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DIPLOMARBEIT

EFFECTS OF BSA ON IN VITRO GLUCURONIDATION STUDIES USING HUMAN MICROSOMES AND RECOMBINANT ENZYMES

Verfasser

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List of abbreviations

4MU	4-Metylumbelliferone
BAK	Benzalkonium chloride
BSA	Bovine serum albumin
CYP 450	Cytochrome P450
FA	Fatty acid
f_u	Fraction unbound
HEK293	Human embryonic kidney cell lines 293
HLM	Human liver microsomes
MM	Michaelis Menten
Sf9	Spodoptera frugiperda
UDP	Uridine diphosphate
UDPGA	UDP- glucuronic acid
UGT	UDP- glucuronosyltransferase

Zusammenfassung

Einleitung UDP-glucuronosyltransferasen (UGTs) bilden eine Familie von u. A. humanen Metabolisierungsenzymen. UGTs transferieren Glucuronsäure vom Cosubstrat (UDP-Glucuronsäure) auf ein Substrat. Dadurch wird das Substrat hydrophiler und somit besser ausscheidbar.

Um die Verfügbarkeit von Arzneistoffen im Körper zu ermitteln und um Nebenwirkungen zu verhindern, werden *in vitro* Tests entwickelt, die es ermöglichen die katalytische Aktivität der UGTs vorherzusagen. Ältere Publikationen zeigten, dass das Zugeben von Rinderserumalbumin (BSA) bei *in vitro* Tests, die Aktivität dieser Enzyme steigerte. Diese Wirkung entsteht wahrscheinlich durch das Entfernen von kompetitiv inhibierenden Fettsäuren. Durch klinische Studien wurde nachgewiesen, dass auf diese Weise erworbene Daten besser mit *in vivo* Daten übereinstimmen.

Ziele Mit dieser Arbeit wollten wir die Wirkung von BSA auf die Glucuronidierung von 4-Methylumbelliferon (4MU) und Entacapon durch eine Gruppe von unerforschten UGT Isoformen (UGTs 1A7, 1A8, 1A10, 2A1 und 2A15) analysieren. Die Glucuronidierung von 4MU durch UGT1A9 wurde durchgeführt, um unser System mit schon publizierten Daten zu vergleichen. Zum selben Zweck wurden auch kommerziell verfügbare humane Lebermikrosomen (HLM) und Darmmikrosomen (HIM) getestet.

Ergebnisse und Diskussion Mit Ausnahme der Kinetiken von 4MU durch HIM, konnten wir zeigen, dass alle getesteten Enzyme von einem nicht-kompetitiven Inhibitor (genauer „mixed type Inhibitor“) beeinflusst wurden. Dadurch dass die Entfernung des Inhibitors (vermutlich eine Mischung an Fettsäuren) eine weitere Bindetasche für das Substrat freistellte, folgerten wir, dass das Substrat und die Fettsäuren um die selbe inhibierende Bindetasche konkurrieren. Angesichts der Tatsache, dass Fettsäuren auch an die katalytische Tasche binden können, vermuteten wir, dass diese, dem Anschein nach ähnliche Taschen, die homologen Taschen eines Enyzmdimers

darstellen. Das Auftreten von Dimeren in UGTs wurde schon in früheren Publikationen postuliert, aber noch nicht vollkommen bestätigt.

Abstract

Introduction UDP-glucuronosyltransferases (UGTs) are a family of metabolizing enzymes present in the human body. The UGTs transfer a moiety of glucuronic acid from the cosubstrate UDP-glucuronic acid (UDP-GA) to the substrate in order to make it more hydrophilic and therefore better excretable from the body.

In vitro tests for the prediction of the catalytic activity of UGTs are being developed to analyze the availability of drugs in the body and to reduce eventual side effects. It has been found, that the addition of BSA to the incubation mixture increases the activity of these enzymes *in vitro*, probably by removing competitively inhibiting fatty acids (FAs). Data retrieved by this method have shown to be more consistent to the data evaluated *in vivo*.

Aims Our objectives were to test the effect of BSA on the glucuronidation of 4-methylumbelliferone (4MU) and entacapone by an untested group of UGTs which includes the UGTs 1A7, 1A8, 1A10, 2A1 and 2B15. The glucuronidation of 4MU by UGT1A9 was performed to compare our system with the previously published data. Also the commercially available HLM and HIM have been tested for the purposes of comparison.

Results and discussion With the exception of the kinetics of 4MU by HIM, all our tested enzymes were shown here to be inhibited by a mixed type inhibitor. Contrary to earlier findings from another laboratory, the inhibitor in our system was not only binding to the same binding site as the aglycone substrate. Given by the observation that the inhibitor(s) removal increased or even unmasked the presence of a second substrate binding site, we concluded that the inhibitor (possibly fatty acids) and the substrate have affinity to both a catalytic and an inhibititory binding site. This suggestion is in agreement with the possibility that UGTs can form homo- and hetero-dimers, and that interactions occur between the two monomers within the dimeric UGT.

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1 Introduction

This thesis talks about UDP-glucuronosyltransferases (UGTs), a family of enzymes present in various organs and tissues of the human body. UGTs catalyze the attachment of the glucuronic acid moiety from the donor UDP-glucuronic acid (UDP-GA) to a suitable nucleophilic group on the aglycone substrate molecule. This conjugation, also called glucuronidation, makes the substrate more water soluble and better excretable from the body. The present work tries to find a way to improve experimental methods for the evaluation of the activity of the UGTs, thereby also answering some questions about the mechanism of these enzymes.

1.1 Pharmacokinetics, (L)ADME scheme

The aim of pharmacokinetic studies is to investigate the pathway of a substance through the human body. There are 4 steps, which can influence the time between the uptake of the substance and its elimination. The drug, or non-drug xenobiotic, has to enter first into the blood circulation (Absorption), from where it can be transported to various tissues and organs (Distribution). The modification of the substrate during the process (Metabolism) is an important step towards possible inactivation and better elimination (Excretion) of the compound. In the last few years, some formulations have been developed that are able to modify the release of the drug. This may add a Liberation step to the pathway, forming the abbreviation (L)ADME.

1.1.1 Metabolism

Many exogenous and endogenous compounds the human body has to deal with are lipophilic (fat soluble). Since the elimination of substances is performed through a hydrophilic medium (mainly urine and bile), lipophilic compounds have to be converted into more polar molecules first. This modification is generally achieved through metabolism. In addition to enhanced excretion by metabolism, most of the compounds lose or decrease their biological activity by

getting modified. The metabolism system is, therefore, an important step for the detoxification. There are some compounds, however, that are activated by biotransformation, even by glucuronidation. One example for this is the analgesic drug codeine that is metabolized by CYP2D6 into the active form morphine (Caraco et al., 1999). Further metabolism by UGT2B7 forms the even 100 times more potent compound morphine-6-glucuronide (Paul et al., 1989). Codeine can, therefore, be defined as a prodrug.

Drug metabolism in the human body is executed by several groups of enzymes that catalyze different reactions. This process is traditionally divided into two “phases”, I and II. The ‘Phase I’ enzymes are in charge of the functionalisation of the compound, where polar functional groups are added, or already present groups are unmasked. Such functional groups include hydroxyl (-OH), carboxyl, ($-CO_2H$) or amino ($-NH_2$) moieties. The most important functionalisation enzymes by number and activity are the Cytochromes P450 monooxygenases (CYP450), which are also the most important drug metabolizing enzymes in general.

The ‘Phase II’ enzymes catalyze conjugation reactions. Polar endogenous compounds such as glucuronic acid, sulfate or glutathione are covalently linked to a suitable functional group of the substrate. An exception in this group are the acetyltransferases that catalyze the transfer of an acetyl group to the substrate. The formed product gets therefore more lipophilic. The main Phase II enzymes are the UDP-glucuronosyltransferases (UGTs).

Since conjugation is performed onto functional groups, phase II enzymes mainly metabolize compounds only after fictionalization by phase I enzymes. However, in many cases compounds already have suitable functional groups and do not require phase I reactions. In addition, many compounds are secreted already after phase I metabolism, without further conjugation, and already hydrophilic compounds can be eliminated without any metabolism at all.

1.2 UDP-glucuronosyltransferases

1.2.1 Function and mechanism

UGTs are involved in the metabolism of a wide range of exogenous compounds (xenobiotics) such as drugs, dietary chemicals and environmental pollutants, as well as endogenous compounds, including bilirubin, bile acids, fatty acids, steroid hormones, thyroid hormones and fat soluble vitamins (Miners and Mackenzie, 1991; Burchell et al., 1995; Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000; Kiang et al., 2005). The reaction in which the glucuronic acid from UDPGA (UDP- Glucuronic acid) is covalently linked to the substrate is referred to as glucuronidation. The linkage can be performed on different functional groups, containing oxygen or nitrogen. O-glucuronidation can occur on alcohols (ROH) or phenols (ArOH), while N-glucuronidation is carried out on either primary amines (RNH_2), secondary amines (RNR'H), or tertiary amines. Aromatic and heterocyclic amines (RNR'R'') can also be conjugated.

