacological, toxicological, and biopharmaceutical research. However, only a few synthetic glucuronide metabolites are commercially available. Small amounts of glucuronide conjugates can be isolated from urine or tissues after administration of the drug aglycone to humans or laboratory animals. However, this method is very laborious given the small amount of purified compound obtained. In addition, the ethical issues are noteworthy, and these cannot be simply addressed by using laboratory animals since they do not necessarily produce similar metabolites as humans do.

The Koenigs-Knorr reaction is the most widely applied method to chemically synthesize alkyl and aryl *O*-glucuronide metabolites [29, 30]. In this reaction the aglycone is coupled with methyl acetobromo- α -D-glucuronate (**11**) (Fig. 4) in the presence of silver salts. The first step of the synthesis yields fully protected β -D-glucuronide (**12**), and the protecting groups are then hydrolyzed with alkali. In addition to the desired β -D-glucuronide (**13**), the reaction often yields the α -anomer (**14**) and ortho ester (**15**) as by-products. If the aglycone has multiple glucuronidation sites, chemical synthesis can yield a mixture of mono- and polyglucuronides unless the unwanted glucuronidation sites are protected. The reaction gives glucuronides in variable yields depending on the catalyst, solvent, aglycone, and the ratio of the starting materials used. Other methods have been used for the synthesis of glucuronides too; the main differences between the reactions are in the glycosyl donor [27, 29, 30]. *N*-Glucuronides can be synthesized by the reaction of glucuronic acid and amine. Modifications of the Koenigs-Knorr reaction have also been used for production of *N*-glucuronidies.

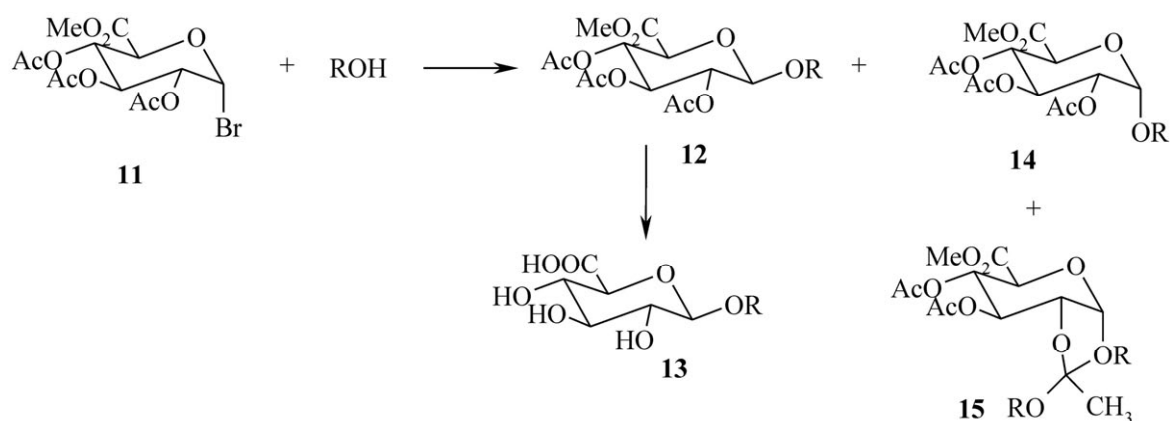


Figure 4 Koenigs-Knorr reaction [29, 30]. Methyl acetobromo- α -D-glucuronate (**11**) is conjugated to aglycone (ROH) yielding protected β -D-glucuronide (**12**) and, as by-product, α -anomer (**14**) and ortho ester (**15**). The protecting groups are hydrolyzed with alkali to produce the β -D-glucuronide (**13**).

Glucuronides can be synthesized with the help of enzymes. Different kinds of enzyme preparations have been used, such as perfused rat liver [31], liver microsomes from human [32-35], bovine [36, 37], dog [32], pig [36], and Aroclor 1254-induced rat [38-40], and even recombinant human UGTs [41]. Enzyme-assisted syntheses have mostly been utilized to produce *O*-glucuronides, but syntheses of *N*-glucuronides have also been described [35, 41]. Enzymes produce mainly monoglucuronides, even regio- or stereoselectively. In addition, the advantage of UGT-catalyzed synthesis of glucuronides is the formation β -anomers only. Immobilization of microsomes has been used to enable easy separation of products from proteins after the synthesis reaction [42-45]. Also, the stability of enzymes is often improved by immobilization, leading to the possibility of increasing the incubation time or even reusing the proteins. Compared to free microsomes, better enzyme activities have sometimes been obtained with immobilized microsomes.

Production of glucuronides for reference material without isolation and purification can sometimes be appropriate, for example in qualitative chromatographic analyses that include the separation of the conjugate and unreacted aglycone. Then mass spectrometry (MS) or NMR spectroscopy instruments could be coupled to chromatography, thus allowing a method to identify and characterize the substances. Even immobilized enzyme reactors have been developed that synthesize, separate, and detect the metabolites on-line [46-48].

Structural Characterization of Glucuronides

Nowadays, mass spectrometry is probably the most often used method to identify glucuronides. The advantage of the MS technique is that only a very small quantity of compound is required to obtain accurate tandem mass (MS/MS) spectra. Using positive ion MS/MS, the typical fragmentation is the neutral loss of the glucuronide moiety (176 Da), and the product ions include the aglycone and the fragments of the aglycone. In negative ion mode, characteristic fragment ions are observed from the glucuronide moiety at m/z 175 [$C_6H_7O_6^-$] and m/z 113 [$C_6H_7O_6 - CO_2 - H_2O^-$]. Thus, the determination of the conjugation site is not generally possible and the glucuronide isomers often have similar MS/MS spectra. However, some exceptions exist in the literature. Conjugation at an aliphatic hydroxyl group can be seen in negative ion mode as a product ion at m/z 193, which is a deprotonated glucuronic acid [$C_6H_9O_7^-$]. This kind of cleavage is not possible for glucuronides conjugated at an aromatic hydroxyl, nor for *N*-glucuronides [33, 37, 49]. Derivatization of glucuronides before MS/MS analysis has also been exploited for determination of the conjugation sites [50, 51].

NMR is required for detailed structural characterization of glucuronides. The site of glucuronidation can be identified by comparing the chemical shifts of the glucuronide to those of the aglycone. The largest changes in chemical shifts upon glucuronidation are found in the atoms located near the conjugation site. Nevertheless, if the aglycone has multiple functional groups at which the glucuronide can be conjugated, and especially if

the functional groups are close to each other in the molecule, it can be difficult to determine the glucuronidation site with confidence. Nuclear Overhauser effect (NOE), rotating Overhauser effect (ROESY), and heteronuclear multiple bond correlation (HMBC) spectroscopy are all useful NMR techniques for verifying the structures and particularly the site of glucuronidation. In addition, the anomeric configuration can easily be determined by NMR. Enzyme-catalyzed reactions produce only glucuronides having a β -configuration, but chemical syntheses can yield both α - and β -anomers. Vicinal couplings between two axial protons, GH1 and GH2, in a glucuronide ring are 8-9 Hz, indicating the β -anomeric configuration, whereas in other cases the coupling would be less than 4 Hz [52-54]. The disadvantage of ^{13}C and two-dimensional NMR studies is that a sufficient amount of compound, in the milligram range, is required.

1.3 Studying Glucuronidation *In Vitro*

In the drug discovery process, metabolism studies of a drug candidate begin with preclinical trials. The aim is to detect poor drug candidates as early as possible in order to avoid unnecessary clinical trials that consume time and money. The preclinical metabolism studies are carried out both *in vivo* with animals and *in vitro*, and the purpose is to predict human pharmacokinetics and drug-drug interaction risks. It is important to expose laboratory animals to the same metabolites as the drug consuming humans would be exposed. However, species differences are large, and it is difficult to predict and extrapolate how the drug would be metabolized in humans. Therefore, knowledge of the characteristics of drug metabolism in laboratory animals is needed.