The reaction catalyzed by UGT occurs on the C_1 atom of the glucuronic acid and follows a second order nucleophilic substitution (S_N2) mechanism (Miners and Mackenzie, 1991; Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000). The heteroatom in the functional group of the substrate presents one or two free electron pairs that can bind to the C_1 atom. The main evidence that the reaction follows the S_N2 nucleophilic substitution mechanism is that it induces an inversion of the previous *α*- conformation into the *β-D*-glucuronide (Figure 1).

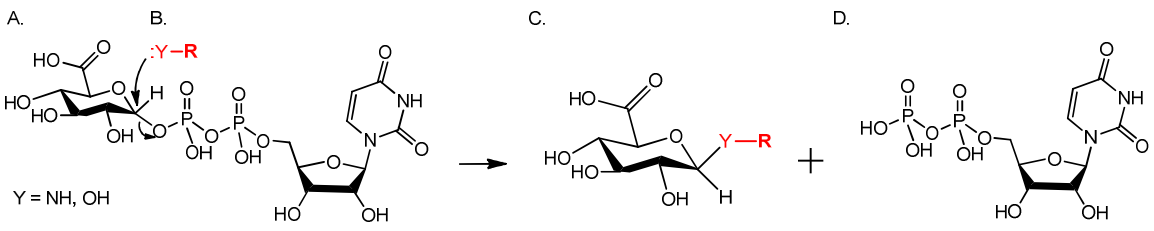


Figure 1. Glucuronidation of substrate by *SN2* mechanism. The nucleophilic heteroatom of the substrate (B.) binds to the *C1* atom of the glucuronic acid, removing the UDP (D.) from the linkage.

1.2.2 Catalytic mechanism

The glucuronidation of a substance by UGTs requires the presence of the cosubstrate UDPGA. Since the reaction yields two products out of two substrates (Figure 1), it can be defined as bi bi mechanism. It is still unclear, however, if the reaction follows a certain order for the binding of cosubstrate and aglycone. There are some data suggesting a compulsory ordered mechanism, where UDPGA has to bind to the free enzyme first (Figure 2) (Luukkanen et al., 2005; Patana et al., 2008), but a random ordered mechanism has not been fully excluded (see 1.3.3).

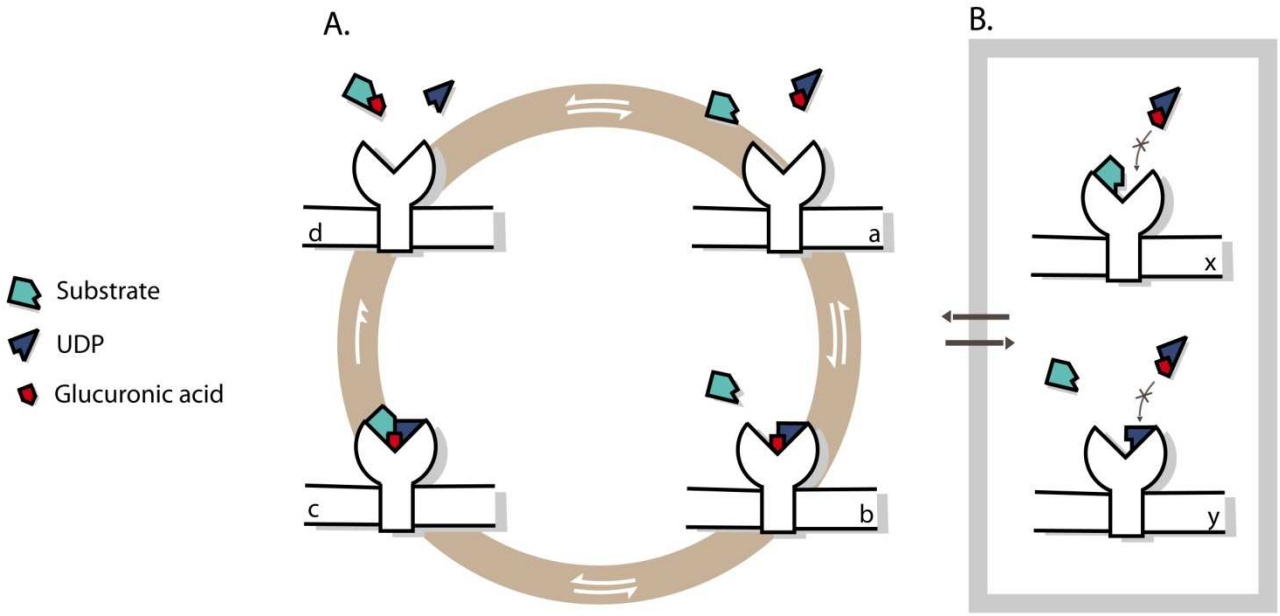


Figure 2. Catalytic cycle of UGTs
A. Glucuronidation by a compulsory ordered bi bi mechanism with UDPGA as first binding substance. Box B. shows two possible "dead end" or non-productive complexes due to either binding of aglycone substrate before UDPGA (x), or binding of the reaction product, UDP, instead of the co-substrate, UDPGA (y). In the case of either "dead end" enzymes further activity is only possible after the dissociation of the inhibiting substance. Complex a. is the conformation believed to be responsible for the atypical kinetic "substrate inhibition" (see 1.3.1)

1.2.3 Structure and Heterogeneity

The UGT protein is about 500-505 amino acids long, following the removal of the first 25 residues, the signal sequence, that directs the newly synthesized protein to the endoplasmic reticulum. The UGTs are probably dimeric proteins and it was suggested that they could form both homo-dimers and hetero-dimers, but more structural evidence is needed to verify this (Finel and Kurkela, 2008). The UGT monomer was suggested to have four different substructures, two large ones, the N-terminal and C-terminal domains, and two smaller ones that follow the C domain, the envelope helices and the transmembrane segment (Laakkonen and Finel, 2009). Apart from the more variable N-terminal domain, all substructures are very similar in all the isoforms, and among the UGT1 as, as well as between UGT2A1 and UGT2A2, they are identical due to exon sharing (Mackenzie et al., 2005). The C-domain contains the binding site for the common co-substrate UDPGA, although some residues in the N-terminal domain may also contribute to the co-substrate binding (Miley et al., 2007; Patana et al., 2007). It is understandable, therefore, that the binding site for the aglycone substrate is located in the N-terminal domain.

The aglycone substrate selectivity of individual UGT isoforms can sometimes be overlapping. The presence of backup proteins allows to metabolize via a secondary pathway in cases of miss functions of single isoforms. Diseases or drug-drug interactions caused by modifications of UGTs are, therefore, not very common. An important exception to this general rule is the Crigler- Najjars syndrome, caused by modifications of UGT1A1. The hemoglobin decomposition product bilirubin is a major substrate of UGT1A1, the only human enzyme that catalyzes its conjugation with glucuronic acid, a pre-requisite for its excretion (Petit et al., 2008). There are several modifications of UGT1A1 that can lower or abolish bilirubin metabolism. This can lead to elevated concentrations of unconjugated bilirubin in the blood and, subsequently, to severe jaundice and kernicterus.

To date 22 active UGT-encoding genes have been identified (Miners et al., 2004; Mackenzie et al., 2005) belonging to 4 different families. The relevant

families for this study, and for drug glucuronidation, are UGT1 and UGT2 that, together, include 19 different isoforms. Families UGT3 and UGT8 are of minor relevance.

The large human UGT1 (This family is commonly called UGT1A, but no other UGT1 subfamilies are known) gene is located on chromosome 2q37. The variability of the different UGT1A proteins is given by the alternative splicing of 9 first exons, with 4 exons that are shared by all of them. Each of the 9 first exons forms, together with the four common exons (2-5), one of the 9 functional transcripts UGT1A1 and UGT1A3-1A10. The four common exons generate the C-terminal part (Mackenzie et al., 2003), while the variable exon 1 is encoding the N- terminus.

The UGT2 family is encoded by separate genes located in chromosome 4q13 and is further divided in two subfamilies, UGT2A and UGT2B (Mackenzie et al., 2005). Those include three (2A1, 2A2 and 2A3) and seven (2B4, 2B7, 2B10, 2B11, 2B15, 2B17 and 2B28) members respectively. Each of the UGT2 genes has all its exons, with the exception of UGT2A1 and UGT2A2 that share the exons that encode the C-terminal half in a similar way to the exon sharing among the UGT1As. The UGT2A subfamily has a lower importance in systemic metabolism. UGT2A1 and UGT2A2 are mainly found in the olfactory epithelium (Jedlitschky et al., 1999; Somers et al., 2007; Sneitz et al., 2009), while UGT2A3 is expressed in the small intestine, liver and adipose tissue (Court et al., 2008). On the other hand, members of the UGT2B subfamily play important roles in the metabolism of drugs and endogenous compounds.

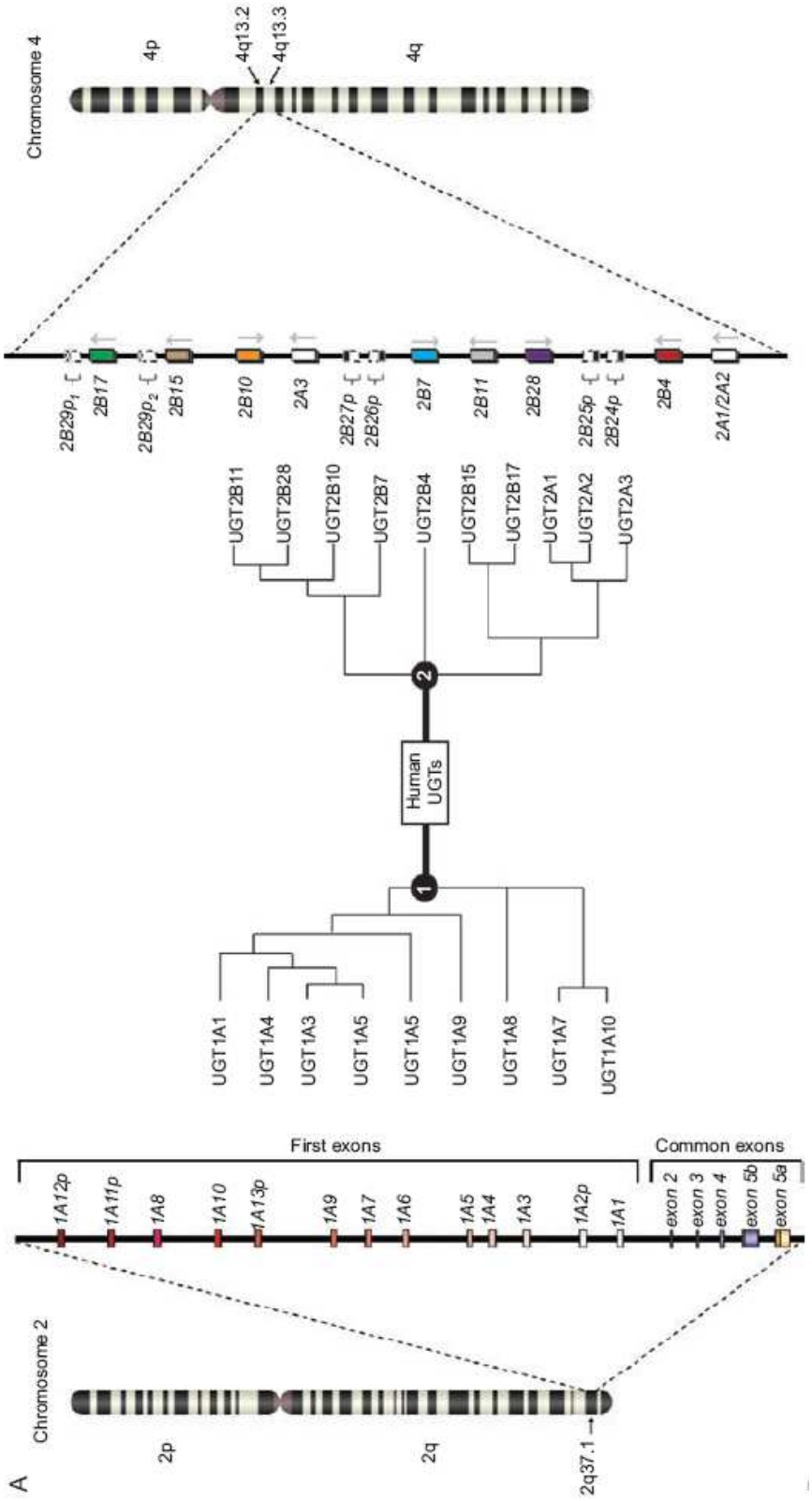


Figure 3. Family tree of human UGTs and location of the exons, needed for their translation (from Guillemette et al., 2010)

1.2.4 Tissue specificity

UGTs can be found in different tissues at different concentrations (Figure 4). The expression vary due to differences between individuals (Nishimura and Naito, 2006; Izukawa et al., 2009) and it is worth noting that the figure below is from mRNA determinations that is not always identical with the level of active proteins in the tissue.

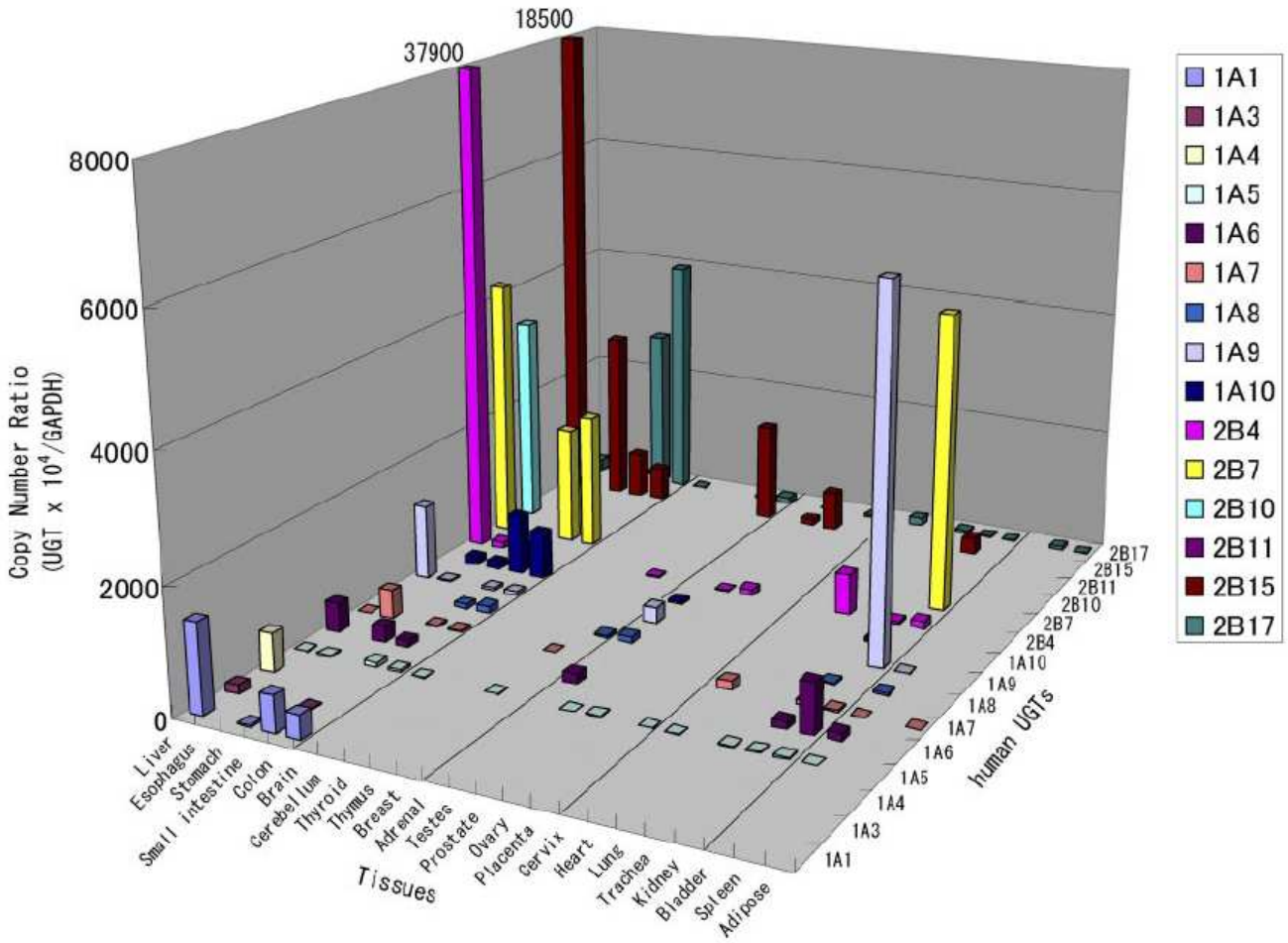


Figure 4. Expression levels of UGT1A and UGT2B families in human tissues. (from Ohno and Nakajin, 2009)

1.3 Enzyme Kinetics

1.3.1 Michaelis Menten equation and atypical kinetics

Enzyme kinetic studies are commonly used to analyze the activity of enzymes, to get insights into their mechanism, and the effect of different substances on the catalysis. The modality of the reaction has first been described by Michaelis and Menten (1913) and developed further by Briggs and Haldane (1925):

Equation 1

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

The assumptions are that the Enzyme (*E*) and the substrate (*S*) form a binary *ES* complex in order to yield the product (*P*) and restore free enzyme. The binary complex is usually stabilized by nonconvalent forces, including electrostatic interactions, hydrogen bonds, hydrophobic forces and van der Waals forces (Copeland, 2000). These weaker bonds allow a reversible equilibrium between the complex and the free forms of enzyme and substrate. This state of balance is described by rate constants k_1 and k_{-1}. The scheme assumes practical irreversible catalysis and, therefore, no reverse reaction is shown for the product formation. The rate constant for the catalysis and product release is given the symbol k_2.