In vitro metabolism studies can be carried out with different kinds of preparations such as perfused organs, tissue slices, isolated cells, subcellular organelles, and purified enzymes. Microsomes are the most commonly-used source of enzymes for oxygenation and glucuronidation research, because the use of this model is simple [55]. Most often the microsomes are derived from liver, but also extrahepatic organs are used. To obtain microsomes, the tissue is homogenized and then fractionated by differential centrifugation. Nuclei, mitochondria, and other denser materials are removed at a lower centrifugation force (10 000 *g*) and soluble enzymes stay in the supernatant, even at a higher centrifugation force (100 000 *g*). The microsomal fraction is the pellet obtained at the higher centrifugation force. The microsomes contain mainly membranes of endoplasmic reticulum and the enzymes bound to them. Although the intracellular architecture is lost, microsomes have the advantage that different enzyme isoforms are represented with their natural variability. Genetic and environmental variations are present but they can be minimized by pooling samples from several individual donors [56]. In addition, the presence of native UGT oligomers, which can affect the enzyme activities, is likely possible in microsomes. Compared to microsomes, whole cell systems have the advantage that they also contain soluble enzymes and transporter proteins [55, 57]. Studies on the coupling of conjugation enzymes and efflux transporters have currently generated a lot of

interest, since their interplay has a significant impact on the bioavailability, detoxification, and interactions of drugs [58].

The inter-individual differences in drug metabolism, and thus in drug action, can be caused by genetic, physiological, pathological, and environmental factors. For example enzyme polymorphism, intestinal and liver diseases, and alcohol abuse can have an effect on the metabolism of a drug. If the enzymes that metabolize a drug are identified, the inter-individual differences can be understood and in some cases even predicted. Enzyme selective antibodies, inhibitors, and probes have been used to identify metabolic enzymes, but unfortunately not many of these are available for UGTs yet. Nowadays the availability of recombinant enzymes has increased their use in metabolism research. In addition to avoiding difficult extrapolation to human metabolism from animal metabolism data, ethical considerations support the development of methods to study drug metabolism *in vitro* with recombinant human enzymes [59, 60]. Recombinant human UGTs have been expressed in mammalian and insect cells [61]. They provide a tool to study substrate specificity, and those findings can be used to evaluate tissue specific glucuronidation and to analyze structure-function relationships of UGTs. Catalytic amino acids within the substrate binding site can be identified with the help of recombinant UGT mutants. Recently, clarifying the effects of polymorphic UGTs on drug treatment has become a significant application of recombinant UGTs. However, it is still uncertain if the results from recombinant enzymes can be extrapolated to microsomes, still less to the *in vivo* situation [57].

Dobutamine, Losartan, Candesartan, Zolarsartan, and Clenbuterol as Subjects of Glucuronidation Research

Based on the published reports, dobutamine [62-64], losartan [65-67], candesartan [51, 68], zolarsartan (GR117289, also called zolasartan) [69], and clenbuterol [70, 71] (Fig. 5) are metabolized via conjugation to glucuronic acid without a phase I metabolism reaction. All these compounds contain several possible glucuronidation sites. The likely formation of glucuronide isomers makes the research on these aglycones interesting.

Dobutamine is a synthetic derivative of the endogenous catecholamine dopamine (Fig. 5). It moderately stimulates α_1 - and β_2 -adrenergic receptors and strongly stimulates β_1 -adrenergic receptors, thus causing an inotropic effect on the heart. It is used for the treatment of cardiac failure. Dobutamine is administered as a continuous infusion since its half-life in plasma is only 2 minutes [72]. Depending on the animal species, the metabolites of dobutamine include 3-*O*-methyldobutamine as well as glucuronide and sulfate conjugates of dobutamine and 3-*O*-methyldobutamine [62-64]. Studies with recombinant UGTs showed that rat 2B1 and human 1A6 and 1A9 glucuronidated dobutamine, but rat 1A6 and human 1A1, 2B7, and 2B15 did not [73, 74]. More than one dobutamine glucuronide has been separated by chromatography and detected [64, 73]

indicating the formation of isomers. However, their structures, especially the glucuronidation sites, have not been determined.

The renin-angiotensin system is an important drug target for the treatment of high blood pressure. The hypertensive effect of angiotensin II can be prevented by inhibiting the angiotensin-converting enzyme that catalyzes its formation. Another strategy is to block the binding of angiotensin II to AT₁-receptors. AT₁ antagonists are called “sartans”, and losartan was the first sartan on market. Candesartan and zolarsartan are structural analogues of losartan: they all have a tetrazole ring and there is a carboxyl group in candesartan and zolarsartan (Fig. 5). Losartan is partly metabolized to an active carboxylic acid derivative. Two different glucuronide metabolites are formed by biotransformation: tetrazole-*N*2-glucuronide by monkeys, rats, dogs, and humans, and *O*-glucuronide by monkeys and rats [65-67, 75]. In addition, losartan *N*1-glucuronide has been synthesized chemically [65]. Candesartan is administered as a prodrug, candesartan cilexetil, that is hydrolyzed during the absorption process. This improves its oral bioavailability. Most candesartan is excreted unchanged [76], but it has been found that rats and dogs produce two candesartan glucuronide metabolites, the tetrazole-*N*2-glucuronide and the *O*-glucuronide (acyl glucuronide) [51, 68]. Zolarsartan is not in clinical use, but it is also an active AT₁ antagonist, both *in vitro* [77] and *in vivo* [78]. Glucuronic acid conjugates of zolarsartan have been found in rat and dog bile [69]. Dogs produced both the tetrazole-*N*2-glucuronide and the *O*-glucuronide (acyl glucuronide) whereas rats produced only the *O*-glucuronide (acyl glucuronide) of zolarsartan.

The sympathomimetic clenbuterol (Fig. 5) selectively stimulates β_2 -receptors, therefore it is a bronchodilator. It is used for treatment of asthma and chronic obstructive pulmonary disease in both human and veterinary medicine. Athletes have abused clenbuterol because of its possible anabolic effect and reduction of body fat. It has also been used illegally in cattle, which has even caused toxic effects to humans consuming liver or meat that contained residues of clenbuterol [79-81]. A part of the clenbuterol dose is excreted as the parent compound, and the amount of unchanged drug depends on the species [70, 82]. The main metabolites include 4-amino-3,5-dichloromandelic acid, 4-amino-3,5-dichlorohippuric acid, and clenbuterol hydroxylamine [70, 82-88]. In addition, *N*- and *O*-glucuronides have been reported as metabolites of clenbuterol [70, 71].

The site-specific glucuronidation of one aglycone by individual recombinant human UGTs provides new insights into substrate specificity of human UGTs. The knowledge of interspecies differences in glucuronidation activity in liver microsomes can be utilized in toxicology tests and, in addition, the suitability of microsomes as catalysts in enzymatic synthesis of metabolites can be assessed. Synthetic glucuronides are needed as reference material for these kinds of assays, but at present only a few glucuronide conjugates are commercially available.