There are two constants used to compare different kinetics. The K_m constant is defined by:

Equation 2

$$K_m = \frac{(k_2 + k_{-1})}{k_1}$$

Even though it is often used as a parameter for the substrate affinity to the enzyme, it must be noticed, that k_2 determines the rate of formation of the product. Therefore, K_m is only equal affinity if k_2 is much smaller than k_{-1}. The

constant V_{max} stands for the maximum reaction velocity, reached at a saturated concentration of substrate. It is defined by the following equation:

Equation 3

$$V_{\max} = k_2 \times [E_0]$$

where $[E_0]$ is the total concentration of enzyme present.

The Michaelis-Menten equation, modified by Briggs and Haldane (1925), combines the two constants, V_{max} and K_m, in a single equation:

Equation 4

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

where v is the rate of product formation.

Visualized as a graph, where the rate of product formation is plotted versus the substrate concentration, the Michaelis-Menten equation forms a hyperbolic curve. The plateau of this curve indicates the V_{max}, whereas the halfway between V_0 and V_{max} can be used to determine the K_m.

As shown in Figure 5, however, it is not always possible to describe kinetics by the simple Michaelis-Menten equation. In many cases atypical kinetics models, such as substrate inhibition, activation, autoactivation or biphasic mechanism (Hutzler et al., 2001), would fit best. Unfortunately, V_{max} and, particularly, K_m can not be used for meaningful comparisons of the reactions that proceed according to different kinetic models.

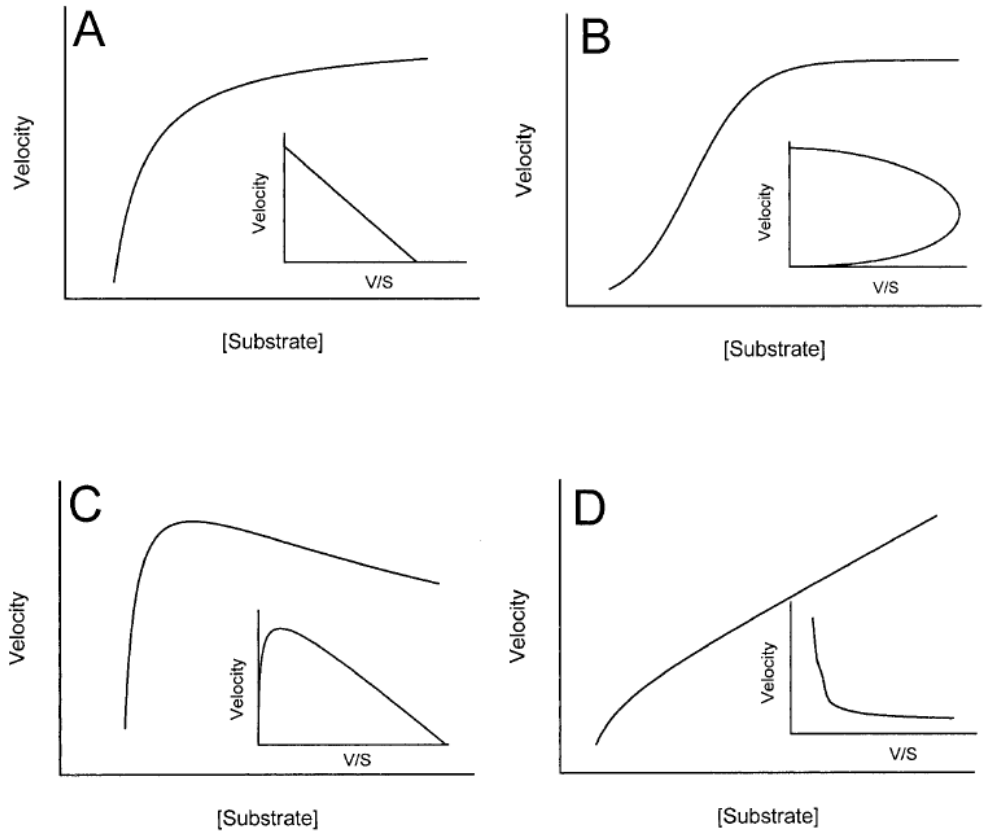


Figure 5. Graphs of Michaelis- Menten (A) and atypical kinetics (B, C, D), where the rate of product formation is plotted against the substrate concentration. The smaller graphs are samples of the Eadie-Hofstee diagram (from Hutzler and Tracy, 2002)

The shapes of the curves in a graph, where the velocity is plotted versus the substrate concentration, are usually not as clear as shown in Figure 5. Sometimes it is difficult or even impossible to detect an atypical kinetic mechanism by looking at the graph of the simple dependence of reaction rate on substrate concentration. Eadie-Hofstee plot is a useful method for better visualization of these phenomena. Atypical kinetics will deviate from the straight line (A) of the Michaelis-Menten kinetics in an Eadie-Hofstee plot, when the rate of product formation is plotted versus *v/[S]* in a typical manner. If the line is curved as shown in diagram C, the enzyme is affected by substrate inhibition. A curved as in B, suggests (auto-) activation. Biphasic kinetics are depicted by a buckle in the middle of the line (D). A combination of these kinetic patterns is also possible. Atypical kinetics occur in enzymes with multiple binding sites and will be further described at chapter 1.3.3.

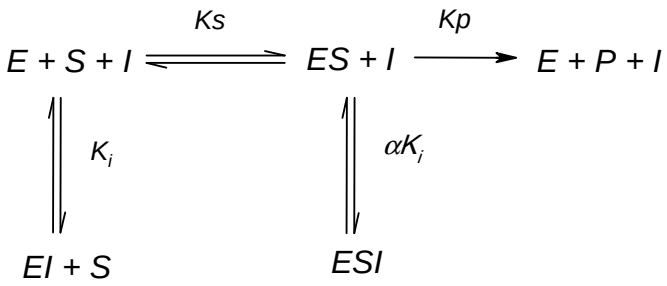
Given the availability of modern computer software, it is not necessary any more to plot linear transformations to evaluate kinetic parameters. Graphs can still be used, however, for fast and easy visualization of kinetics analyses.

1.3.2 Enzyme inhibition

The studies of enzyme inhibition are very important for understanding the enzyme structure and mechanism, for the development of new drugs and for evaluation of drug-drug interactions. An inhibitor is a substance that decreases the activity of an enzyme towards its substrate. Given by the strength of inhibitor binding the enzyme, we can distinguish between reversible and irreversible inhibition. A reversible inhibitor is present in an equilibrium between the bound and free states, whereas an irreversible inhibitor forms a permanent complex with the enzyme. A reactivation of the enzyme is in the latter case not possible. The influence of irreversible inhibitor on the cell or organ lasts until new enzyme molecules are available.

1.3.2.1 Reversible inhibition models

Equation 5



The classification into competitive, noncompetitive and mixed-type inhibition is given by the affinity of the inhibitor to the free enzyme and/or the *ES* complex (Copeland 2005). It is possible to use the constant α to describe the different binding behaviors. An inhibitor that binds to the same binding site as the substrate is defined as competitive. This would result in $\alpha = \infty$. The dissociation constant between the inhibited and the free enzyme is K_i. In some cases, however, also the inhibitor itself can be processed. This means that two

different substrates of the same enzyme can act as competitive inhibitors to each other.

If $\alpha << 1$, the inhibition type is defined as uncompetitive. The inhibitor binds only to the ES complex, using a different binding site than the substrate. As a result of inhibitor binding, the enzyme-substrate complex changes its conformation, losing or decreasing its affinity to the substrate or reducing its catalytic activity. This mechanism is commonly referred to as allosteric modulation. The dissociation constant for the ternary *ESI* complex is αK_i.

A noncompetitive inhibitor can bind to both, the free enzyme and the *ES* complex, also by acting as an allosteric modulator. There is still not a standardized nomenclature about this mechanism. Some scientific publications assign the term noncompetitive only to reactions where $\alpha = 1$, where the inhibitor has the same affinity to the free enzyme as to the ES complex. If $\alpha > 1$ (higher affinity to E) or $\alpha < 1$ (higher affinity to ES), but $\alpha \neq 1$, the term mixed type inhibition, could be more precise. In any case, the binding affinity of the inhibitor must be described by both, K_i and αK_i.

Performing steady-state kinetic experiments, where increasing concentrations of substrate are added to the reaction and determining the initial reaction velocity (V), it is possible to compare the activity of an enzyme in presence and absence of inhibitors. The effect of inhibitor presence on the K_m and V_{max} constants can tell the mechanism and magnitude of inhibition. The different inhibition types and their affect on the two parameters are shown in Table 1.

Table 1. The effect of inhibitors on kinetic parameters

Parameter	**Type of Inhibition**				
	Competitive ***α* = ∞**	**Noncompetitive/ Mixed Type** ***α > 1***	**Noncompetitive** ***α = 1***	**Noncompetitive/ Mixed Type** ***α < 1***	**Uncompetitive** ***α << 1***
K_m	Increases linearly with increasing *[I]*	Increases curvilinearly with increasing *[I]*	No effect	Decreases curvilinearly with increasing *[I]*	Decreases curvilinearly with increasing *[I]*
V_{max}	No effect	Decreases curvilinearly with increasing *[I]*	Decreases curvilinearly with increasing *[I]*	Decreases curvilinearly with increasing *[I]*	Decreases curvilinearly with increasing *[I]*
Inhibited enzyme state	E	E > ES	E = ES	E < ES	ES

As mentioned above, it is possible to draw a graph where the rate of product formation (*V*) is plotted versus the substrate concentration *[S]*. Comparing the kinetics of the inhibited and not inhibited enzyme it is possible to see changes of K_m and V_{max}. Nevertheless, it is not always easy to evaluate the two parameters by the hyperbolic curve. Therefore, linearizations, such as the double reciprocal Lineweaver-Burk plot, have been developed to facilitate the estimation of the kinetic constants and inhibition types (Cortes et al., 2001). If *1/V* is plotted versus *1/[S]*, $1/V_{max}$ is located at the point of interception of the line with the *y*-axis. K_m can be evaluated ether from the slope of the line, or from the interception point with the abscissa (*x*-axis) (Figure 6).

Keeping in mind that a competitive inhibitor does not affect V_{max}, one can assume that the line of an inhibited enzyme intercepts the line of the uninhibited enzyme at *1/[S] = 0*. If the lines are crossing each other on the x-axis, the inhibitor is noncompetitive (*α = 1*), whereas if the graph displays two parallel lines, an uncompetitive inhibitor is inhibiting the tested enzyme. For the mixed type inhibition, the intersection occurs in quadrant II (*y*|-*x*) or quadrant III (-*y*|-*x*) if *α >1* or *<1*, respectively.

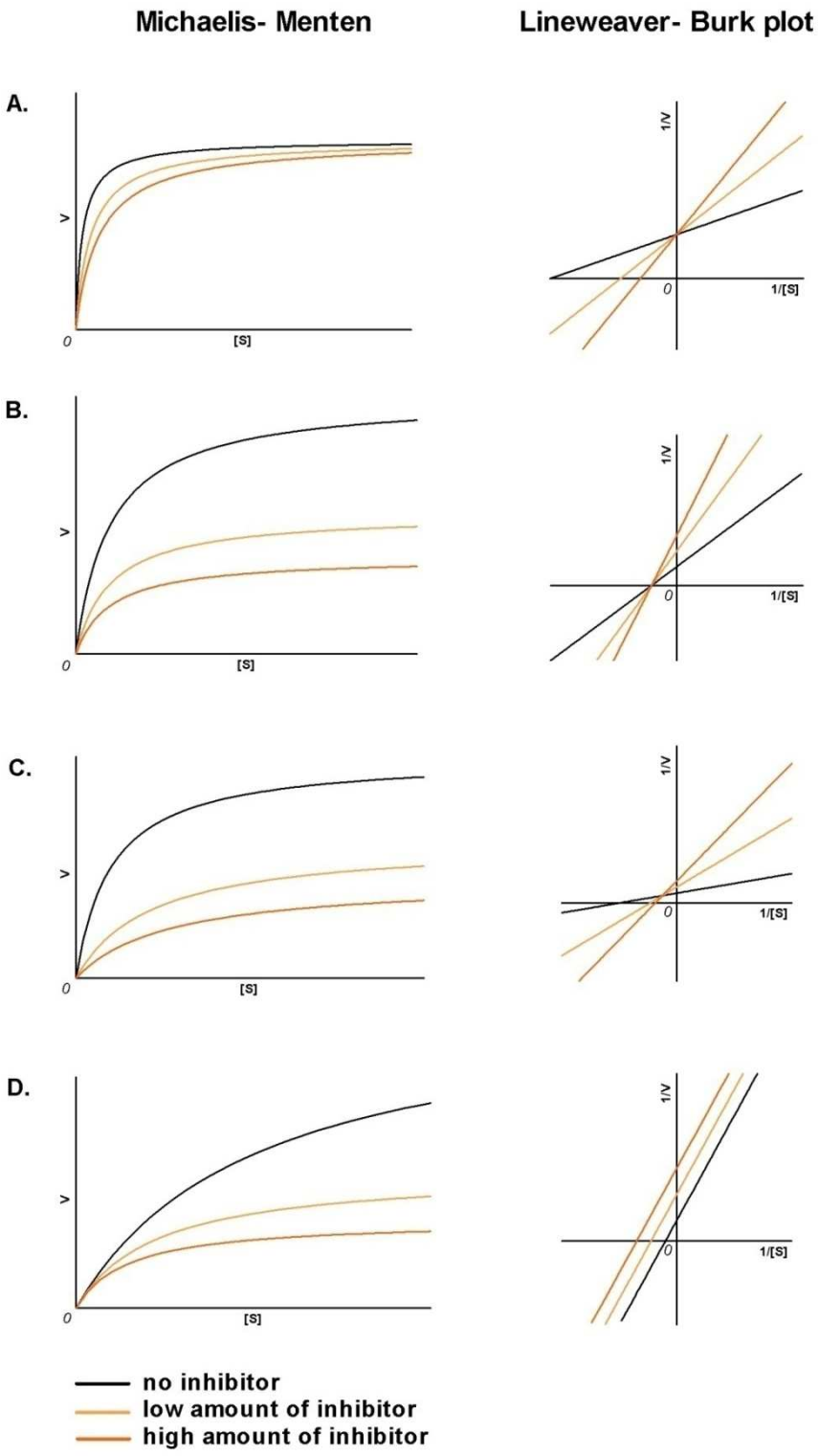


Figure 6. Graphs of A. competitive, B. noncompetitive (α = 1), C. mixed type (α > 1), and D. uncompetitive inhibition.

1.3.3 Enzyme reactions with multiple substrates or multiple site kinetics

The reaction mechanism by which one substrate is needed to form one product, is useful for understanding enzyme kinetics, but is not very common in nature. It is common to encounter enzymatic reactions involving two or more substrates and at least two products (Copeland 2000). The amount of chemical entities present in the reaction is denominated by the Latin prefixes uni, bi, ter and so on. A simple model for a good demonstration is the bi bi mechanism, where two substrates are needed to form two products.

Equation 6

$$E + AX + B \rightleftharpoons E + A + BX$$

where X is the moiety that is being transferred from substrate A to substrate B. This simple model is at the basis of the explanation for at least three different mechanisms, namely, random ordered, compulsory ordered (Figure 7) and double-displacement or “Ping- Pong” bi bi mechanism.

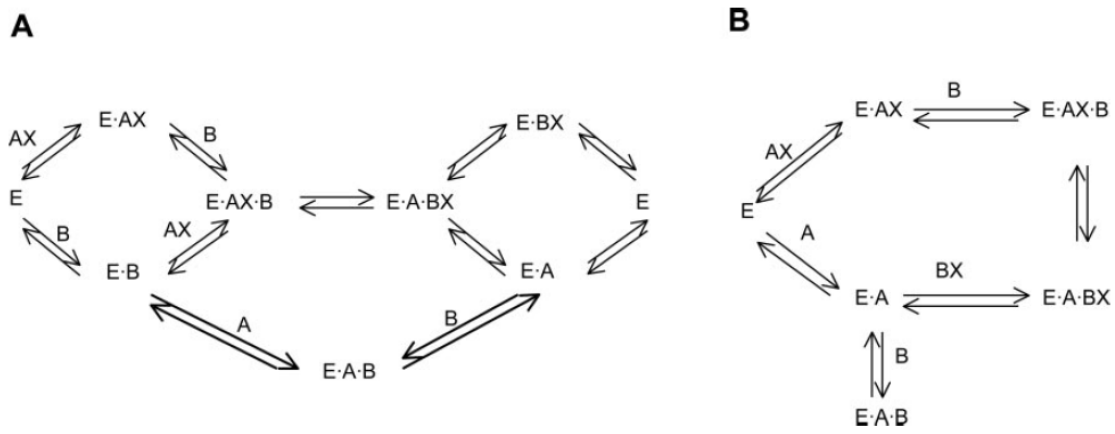


Figure 7. Schemes of a random ordered (A) and compulsory ordered mechanism (B) (from Luukkanen et al., 2005)

In contrast to the random ordered mechanism, a compulsory order reaction has a defined sequence of binding and/or leaving of the substrates and products, respectively. Given by that, an inhibition by the second-binding substrate, *B*, can occur and be visible at higher concentrations. *B* can in this case bind to the

free enzyme, inhibiting the binding of substrate *A* and preventing turn over. This mechanism can explain the atypical kinetic substrate inhibition (Figure 5).