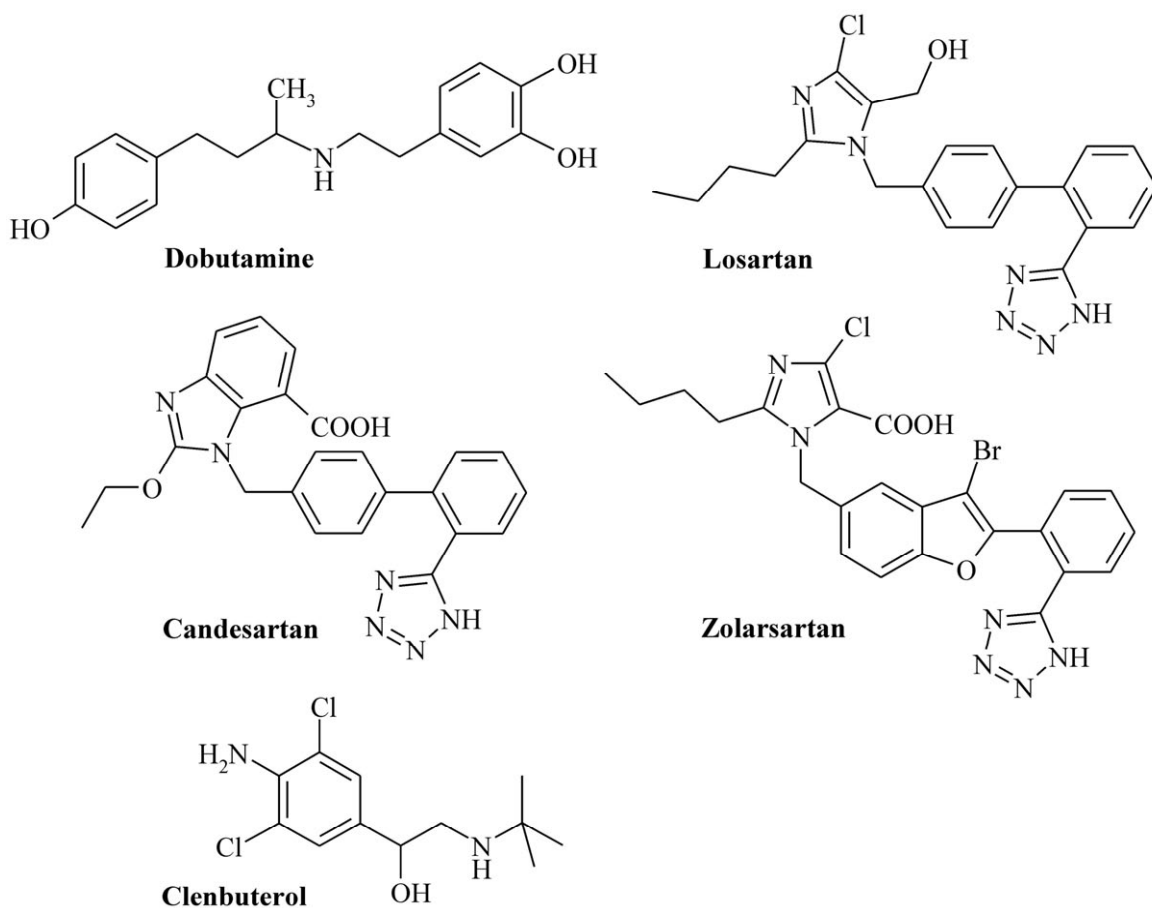


Figure 5 Structures of the studied compounds. The sartans, losartan, candesartan, and zolansartan, are structural analogues.

2 Aims of the Study

The primary aim of the study was to investigate the ability of human recombinant UGTs and liver microsomes from different animals to catalyze glucuronide isomers. For this purpose suitable aglycones were selected and their glucuronide isomers were synthesized and characterized.

The detailed aims of the research were

- to synthesize glucuronide regioisomers of dobutamine (I), losartan (II), candesartan (II), and zolansartan (II) using enzyme-catalyzed reactions and to characterize the structures of the synthesis products
- to synthesize glucuronide isomers of clenbuterol (IV) using the Koenigs-Knorr reaction and characterize the structures of the synthesis products
- to screen the activity of recombinant human UGTs and liver microsomes from various species in the production of different glucuronide isomers of dobutamine (I), losartan (II), candesartan (II), zolansartan (II), and clenbuterol (IV)

3 Materials and Methods

The materials and methods used in this study are summarized in this section. More detailed descriptions can be found in the original publications (I–IV).

3.1 Chemicals and Enzyme Preparations

Chemicals and enzyme preparations used in the study are listed in Tables 2 and 3, respectively. All the other reagents and solvents were of high performance liquid chromatography (HPLC) or analytical grade.

Table 2. *Chemicals used in the study.*

Chemical	Producer	Publication
Dobutamine (hydrochloride salt)	Eli Lilly & Co, Indianapolis, IN, USA	I
Candesartan	AstraZeneca, Mölndal, Sweden	II, III
Clenbuterol (hydrochloride salt)	Sigma-Aldrich St. Louis, MO, USA	IV
Losartan (potassium salt)	Merck, Rahway, NJ, USA	II, III
Methyl acetobromo- α -D- glucuronate	Sigma-Aldrich St. Louis, MO, USA	IV
Saccharic acid 1,4-lactone	Sigma-Aldrich, St. Louis, MO, USA	I, II, III, IV
Silver carbonate	Sigma-Aldrich, St. Louis, MO, USA	IV
Uridine-5'- diphosphoglucuronic acid (trisodium salt)	Sigma-Aldrich, St. Louis, MO, USA	I, II, III, IV
Zolarsartan	GlaxoSmithKline, Hertfordshire, UK	II, III

Table 3. *Enzyme preparations used in the study.*

Enzyme preparation	Producer	Publication
Aroclor 1254-induced male Sprague-Dawley rat liver microsomes	Faculty of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki ^a	I, II, III, IV
Bovine liver microsomes	Faculty of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki ^a	III, IV
Dog liver microsomes	Orion Corporation, Espoo, Finland ^b	IV
Human UGTs 1A1, 1A3–1A10, 2B4, 2B7, 2B10, 2B15, 2B17, and 2B28	Faculty of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki ^c	I (not 1A5, 2B10, 2B17, 2B28), II (only 1A3, 1A8, 1A10), III, IV
Male New Zealand white rabbit liver microsomes	In Vitro Technologies, Baltimore, MD, USA	I, III, IV
Moose liver microsomes	Faculty of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki ^a	II, III, IV
Pig liver microsomes	Faculty of Pharmacy, Division of Pharmaceutical Chemistry, University of Helsinki ^a	I, II, III, IV
Pooled human liver microsomes	BD Gentest, Woburn, MA, USA	I, III, IV

^aPrepared as described in Ref. [89].

^bPrepared as described in Ref. [90].

^cPrepared as described in Refs. [91-93].

3.2 Glucuronide Syntheses (I, II, IV)

Glucuronides were synthesized by enzymatic (I, II) and traditional chemical (IV) methods.

Enzyme-Assisted Syntheses of Glucuronides (I, II)

Three dobutamine glucuronide regioisomers were synthesized in one reaction mixture using pig liver microsomes as catalyst (I). Regioisomers of losartan, candesartan, and zolarsartan glucuronides were synthesized in separate reactions using liver microsomes from different animals or recombinant human UGTs as catalysts (II). All reaction mixtures contained 5 mM saccharic acid 1,4-lactone, 5 mM UDPGlcA, 5 mM MgCl₂, and 50 mM phosphate buffer (pH 7.4). The protein concentration of the enzyme catalyst was 1–2.5 mg/ml in the reaction mixture. The aglycone concentrations varied between 0.2 and 1.5

mM, and in reactions for dobutamine glucuronides, losartan *N*1-glucuronide, and zolarsartan *N*2-glucuronide, aglycone was added in two portions, at the beginning and in the middle of the incubation time. Reactions were incubated at 37 °C under continuous stirring for 5–48 hours. Proteins were removed from the reaction mixtures either by precipitation and centrifugation or directly by centrifugation (26,700 *g*, 10 min, 6 °C), and the supernatants were solid-phase extracted (SPE). SPE elution solvents were evaporated and the crude products were dissolved in the HPLC eluent that was used in fractionation. The glucuronides were fractionated using an Agilent 1100 HPLC equipped with an autosampler, a UV multiple wavelength detector, and a fraction collector (Agilent Technologies, Waldbronn, Germany). The collected glucuronide fractions were evaporated, and salts were removed from dobutamine, losartan, and candesartan glucuronides by SPE. Finally all glucuronides were lyophilized.