While in the mechanisms described above the ternary complex *E•AX•B* has to be formed to create the products, in the double-displacement mechanism *A* and *B* are never bound to the enzyme at the same time. A dissociation of *A* has to occur to allow the binding of *B*.

In some cases, a substrate can bind to different sites, forming the complex *SES*. This could either increase or decrease the enzyme activity, described as positive or negative homotropic cooperativity respectively (Houston and Kenworthy, 2000). This mechanism may also be involved in atypical kinetics due to substrate inhibition. It is possible that the SES complex can still generate product, but at lower rates than the ES complex. This type of substrate inhibition is generally referred to as partial inhibition. If the substrate is enhancing the activity, instead, the mechanism is shaped by autoactivation.

1.4 *In vivo – in vitro* correlation

In vivo – in vitro correlations should allow a prediction of drug metabolism in the body at quantitative and qualitative levels from in vitro assays with suitable samples such as recombinant enzymes or liver microsomes. Since it is not possible and not ethical to execute extensive characterizations of human drug metabolism *in vivo*, *in vitro* approaches have been developed to enhance the safety and efficacy of pharmaceutical therapies (Houston, 1994; Miners and Birkett, 1998; Iwatsubo et al., 1997; Boase and Miners, 2002; Miners, 2002). Most of the drug-metabolizing enzymes, such as the CYPs and UGTs, often exhibit partial overlapping activities. To examine such overlaps and also to learn about the effects of genetic polymorphism in these enzymes, it is necessary to prepare single isoforms and test their behavior *in vitro*. Also the analysis of mixtures of enzymes present in the respective tissues can be a helpful tool to investigate the metabolism rates in different organs.

A common parameter for comparisons of metabolic activities is the intrinsic clearance CL_{int} that is defined by the kinetic parameters:

Equation 7

$$CL_{\text{int}} = \frac{V_{\max}}{K_m}$$

CL_{int} is, however, just a measure of the efficiency of the metabolic enzymes. To obtain complete *in vivo* data by calculations, other parameters, such as the blood flow through the organ, the uptake into the organ and the fraction unbound f_u, have to be taken in consideration.

1.4.1 The “albumin effect”

Despite the importance of the *in vivo – in vitro* correlation for drug development, several publications claimed a general trend of under- prediction for CYPs and UGTs metabolism, when in vitro CL_{int} derived from liver microsomes or recombinant enzymes are used to forecast the in vivo CL_{int} (Houston and Carlile, 1997; Iwatsubo et al., 1997; Ito et al., 1998; Naritomi et al., 2001; Miners et al., 2004; Engtrakul et al., 2005; Ito and Houston, 2005; Cubitt et al., 2009). Interestingly, it was found that in the presence of bovine serum albumin (BSA) during microsomal incubations, the in vitro CL_{int} seems to get closer to the true rates of in vivo metabolism (Ludden et al., 1997; Carlile et al., 1999; Baba et al., 2002; Tang et al., 2002; Wang et al., 2002; Rowland et al., 2006; Rowland et al., 2008a; Rowland et al., 2009; Uchaipichat et al., 2006; Raungrut et al., 2010). When it comes to UGTs, (Rowland et al., 2008b) it was shown that increased activity of UGT2B7 could be achieved by the addition of BSA, probably due to its activity in the removal of fatty acids (FA) that may be released during microsomes preparation. FA are metabolized via different CYPs from the CYP2 and CYP4 families (Hoagland et al., 2001, Maier and Roman, 2001) and UGTs from the UGT1A and UGT2B families (Turgeon et al., 2003, Little et al., 2004, Little et al., 2002). Being substrates of these enzymes, they could also bind and act as competitive inhibitors with respect to other substrates (Tsoutsikos et al., 2004; Rowland et al., 2006). As shown in Table 2, in most of the recombinant UGT isoforms that were tested in this study, BSA seemed to remove (also) a competitive inhibitor.

Table 2. Effect of BSA on recombinant UGT isoforms

Enzyme	Substrate	Inhibitor type	Kinetics model
1A1	4-Metylumbelliferone[1]	no inhibitor	Control: Michaelis Menten BSA: two site model – autoactivation and substrate inhibition (Segel 1975; Houston and Kenworthy, 2000)
1A4	Lamotrigine[4]	mixed type inhibitor	Control: Michaelis Menten BSA: Michaelis Menten
1A6	4-Metylumbelliferone[1]	no inhibitor	Control: Michaelis Menten BSA: two site model – autoactivation (Segel 1975, Huston and Kenworthy 2000)
1A9	4-Metylumbelliferone[1]	competitive inhibitor	Control: Michaelis Menten BSA: Michaelis Menten
	Propofol[1]	competitive inhibitor	Control: Michaelis Menten BSA: Michaelis Menten
2B4	Codeine[3]	competitive inhibitor	Control: Sigmoidal kinetics BSA: Sigmoidal kinetics (amplified)
2B7	Zidovudine[2,4]	competitive inhibitor	Control: Michaelis Menten BSA: Michaelis Menten
	4-Metylumbelliferone[4]	competitive inhibitor	Control: Hill Equation – positive cooperativity BSA: Hill Equation – positive cooperativity
	Codeine[3]	competitive inhibitor (V_{max} decreased with BSA)	Control: Sigmoidal kinetics BSA: Sigmoidal kinetics (amplified)

1(Rowland et al., 2008); 2(Uchaipichat et al., 2006); 3(Raungrut et al., 2010); 4(Rowland et al., 2006)

1.4.2 Physiological function of albumin

Albumin is the most abundant plasma protein in humans and other mammalians (Peters, 1996). Around 3.5 – 5.0 g/dl are present in the human plasma (Ganong, 1989). Its functions include maintaining the oncotic pressure, buffering pH and transporting endogenous compounds such as metals, ions, fatty acids, amino acids, bilirubin, enzymes, thyroid and steroid hormones, and exogenous compounds including drugs, environmental chemicals and dietary fatty acids. Binding to albumin keeps the substances from being rapidly filtered through the glomeruli and provides a stable reservoir. It is also preventing the occurrence of high local concentrations of compounds and therefore reducing the risk of intoxications.

1.4.3 Fatty acids

Esterified long chain fatty acids are the main components of the phospholipid membranes. Short- and medium-chain fatty acids are generally not esterified and bound to albumin, or other lipid binding proteins. The binding to albumin can reach from 3 to 30 molecules of fatty acids per molecule of albumin, depending on the concentrations in the plasma (Guyton and Hall, 2006). Fatty acids can be used as a source for energy. They can also act as second messenger within cells for the transduction of external signals. Their most important function is, however, to serve as precursors for biologically active signaling molecules, called eicosanoids. These compounds include hydroxyeicosatetraenes, prostanoids (prostaglandins, prostacyclins and tromboxanes), leukotrienes, resolvins and isoprostanes (Ware and Bruckner 1992)

1.5 Aglycone UGT Substrates Used in the Study

1.5.1 4-Methylumbelliferone

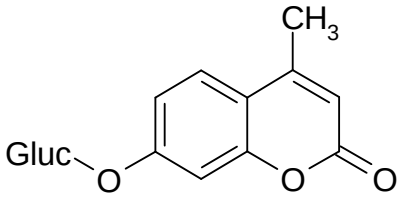


Figure 8. 4-Methylumbelliferone glucuronide

4-Methylumbelliferone (4MU) is a coumarin derivate, often used for *in vitro* studies of UGT enzymes, as it is glucuronidated by a wide range of isoforms, such as UGT1A1, 1A3, 1A6, 1A7, 1A9, 1A10, 2B7 and 2B15 (Ismail et al., 2010). Despite the wide use of coumarin derivatives for their antimicrobial and, more important, anticoagulative proprieties, 4MU has no pharmacological relevance.