Chemical Synthesis of Glucuronides (IV)

Clenbuterol glucuronide diastereomers were synthesized by the Koenigs-Knorr reaction (Fig. 6). An alkaline aqueous solution of clenbuterol hydrochloride was extracted with diethyl ether to obtain the free base of clenbuterol. The ether was evaporated and the residue was dissolved in toluene. Methyl acetobromo- α -D-glucuronate and Ag₂CO₃ were added to the solution. After stirring at 75 °C for 25 hours, the reaction mixture was allowed to cool to room temperature. The mixture was filtered and the filtrate was evaporated to give a yellow residue. The residue was dissolved in methanol, and an equivalent volume of 1 M LiOH aqueous solution was added. The reaction mixture was stirred at room temperature for 28 hours, and finally the solvents were evaporated in vacuum and the crude products were dissolved in the eluent that was used in fractionation. The clenbuterol glucuronides were fractionated by a Waters preparative liquid chromatograph (Waters Corporation, Milford, MA, USA) equipped with a binary gradient pump, an autosampler, an active flow splitter, an HPLC pump, a UV diode array detector, an ion spray source, a MS detector (Waters micromass ZQ), and a fraction collector. Two clenbuterol glucuronide fractions were collected, evaporated, and lyophilized.

Purities of Synthesis Products

Purities of the synthesis products were assessed using an Agilent 1100 Series HPLC equipped with an autosampler and Agilent 1100 Series fluorescence or UV diode array detectors (Waldbronn, Germany). The amounts of impurities were calculated using the fluorescence excitation and emission wavelengths of 285 nm and 313 nm for dobutamine glucuronides (I) and UV wavelengths of 256 nm, 210 nm, 306 nm, and 254 nm for losartan, candesartan, zolarsartan, and clenbuterol glucuronides (II, IV), respectively.

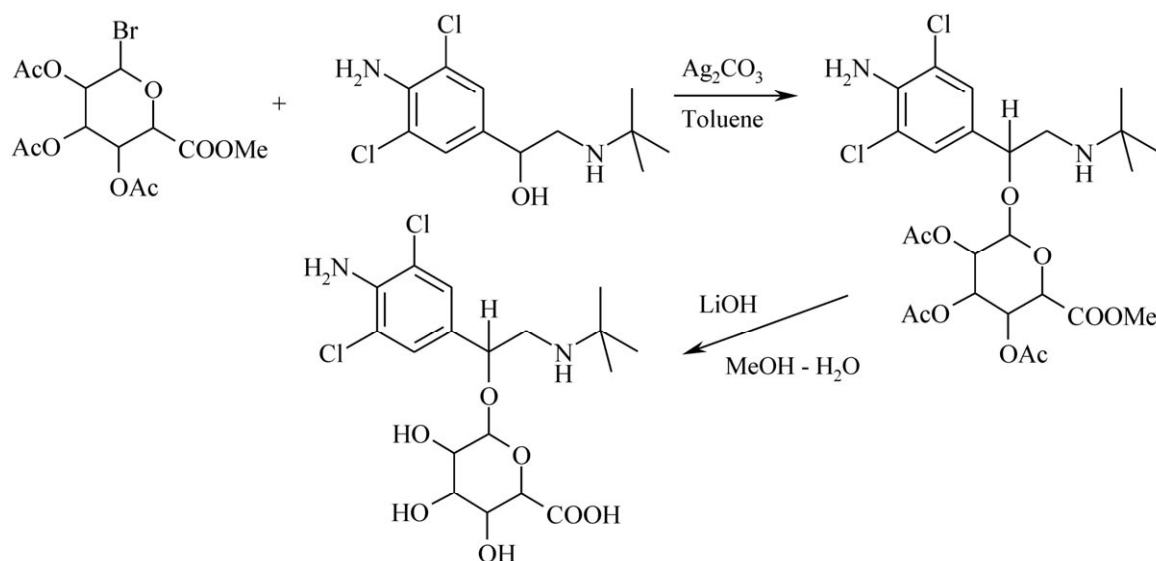


Figure 6 Synthesis of clenbuterol glucuronides from racemic clenbuterol by the Koenigs-Knorr reaction.

Structural Characterization

The mass spectrometric structure characterizations of isolated glucuronides were carried out on the instruments given in Table 4. The dobutamine glucuronides (I) were measured by direct infusion, whereas HPLC with a short analysis time was used for other glucuronides (II, IV) before MS analyses. The experiments were performed both in positive and negative ion modes.

Table 4. MS instrumentation used in the study.

Mass spectrometer	Producer	Compounds	Publication
API 3000 triple quadrupole	MDS Sciex, Toronto, Ont, Canada	Dobutamine glucuronides, Losartan <i>O</i> -glucuronide, Losartan <i>N2</i> -glucuronide, Candesartan glucuronides, Clenbuterol glucuronides	I, II, IV
Quadrupole time-of-flight	Q-TOF Micro Waters/Micromass, Manchester, UK	Losartan <i>N1</i> -glucuronide, Zolarsartan glucuronides	II

The NMR characterizations of isolated glucuronides were carried out on the instruments given in Table 5. For comparison, NMR spectra were recorded for aglycones as well. Interglycosidic correlations for determination of glucuronidation sites were observed from NOE, ROESY, and/or HMBC spectra (Table 6).

Table 5. *NMR instrumentation used in the study.*

NMR spectrometer	Producer	Note	Publication
Varian 300 MHz mercury plus	Varian, Inc., Palo Alto, CA, USA		II, IV
Varian Unity 500 MHz	Varian, Inc., Palo Alto, CA, USA	gHX nano-NMR probe for the heteronuclear experiments	I
Varian Unity Inova 600 MHz	Varian, Inc., Palo Alto, CA, USA	cold probe	II, IV

Table 6. *NMR experiments for structure characterization of synthesized glucuronides.*^a

Glucuronide	Experiment	Publication
Phenolic dobutamine <i>O</i> -glucuronide	1D ¹ H, 1D ¹³ C, DQFCOSY, NOESY, HSQC, HMBC	I
Catecholic dobutamine <i>para O</i> -glucuronide	1D ¹ H, DQFCOSY, NOESY, HSQC, HMBC	I
Catecholic dobutamine <i>meta O</i> -glucuronide	1D ¹ H, 1D ¹³ C, DQFCOSY, NOESY, HSQC, HMBC	I
Losartan glucuronides	1D ¹ H	II
Candesartan glucuronides	1D ¹ H, NOESY, HMBC	II
Zolarsartan <i>O</i> -glucuronide	1D ¹ H	II
Zolarsartan <i>N</i> -glucuronides	1D ¹ H, ROESY	II
Clenbuterol <i>O</i> -glucuronides	1D ¹ H, HSQC, HMBC	IV

^aDQFCOSY, double quantum filtered correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; ROESY, rotating Overhauser effect spectroscopy.

3.3 Enzyme Activity Assays (I, III, IV)

Glucuronidation screening assays of dobutamine (I), losartan (III), candesartan (III), zolarsartan (III), and clenbuterol (IV) with recombinant human UGTs and liver microsomes from various species were carried out in 250 μ l reactions containing various concentrations of aglycone, 5 mM saccharic acid 1,4-lactone, 5 mM UDPGlcA, 5 mM MgCl₂, 50 mM phosphate buffer (pH 7.4), and 625 μ g protein (recombinant UGT membrane or microsome). The substrates were added as dimethylsulfoxide solution (2% in the final reaction volume). Enzyme reactions were incubated at 37 °C for four hours.

Kinetic analyses of the formation of losartan *N*2-glucuronide were carried out with human liver microsomes and recombinant UGTs 1A1, 1A3, and 2B7 (III). Concentration of losartan in the samples varied between 2 and 600 μ M. The protein concentration and incubation time were within the linear range with respect to both parameters. Otherwise

the reactions were carried similarly to the screening assays described above. The kinetic parameters were obtained by fitting the observed velocities to kinetic models using GraphPad Prism 4.02 for Windows (GraphPad Software Inc., San Diego, CA, USA). The kinetic models were Michaelis–Menten ($v = V_{\max} [S] / (K_m + [S])$) and the substrate inhibition equation ($v = V_{\max} / (1 + (K_m / [S]) + ([S] / K_{si}))$).

The enzyme reactions were analyzed by an Agilent 1100 Series HPLC equipped with an autosampler and the Agilent 1100 Series fluorescence or UV multiwavelength detector (Waldbronn, Germany).

4 Results and Discussion

The results obtained in this work are described and discussed briefly in this section. More details can be found in the original publications (I-IV).

4.1 Glucuronide Syntheses

Altogether, 13 different glucuronides were synthesized from dobutamine, losartan, candesartan, zolansartan, and clenbuterol. Clenbuterol glucuronides were synthesized by the Koenigs-Knorr reaction (IV), whereas the others were prepared using enzyme-assisted synthesis (I, II).

Yields and Purities

The yields and purities of the synthesized glucuronides are presented in Table 7. The amounts of glucuronides obtained in this study were sufficient for two-dimensional NMR analyses to characterize the detailed structures of the synthesis products. The synthesized glucuronides were also used as reference compounds in enzyme screening assays (I, III, IV). Over 90% purity was observed for nine glucuronides and those could also be used as standards in quantitative analytical studies. The synthesized losartan *N*₂-glucuronide was used as a standard compound for enzyme kinetic analyses (III). In this study the purities were assessed using HPLC with fluorescence (I) or UV (II, IV) detection, assuming that the impurities have the same fluorescence or absorbance as the glucuronide isomer. Impurities that did not have a chromophore were not detected. For more exact results of the purities, evidence obtained with other methods could have been included. Residues of the aglycone and other isomers were identified on the basis of their retention times, but the origin of the other impurities found were not studied in detail. It is obvious that some of the detected impurities were formed by degradation after isolation of the glucuronides, since the purity analyses were performed by liquid chromatography with a C18 reversed phase column, as was the separation done when isolating the glucuronides by fractionation. Contamination of the synthesis products is also possible.

Table 7. *The yields and purities of synthesized glucuronides.*

Glucuronide (Publication)	Enzyme source / method ^a	Yield	Purity ^b
<u>Dobutamine (I)^c</u>			
Phenolic <i>O</i> -glucuronide	Pig liver microsomes	1.7 mg (4.6%)	> 97%
Catecholic <i>para O</i> -glucuronide		3.5 mg (9.3%)	> 97%
Catecholic <i>meta O</i> -glucuronide		6.2 mg (16.6%)	> 97%
<u>Losartan (II)</u>			
<i>O</i> -Glucuronide	Bovine liver microsomes	0.5 mg (1.1%)	> 97%
<i>N1</i> -Glucuronide	UGT1A10	0.9 mg (2.2%)	> 97%
<i>N2</i> -Glucuronide	Moose liver microsomes	2.0 mg (7.4%)	> 97%
<u>Candesartan (II)</u>			
Acyl <i>O</i> -glucuronide	UGT1A8	0.9 mg (2.8%)	> 85%
<i>N2</i> -Glucuronide	UGT1A3	1.5 mg (4.0%)	> 90%
<u>Zolarsartan (II)</u>			
Acyl <i>O</i> -glucuronide	Pig liver microsomes	1.6 mg (4.3%)	> 60%
<i>N1</i> -Glucuronide	Rat liver microsomes	3.6 mg (34.6%)	> 80%
<i>N2</i> -Glucuronide	UGT1A3	6.8 mg (33.9%)	> 97%
<u>Clenbuterol (IV)^c</u>			
<i>O</i> -Glucuronide 1	Koenigs-Knorr	1.6 mg (0.9%)	> 90%
<i>O</i> -Glucuronide 2		1.3 mg (0.7%)	> 75%

^aClenbuterol glucuronides were synthesized by the Koenigs-Knorr reaction, whereas the others were prepared using enzyme-assisted synthesis.

^bDetermined by HPLC-UV, except dobutamine glucuronides, which were determined by HPLC-fluorescence.

^cAll glucuronide isomers from the aglycone were synthesized in one reaction mixture.

In this study, the percentage yields of the glucuronides were often low (Table 7). Large variation in yields of glucuronide syntheses have been described: 4–78% for enzyme-assisted syntheses [35, 36, 38-40] and 0.6–82% for Koenigs-Knorr reaction [30]. Compared to these results the yields obtained in this study were not as high as could have been expected. Higher yields could have been obtained by further optimization of the reactions. The preliminary experiments for the enzyme-assisted syntheses included tests for best biocatalyst, optimal concentration of aglycone and protein, and incubation time (I, II). An interesting detail was observed when optimizing the incubation time of enzyme-assisted syntheses (II), namely that recombinant human UGTs were active for a much longer time than liver microsomes. This would be nice to study more systematically to find a possible explanation. Other researchers have found that glucuronidation activity can also be affected by the presence of alamethicin [19, 94-96] and bovine serum albumin [97, 98], the concentrations of detergents [94, 96, 97], UDPGlcA [40, 97, 99], and buffer,

as well as pH [94, 100]. In the chemical synthesis of clenbuterol glucuronides (IV), optimized reaction conditions, or even a chemical method other than the Koenigs-Knorr reaction could have been used to increase the yields. Thorough optimization of reaction conditions for enzymatic and chemical glucuronide syntheses, however, would increase the time and money consumed. As shown in this study, optimization of the most important factors is sufficient for easy and efficient synthesis of glucuronide metabolites in milligram scale.

It was remarkable that neither hydrolysis nor acyl migration were detected in the syntheses of candesartan and zolersartan *O*-glucuronides (II), even though acyl glucuronides are usually considered to be relatively labile [24, 25]. Acyl glucuronides require special attention in drug research because of their possible toxicity [21, 24-27]. Studying the instability of acyl glucuronide metabolites of drug candidates is also important because such glucuronides are susceptible to enterohepatic circulation, or they may otherwise have prolonged action.

Both enzyme-assisted and traditional chemical syntheses of glucuronide metabolites had their advantages and disadvantages in this study. Enzyme-catalyzed reactions of losartan *N*2-glucuronide by moose liver microsomes, candesartan *O*-glucuronide by human UGT1A8, and candesartan *N*2-glucuronide and zolersartan *N*2-glucuronide by human UGT1A3 were regiospecific (III). In these cases, optimized SPE for the isolation of the synthesis products could have been a reasonable alternative to HPLC fractionation (II). After incubation, careful removal of proteins from the reaction mixtures and the subsequent SPEs were time consuming, but they were important for successful HPLC fractionation. Preliminary experiments in small scale needed to be performed before each large scale enzyme-assisted synthesis, because the yields were strongly dependent on the specific activity of the enzyme preparation that was used as catalyst. In this study the Koenigs-Knorr reaction produced sufficient amounts of glucuronides without any optimization of the reaction conditions (IV). The synthesis consisted of two steps and was fairly simple and straightforward. On the other hand, the reaction mixture included various compounds due to formation of intermediate products, which were the protected glucuronides from the first step as well as the partially and fully deprotected glucuronides from the second step. In addition, the reaction monitoring was difficult.

Taken together, it can be seen that synthesis of milligram amounts of glucuronides by enzyme-assisted methods and the Koenigs-Knorr reaction is relatively easy. Based on the research done in this study, to synthesize glucuronide metabolites the enzymatic method is more convenient if the affinity of the aglycone substrate for the enzyme is high, but otherwise the traditional chemical method would generally be preferred.

Structural Characterizations

The MS spectra of the synthesis products in positive and negative ion modes showed protonated and deprotonated glucuronides, respectively (I, II, IV). The compounds were identified as glucuronides on the basis of m/z values, which were 176 Da higher than those of the corresponding aglycones. These glucuronide ions were selected as precursors for the MS/MS analysis. In most cases the MS/MS spectra did not show any diagnostic ions for the determination of the glucuronidation site. The only exceptions were obtained in the negative ion mode MS/MS spectra of clenbuterol glucuronides, which showed abundant product ion at m/z 193, corresponding to deprotonated glucuronic acid (IV). This gave strong evidence that both clenbuterol glucuronides were *O*-glucuronides [33, 37, 49].