1.5.2 Entacapone

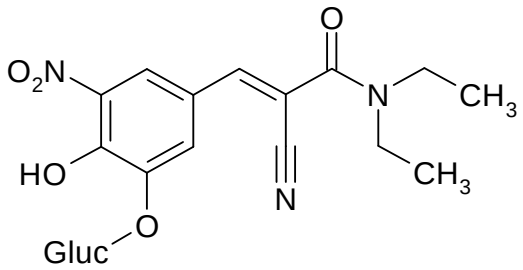


Figure 9. Entacapone glucuronide

Entacapone is a synthetic drug used in combination with levodopa to treat Parkinson's disease. Its effect is based on the inhibition of the catechol-O-methyl transferase (COMT) and therefore reducing its ability to inactivate levodopa and dopamine.

Entacapone is glucuronidated, at very different rates, by UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9 and 1A10 (Luukkanen et al., 2005). In the human liver, it is almost exclusively metabolized by UGT1A9. Polymorphism of UGT1A9 can induce hepatic dysfunctions (Martignoni et al., 2005), when Entacapone is administrated regularly. An epidemiologic study is still needed to confirm this polymorphism effect, however.

2 Aims and significance

As described above in section 1.4, there is a relevant need to improve *in vitro* experiments for UGTs. The addition of BSA was shown to be an important help to achieve this goal, but not all the isoforms present in the human body have been tested yet (Table 2). It is also noteworthy that all the published data until now are from a single laboratory, and they have used recombinant UGTs and liver microsomes that were prepared in their lab and, therefore, are not (easily) available for other researchers (Rowland et al., 2008; Uchaipichat et al., 2006; Raungrut et al., 2010; Rowland et al., 2006).

Our objectives were to analyze the effect of albumin on our recombinant UGTs, expressed in baculovirus-infected Sf9 insect cells, and to compare it with commercial available human liver microsomes (HLM) and human intestinal microsomes (HIM). Moreover, the effect of BSA addition on the glucuronidation of 4MU by recombinant UGT 1A9 has already been published, meaning that our results could be compared to their (Rowland et al., 2008a).

We have decided to use 4-methylumbeliferone and entacapone as substrates since they are known to be metabolized by a broader range of isoforms.

This thesis provides important new knowledge on the effect of BSA on an unexplored group of recombinant UGTs. The acquired information can be a significant help in the future for improving *in vitro* experiments and better understanding of the mechanisms of UGT enzymes activity.

3 Materials and Methods

3.1 Materials

Magnesium chloride hexahydrate and perchloric acid were obtained from Merck (Darmstadt, Germany). Formic acid (98–100%) was obtained from Riedel-de Haeën (Seelze, Germany). Methanol, Acetonitrile, Glycerol, Alamethicin (from *Trichoderma Viride*), UDPGA (triammonium salt, 98–100%, CAS number 63700-19-6), Bovine Serum Albumin (Essentially fatty acid free, CAS number 9048-46-8), 4-Methylumbeliferone (4MU; CAS number 90-33-5) and 4MU-glucuronide (CAS number 6160-80-1)where all purchased from Sigma-Aldrich (St.Louis, MO, USA). Entacapone was obtained from OrionPharma (Espoo, Finland) and the Entacapone glucuronide was synthesized in our laboratory. High performance liquid chromatography (HPLC)-grade solvents were used throughout the study.

3.2 Enzyme sources

Recombinant human UGTs were expressed as Histagged proteins in baculovirus-infected Sf9 insect cells as described previously (Kurkela et al., 2007; Sneitz et al., 2009 and references therein). The relative expression level of each recombinant UGT isoform was immunodetected using tetra-His antibodies (QIAGEN, Hilden, Germany) as described earlier (Kurkela et al., 2007). Protein concentrations were determined by the BCA method (Pierce Biotechnology, Rockford, IL). Pooled human liver microsomes [HLM (lot 18888)] and human intestinal microsomes [HIM (lot 28095)] were purchased from BD Gentest (Woburn, MA, USA).

3.3 Binding assay for BSA

For the detection of the binding of 4MU and Entacapone to BSA we have used Amicon Ultra Centrifugal Filters (0.5mL, Ultracel – 10K Membrane) from Millipore (Carrigtwohill, Co. Cork, Ireland).

3.3.1 Unspecific binding of 4MU to the filter membrane

The pretreatment consisted of filtering 500µl milliQ water, 50 mM phosphate buffer pH 7.4, 1µM 4MU, 10µM 4MU, Tween 20, Tween 80 or benzalkonium chloride (BAK) in order to impregnate the filters. The pretreated filters and the control (not pretreated) were tested using 10, 100 and 1,000µM of 4MU (Table 6 and Table 7). Two or three filtrates per sample with volumes of either 50 or 100µl were collected to analyze the 4MU concentration by HPLC (see section 3.6). The unspecific binding was calculated comparing the concentration of 4MU in the filtrate to the one in the reservoir.

3.3.2 Binding assay of 4MU

Solutions of 4MU within the concentration range of 2 - 160µM, in 50 mM phosphate buffer pH 7.4, were incubated in presence of 0.1% BSA at 37℃ for 60 min. From each test solution, 450µL were transferred into the reservoir of an Ultra Centrifugal Filter, previously pretreated with TW 20 (see section 4.1.1), and centrifuged for 0.5min at 5'000*g*. The feed and the filtrate were collected and ice cold perchloric acid was added to precipitate the BSA. The tubes were transferred on ice for approximately 20 min and then centrifuged for 10 min at 16.000 x *g*. The supernatant in the tubes was collected and used for the analysis by HPLC (see section 3.6). Controls were performed in absence of BSA. Two different formulas were used to evaluate the f_u:

a) Filtrate of the control / Filtrate of the test tubes
b) Feed of the test tubes / Filtrate of the test tubes

Given by the unfinished results, the f_u for 4MU used in this thesis are taken from (Rowland et al., 2008). Binding data for Entacapone are not available in the literature, yet. The data displayed in the kinetics of Entacapone is, therefore, not corrected for binding and due to this, the K_m values are to be lower in presence of BSA. The V_{max} values, in any case, should represent the correct value since it is expected to be independent of the exact substrate concentration in the solution, even if it might appear to be reached at higher substrate concentrations in the presence of BSA.

3.4 "Washing" of UGT1A7-containing membranes with 1% BSA

Recombinant UGT1A7 membranes were pretreated with 1% BSA in phosphate buffer for 30 min, either on ice or at 37°C. The control samples for this experiment were incubated with phosphate buffer without BSA at the corresponding temperatures.

The effect of washing was determined by comparing the enzymes pretreated with 1% BSA to the corresponding control. A second set of controls was prepared by adding 0.1% BSA to the reaction mixture of the first controls. The enzymes were incubated in presence of 400µM Entacapone according to section 3.5 and the formed Entacapone glucuronide was analyzed as described in section 3.6.

3.5 Incubation conditions for recombinant UGTs, HLM and HIM with 4MU and Entacapone

The substrates were dissolved and diluted in pure methanol to obtain the desired concentrations. The different dilutions were added into Eppendorf tubes and centrifuged under vacuum conditions to evaporate the methanol. 90 µL of reaction mixture, containing 50 mM phosphate buffer pH 7.4, magnesium chloride, UGT enzymes at different concentrations (Table 3, Table 4 and Table 5) and when needed 0.05% to 2% of BSA, was added to the tubes. The mixture for HLM and HIM contained in addition alamethicin at a final concentration of 5% of the microsomal protein concentrations. After the total dissolution of substrate (approximately 20 min), the tubes were transferred into a heating block at a temperature of 37°C for a pre-incubation of 5 min. 10 µL of the appropriate dilution of UDPGA was then added to the mixture to start the reaction and 10µL of ice cold perchloric acid were used to stop it after the incubation time, as listed in Table 3-5. The stopped solutions where put on ice for approximately 20 min and then centrifuged then for 10 min at 16,000 x *g*. The supernatants were collected and subjected to the respective liquid chromatography analysis (see section 3.6).

Table 4 and Table 5 show different incubation conditions for the 4MU and Entacapone kinetics and their respective UDPGA kinetics. The protein concentrations and incubation times for the kinetic analyses reactions were selected based on preliminary assays to ensure that product formation was within the linear range with respect to protein concentration and incubation time, and that the substrate consumption was less than 10%. The substrate concentrations for the 4MU and Entacapone screenings were close to the K_m values that were earlier measured in our lab, or reported in the publication of Luukkanen et al., 2005, respectively. All the concentration points were tested in triplicates, except the preliminary tests, where the values represent the average of two measurements.