Detailed structures of the synthesized glucuronides (Fig. 7) were derived from their NMR spectra (I, II, IV). Interglycosidic correlations for determination of the glucuronidation sites were observed from NOE, ROESY, and/or HMBC spectra. The HMBC spectrum of clenbuterol *O*-glucuronide 1 is shown as an example in Figure 8 (IV). Racemic dobutamine (I) and clenbuterol (IV) aglycones were used as the starting material for their glucuronide syntheses. Both syntheses yielded glucuronide diastereomers. HPLC with a C18 reversed phase column was used in the isolation of the glucuronidies, but it did not separate dobutamine diastereomers whereas clenbuterol diastereomers were separated easily. It would have been interesting to identify the two clenbuterol *O*-glucuronide diastereomers, but that would have required pure enantiomers of clenbuterol aglycone.

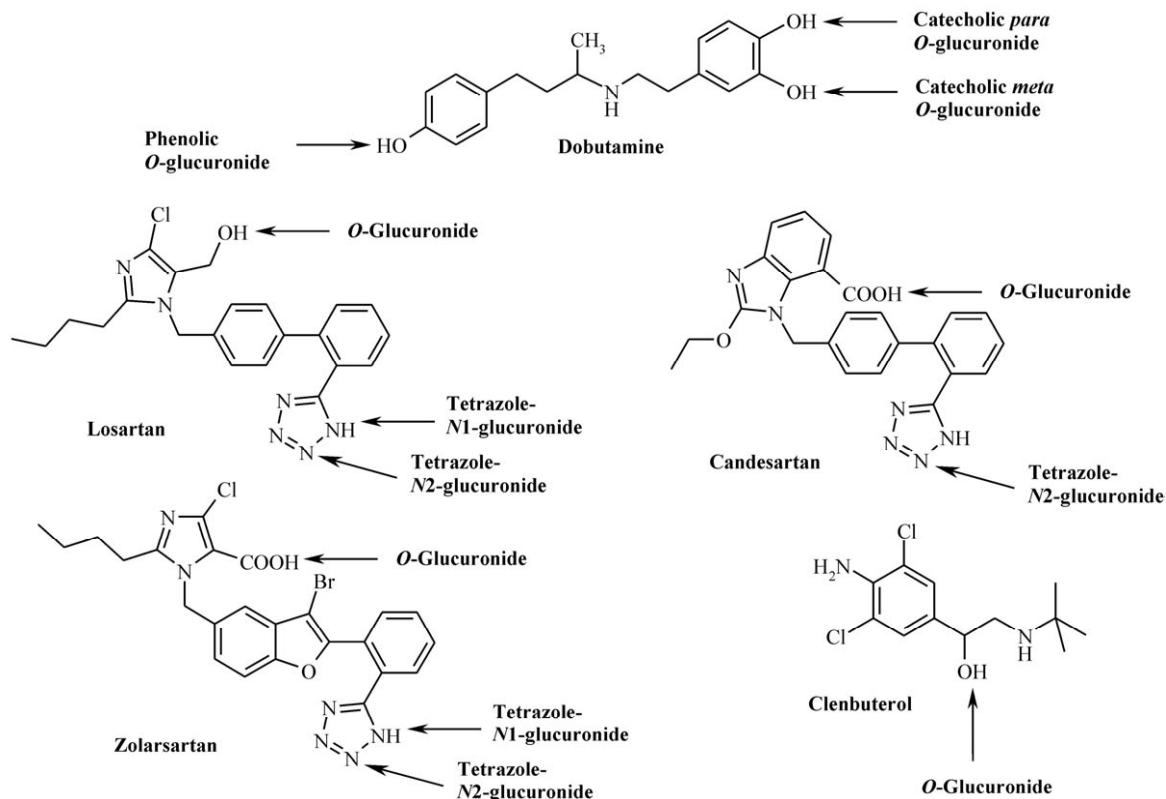


Figure 7 Glucuronidation sites of the synthesized glucuronides.

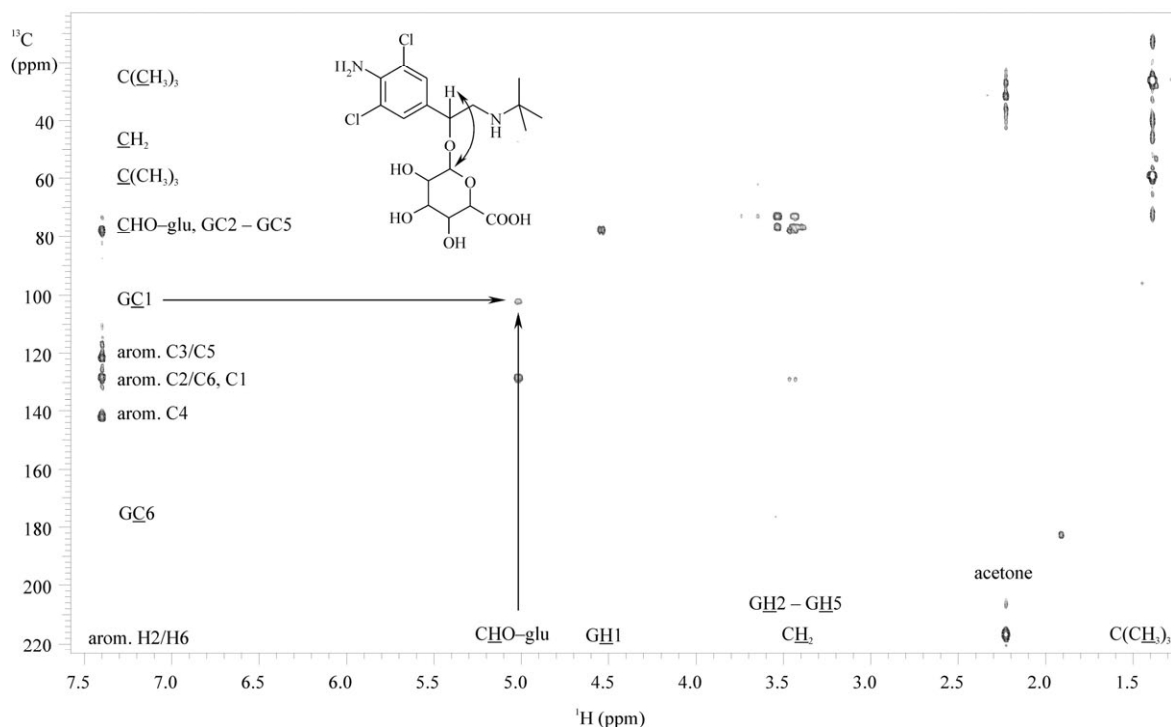


Figure 8 *HMBC spectrum of clenbuterol O-glucuronide 1 (I). The arrows indicate the correlation between the glucuronic carbon GC1 (102 ppm) and the benzylic proton CHO-glucuronide (5.01 ppm) that confirmed the compound as O-glucuronide of clenbuterol.*

4.2 Enzyme Activity Assays

The glucuronidation of dobutamine, losartan, candesartan, zolersartan, and clenbuterol was studied with liver microsomes from various species and with human recombinant UGTs (I, III, IV).

Liver Microsomes

The analyses of the glucuronidation activity of liver microsomes from various animals revealed that there are major differences in glucuronidation properties between species (Table 8). Compared to animal liver microsomes, human liver microsomes were typically inefficient at glucuronidating the studied aglycones. It would have been useful to find that some of the studied animal liver microsomes were good models for predicting drug glucuronidation in humans in general, qualitatively or semi-quantitatively. However, that kind of trend was not observed in this study. Of the species studied here, rat is probably the most common laboratory animal. The glucuronidation profile of rat and human, however, were quite different. The interspecies differences in glucuronidation need to be carefully taken into account when designing metabolism experiments with drugs that are

intended for humans. UGTs in the livers of different animals do not have identical function, activity, substrate specificity, or distribution.

Table 8. *Relative formation of different glucuronides from dobutamine (1 mM) (I), losartan (1mM) (III), candesartan (1 mM) (III), zolarsartan (1 mM) (III), and clenbuterol (1.5 mM) (IV) by liver microsomes from various species.^a*

Glucuronide	Human	Rabbit	Rat	Pig	Moose	Bovine	Dog
<u>Dobutamine</u>							
Phenolic <i>O</i> -glucuronide	+	++	+++	+++	–	–	–
Catecholic <i>para O</i> -glucuronide	+	+++*	+++	++	–	–	–
Catecholic <i>meta O</i> -glucuronide	++	++	++	+++	–	–	–
<u>Losartan</u>							
<i>O</i> -Glucuronide	+	++	+	+	+	+	–
<i>N1</i> -Glucuronide	+	+	+	+	n.d.	n.d.	–
<i>N2</i> -Glucuronide	++	++	++	++	+++*	++	–
<u>Candesartan</u>							
Acyl <i>O</i> -glucuronide	+	++	+++*	+	++	+	–
<i>N2</i> -Glucuronide	+	+	++	+	+	+	–
<u>Zolarsartan</u>							
Acyl <i>O</i> -glucuronide	++	+++	++	+++*	++	+++	–
<i>N1</i> -Glucuronide	n.d.	n.d.	+++	++	+	+	–
<i>N2</i> -Glucuronide	+	n.d.	n.d.	+	n.d.	n.d.	–
<u>Clenbuterol</u>							
<i>O</i> -Glucuronide 1	+	+	++	+	+	+++*	n.d.
<i>O</i> -Glucuronide 2	n.d.	++	+	n.d.	+	+	+++

^a+++* = Largest peak area of glucuronide isomer in HPLC chromatograms obtained from microsome screening assays using one aglycone; + = < 10%, ++ = 10–50%, +++ = > 50%, a percentage of the largest peak area (+++*) of glucuronide isomer; n.d. = not detected; – = not studied.

Recombinant Human UGTs

The fact that UGTs have overlapping substrate specificities was shown again in this study. Glucuronidation of dobutamine, losartan, candesartan, and zolarsartan was catalyzed by several human UGT isoforms (Fig. 9) (I, III). Clenbuterol was glucuronidated only by UGTs 1A7, 1A9, and 1A10 (IV). Human UGTs 1A7–1A10 exhibit 83% identity at the amino acid sequence level [3], and for UGTs 1A7–1A9 this was reflected in their similar

ability to form glucuronide isomers of the studied aglycones. The versatility of UGT1A10 was an exception in this group (Fig. 9). It was able to efficiently glucuronidate both catecholic hydroxyls of dobutamine, not only the hydroxyl in the *meta* position (I). It was also the only isoform that catalyzed the formation of *N*1- and *N*2-glucuronide of losartan at significant rates (III). UGT1A10 also glucuronidated zolarsartan quite differently than UGTs 1A7–1A9; it effectively produced *O*-glucuronide and to a lesser degree *N*1-glucuronide of zolarsartan, whereas UGTs 1A7–1A9 produced only traces, if any, of zolarsartan glucuronides (III).

The high regioselectivity of human UGT1A3 towards the tetrazole *N*2 of sartans was remarkable (Fig. 9) (III). UGT1A4 has been considered to be the main *N*-glucuronidation isoform, followed by UGT1A3 [6, 16, 17, 101, 102]. The results of the present study revealed that many UGTs are able to catalyze the formation of tetrazole-*N*-glucuronides, although UGT1A4 exhibited no significant activity towards any of the three sartans studied here. A probable reason for this is the nature of the tetrazole ring. Tetrazole is acidic, with a pK_a value of 4.9, whereas amines, which are the more common *N*-glucuronidation substrates of UGT1A4, are basic. In addition to tetrazoles, carboxylic acids are often glucuronidated by UGT1A3 [11, 21, 24, 101, 103, 104], which can be explained by the fact that tetrazoles have been used as non-classical isosteres for carboxylic acid moieties in bioactive molecules [105, 106]. It has been previously published that UGT1A3 catalyzes *N*2-glucuronidation of tetrazoles [104, 107], and so the results obtained in this study with different tetrazoles strengthens this observation. Based on these results, it is likely that other tetrazole-containing compounds, for example irbesartan [108, 109], are actively glucuronidated to tetrazole-*N*2-glucuronides by this enzyme.

Zolarsartan could be used as a specific probe for the presence of active UGT1A3 in human tissue samples, since UGT1A3 was the only isoform that catalyzed the formation of zolarsartan *N*2-glucuronide (Fig. 9) (III). However, the glucuronidation is only a minor metabolic route for zolarsartan and the amounts of zolarsartan glucuronides were very small in the incubation reactions. In practice this would result in insuperable difficulties. For example, determining the kinetic parameters for zolarsartan would be very challenging; at the least very sensitive analytical equipment and methods would be needed. In any case, the results of this study could be exploited in developing a derivative with a biphenyl tetrazole-ring structure that could act as a human UGT1A3-selective probe compound with high affinity.

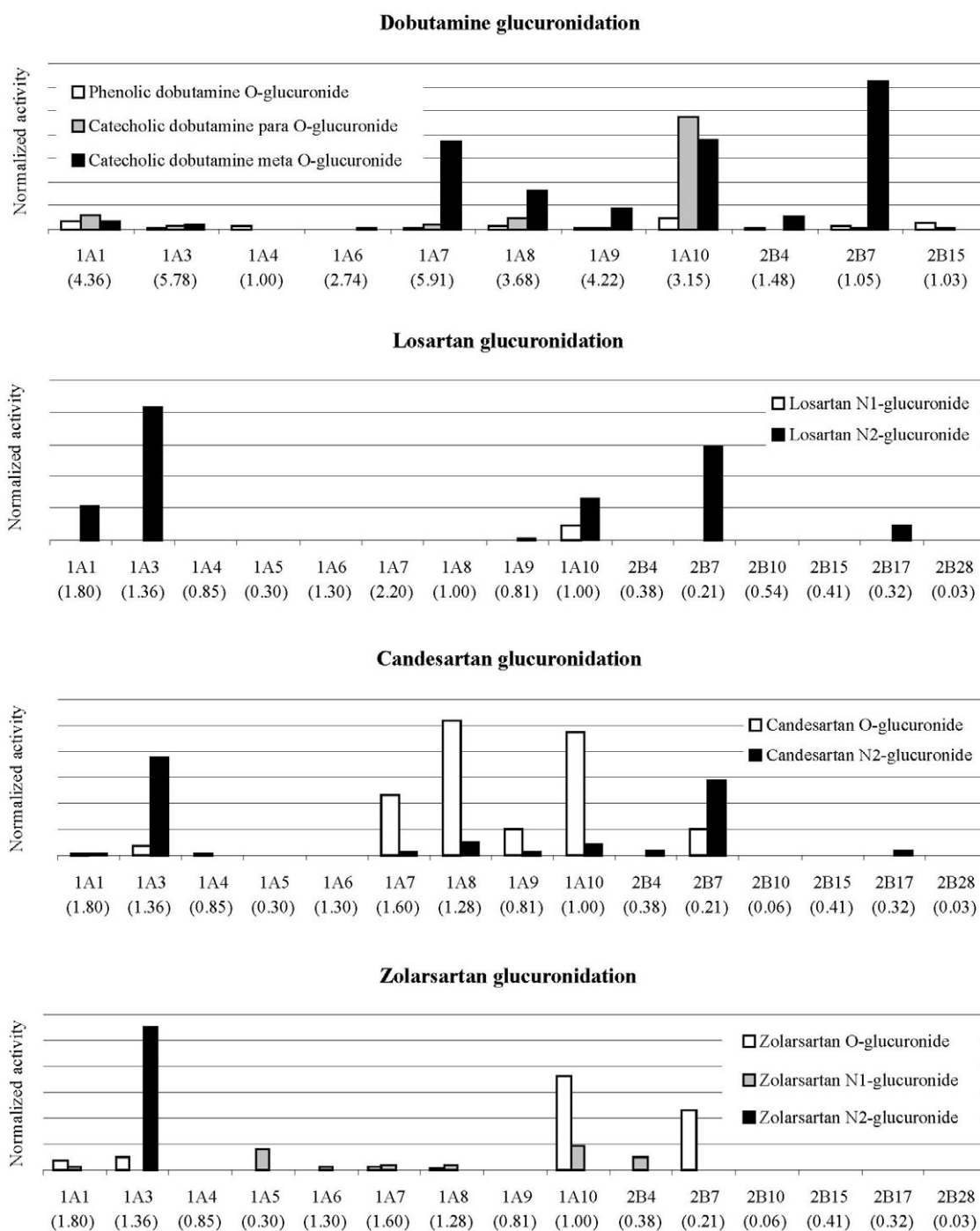


Figure 9 *Glucuronidation activities of recombinant human UGTs towards dobutamine (I), losartan (III), candesartan (III), and zolarsartan (III). The normalized activity (activity corrected for expression level of individual UGTs) is presented and relative expression levels of the different recombinant UGTs are given in parenthesis under the enzyme name. Activity results are averages of duplicate samples, where the aglycone and protein concentrations were 1 mM and 2.5 mg/ml, respectively, and the incubation time was four hours.*

Predicting glucuronidation in human liver microsomes based on the data obtained with recombinant hepatic UGTs was difficult in this study. Although the profile of the glucuronide isomer formation of dobutamine, losartan, candesartan, and clenbuterol seemed to correlate between the hepatic UGTs and human liver microsomes, the glucuronidation of zolarsartan was a bit more complicated (I, III). Despite the high zolarsartan glucuronidation activity of UGT1A3, it only has minor involvement in human liver microsomes where *N*2-glucuronide was a minor product (Table 8) (III). This situation was also seen in losartan tetrazole-*N*2-glucuronidation when the kinetics was examined using human liver microsomes and UGTs 1A1, 1A3, and 2B7 (Fig. 10) (III). UGT1A3 exhibited clear substrate inhibition kinetics, a phenomenon not seen in human liver microsomes. The kinetic analyses of UGTs 1A1 and 2B7 suggest that both of them contribute significantly, and rather similarly, to losartan glucuronidation by human liver microsomes. The explanation for both these observations may be that although UGT1A3 is expressed in the human liver, its expression level is much lower than that of UGTs 1A1 and 2B7 [110].

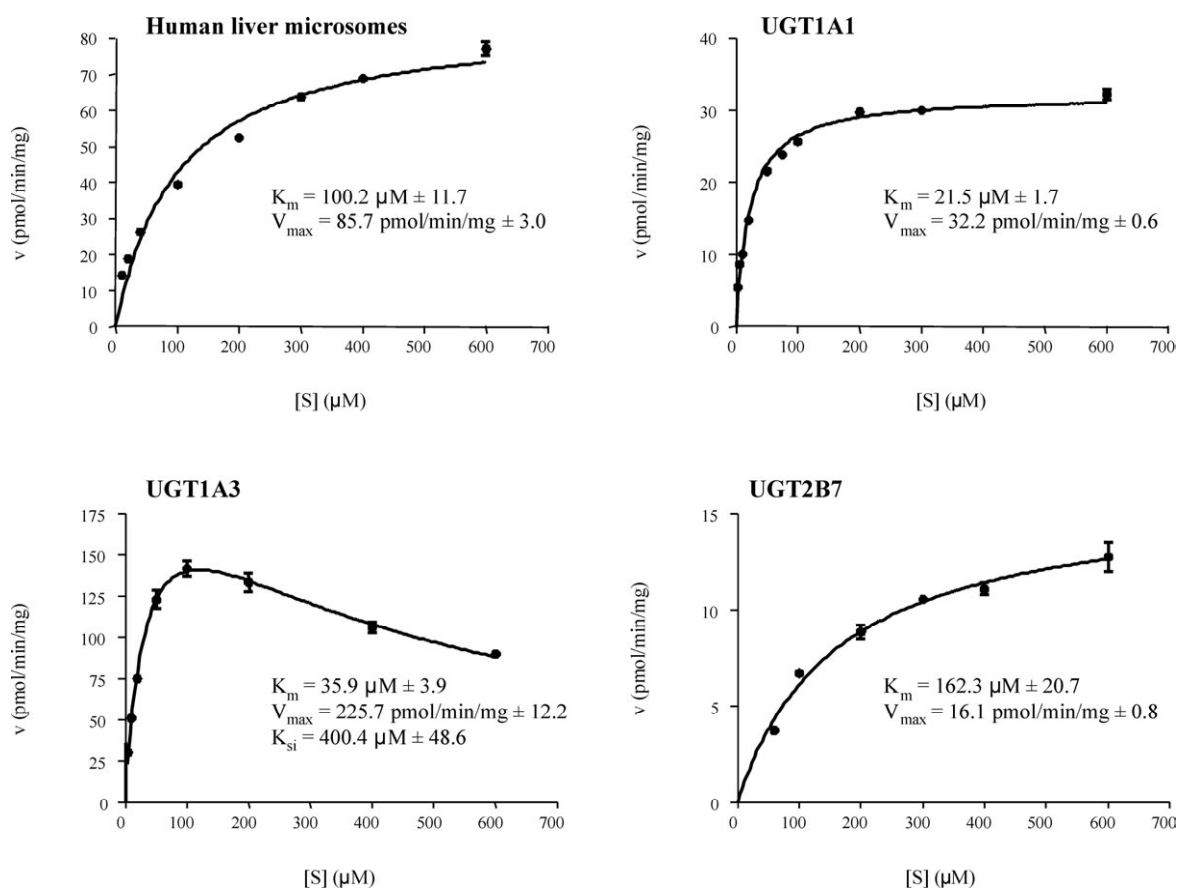


Figure 10 Kinetics of losartan glucuronidation by human liver microsomes, and recombinant human UGTs 1A1, 1A3, and 2B7. The data points were fitted to the Michaelis-Menten (human liver microsomes and UGTs 1A1 and 2B7) or the substrate inhibition (UGT1A3) equations (III).

4 Conclusions

Drug metabolites are needed for pharmaceutical investigations to study the properties of these compounds, as well as the characteristics of the catalyzing enzymes. In this study, glucuronide regio- and stereoisomers were synthesized in milligram scale using both enzymatic and traditional chemical synthesis methods. Two of the studied aglycones, candesartan and zolarsartan, were carboxylic acids that were glucuronidated to acyl glucuronides. The possibility that acyl glucuronide metabolites can be toxic must be taken into account in drug development. In the enzyme-assisted syntheses of *O*-glucuronides of candesartan and zolarsartan neither hydrolysis nor acyl migration were detected, although acyl glucuronides are usually considered to be relatively labile. MS and NMR were used for identification and structural characterization of the synthesized glucuronides. Different NMR techniques, such as NOE, ROESY, and HMBC, were essential for unambiguous determination of the glucuronidation sites of the isomers.

The glucuronidation screening assays with liver microsomes from different species revealed that the results obtained with animal models do not necessarily tell much about the glucuronidation of the drug in humans. Since no crystallographic structure of any UGT is available, the functions and catalytic mechanisms of these enzymes have to be investigated by indirect methods. In this study, new knowledge about substrate specificities of the UGTs was gained from the screening assays, particularly among glucuronide regioisomers. The results of this study have been exploited to study the differences between UGTs 1A9 and 1A10, two human UGTs that are very similar at the level of amino acid sequence, but differ greatly in substrate specificity.

In drug discovery, *in vitro* metabolism studies of a drug candidate are part of the non-clinical development. However, the compounds studied in this work, except zolarsartan, are already in clinical use. Would the drug discovery process of these drugs have been changed, if the results obtained in this study were already available at that time? Probably not, since all the studied drugs were glucuronidated by several UGTs, reducing the risk of drug-drug interactions and adverse reactions caused by polymorphic UGTs. Therapeutically notable is the formation of candesartan acyl glucuronide, because the metabolite can be toxic. Therefore the binding of candesartan acyl glucuronides to the macromolecules and the formation of candesartan acyl glucuronides *in vivo* would be worth further research.

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