Table 3. The incubation conditions for the screenings of recombinant UGTs, HLM and HIM with 4MU and Entacapone as the aglycone substrates

	Incubation time (min)	Amount of enzyme (µg)	Substrate concentration (µM)
4MU			
1A1	20	10	50
1A3	20	25	20
1A6	20	10	20
1A7	20	3	25
1A8	20	25	200
1A9	20	10	20
1A10	20	10	25
2A1	20	10	50
2A2	20	10	50
2B4	20	50	100
2B7	20	25	100
2B15	20	10	50
2B15 (comm)	20	10	50
HLM	20	5	50
HIM	20	5	50
Entacapone			
1A1	60	10	100
1A3	60	10	200
1A7	10	2	10
1A8	60	10	50
1A9	10	2	10
1A10	60	10	50
2A1	60	5	50

Table 4. The incubation conditions for the enzyme kinetics of recombinant UGTs and HLM with 4MU; 4MU kinetic analyses were performed in the presence of 5 mM UDPGA; the kinetics of UDPGA were carried out in the presence of 50 µM 4MU for UTG 1A9, or 20 µM 4MU for UGTs 1A7 and 2A1.

	Incubation time (min)	Amount of enzyme (µg)	Substrate concentration range (µM)/ amount of different concentrations
4MU kinetics			
1A7	20	2	10 - 750/ 8
1A9	20	2	10 – 750/ 8
1A10	20	2	5 – 800/ 10
2A1	20	2	5 – 500/ 8
2B15	20	2	10 – 1000/ 10
HLM	10	2	10 – 1000/ 10
UDPGA kinetics			
1A7	20	2	50 – 5000/ 8
1A9	20	2	50 – 5000/ 8
2A1	20	2	50 – 5000/ 8

Table 5. Incubation conditions for the enzyme kinetics of recombinant UGTs, HLM and HIM with Entacapone; Entacapone kinetics were performed in the presence of 5 mM of UDPGA; the kinetics of UDPGA were carried out with either 75 µM Entacapone (UTG1A9) or 500µM Entacapone (UGTs 1A7, 1A8 and 1A10).

	Incubation time (min)	Amount of enzyme (µg)	Substrate concentration range (µM)/ amount of different concentrations
Entacapone kinetics			
1A7	10	2	5 - 750/ 10
1A8	60	3	10 – 600/ 8
1A9	10	2	5 – 750/ 10
1A10	60	10	10 – 500/ 8
HLM	15	2	10 – 750/ 10
HIM	60	5	10 – 750/ 8
UDPGA kinetics			
1A7	10	2	50 – 5000/ 8
1A8	60	3	500 – 5000/ 8
1A9	10	2	50 – 5000/ 8
1A10	60	7	50 – 5000/ 8

3.6 Analytical methods

The concentration of 4MU D-glucuronide was analyzed with an HPLC system consisting of the Agilent 1100 series degasser, binary pump, autosampler, thermostated column compartment, multiple wavelength UV detector, and fluorescence detector (Agilent Technologies, Palo Alto, CA). The resulting chromatograms were analyzed with Agilent ChemStation software (revision B.01.01) on Windows 2000 Professional workstation. The column used for this instrument was the Chromolith SpeedROD, RP-18e (50×4.6 mm; Merck) and the column temperature was 40℃. The mobile phase c onsisted of 80% 50 mM Phosphate Buffer, pH 3.0 (A) and 20% methanol (B) and a flow rate of 2 ml/min, were kept constant during the entire run that was stopped after 10 min. The retention time of the 4MU glucuronide was 1.5 min. The detection was performed using fluorescence detection with excitation at 316 nm and emission at 382 nm.

The formation of Entacapone D-glucuronide was determined using the UPLC system Waters Acquity UPLC (Waters, Milford, MA), equipped with column manager, sample manager, binary solvent pump, and photodiode array UV detector. The resulting chromatograms were analyzed with Empower 2 software (Build 2154; Waters) on Windows XP Professional. The UPLC column was Acquity UPLC BEH C_{18} (1.7 µm, 2.1×100 mm; Waters), equipped with a precolumn, and the column temperature was 40℃. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B), and the flow rate was 0.5 ml/min throughout. UV absorbance at 309 nm was used for detection. The gradient in this method was as follows: 0 to 3 min of 20% B; 3 to 3.20 min of 30% B; 3.20 to 4.10 min of 80% B; and 4.10 to 6 min of 20% B. The Entacapone glucuronide retention time was 2.2 min.

3.7 Enzyme kinetic analysis

Kinetic analyses were performed using the GraphPad Prism program, version 5.01 for Windows (Graph- Pad Software Inc., San Diego, CA). The best model was selected based on the corrected Akaike's information criterion (AICc), the calculated r^2 values, residuals graph, parameter S.E. estimates, 95% confidence intervals, and visual inspection of Eadie-Hofstee plots. Data were fitted with the following models:

1. Michaelis Menten equation

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

where *v* is the initial velocity of the enzyme reaction, V_{max} is the maximum velocity, *[S]* is the substrate concentration, and K_m is the velocity at 0.5 of V_{max}.

2. Substrate inhibition model equation

$$v = \frac{V_{max}[S]}{K_m + [S](1 + \frac{[S]}{K_i})}$$

where K_i is the descriptor for the inhibitory effect of the substrate.

Various equations were used to describe the two-site kinetic models. Equations no. 3 and 4 were applied for kinetics where the substrate seemed to act as noncompetitive/mixed type inhibitor.

3. Two site model equation I (Galetin et al., 2002; Zhou et al., 2010)

$$v = \frac{V_{max}(\frac{[S]}{K_S} + \frac{b[S]^2}{K_S^2})}{1 + \frac{[S]}{K_S} + \frac{[S]^2}{K_S^2}}$$

where *b* is the constant that affects the product formation and K_s is the affinity constant for the binding of the substrate.

4. Two site inhibition model equation II (Houston and Kenworthy, 2000)

$$v = \frac{V_{max}(\frac{[S]}{K_S} + \frac{b[S]^2}{aK_s^2})}{1 + \frac{2[S]}{K_S} + \frac{[S]^2}{aK_S^2}}$$

where *a* is the constant that effects the affinity of the substrate to the enzyme.

For the two-site kinetic models, where autoactivation occurred, data were fitted to the allosteric model equation:

5. Allosteric sigmoidal model (Hill equation)

$$v = \frac{V_{max}[S]^h}{[S]_{50}^h + [S]^h}$$

where S_{50} is the velocity at 0.5 V_{max} and h is the Hill coefficient.

As already mentioned, it is not possible to perform exact comparisons between atypical kinetics according to the K_m value, and the parameter V_{max} should also be used carefully in such comparisons.

4 Results

4.1 Preliminary Tests

Preliminary tests where needed to establish a standardized method for following the kinetics of different glucuronidation reactions in which different substrates and different UGT isoforms were examined. Tests that were previously performed by others in our laboratory are not reported in this thesis.

4.1.1 Unspecific binding of 4MU to Ultra Centrifugal Filters

4.1.1.1 Pretreatments with water, buffer and 4MU

The filter seemed to bind 4MU at saturating concentrations. After several rounds of filtrations, higher concentrations of substrates were present in the filtrate (Table 6). At higher initial concentrations of substrate, lower percentages of loss occurred. Although only tested with 100 µM 4MU, the pretreatment with 50 mM phosphate buffer pH 7.4 seemed to decrease nonspecific binding. There were no statistically significant differences, however, between the filters washed with water and the filters washed with phosphate buffer.

The pretreatment with 4MU lowered the unspecific binding of the filters compared to the other pretreatments. Nevertheless, there were no differences between the 50 and 100 µM of 4MU.

4.1.1.2 Pretreatments with Tween 20, Tween 80 and BAK

The mild detergents Tween 20 and Tween 80 lowered the unspecific binding of the filter compared to the control (Table 6 and Table 7), and BAK further enhanced this effect. When 100 µl of pre-filtrate (filtrate 1) were removed, the concentration of 4MU in the filtrate (filtrate 2) was the same as in the reservoir.

A second test revealed that the pretreatment with Tween 20 also eliminated the unspecific substrate binding to the filter when 50µl of pre-filtrate were removed (Table 7).

Table 6. Test for the pretreatment of the Ultra Centrifugal filters I; the volume of each filtrate was approximately 50µl

Concentration of 4MU	**Solution for pretreatment**	**Fraction unbound, f_u**		
		Filtrate 1	**Filtrate 2**	**Filtrate 3**
10µM	No pretreatment	N.A.	N.A.	0.78
	mQ water	0.12	0.58	0.74
	50mM PB	0.13	0.59	0.76
100µM	No pretreatment	0.11	0.61	0.76
	50mM PB	0.17	0.65	0.87
	1µM 4MU	0.20	0.77	0.93
	10µM 4MU	0.26	0.78	0.93
1000µM	No pretreatment	N.A.	N.A.	N.A.
	Pretreated with mQ water	0.44	0.97	N.A.
	Pretreated with 50mM PB	0.49	0.92	N.A.

Table 7. Test for the pretreatment of the Ultra Centrifugal filters II; the f_u are evaluated for a 4MU concentration of 100µM

Solution for pretreatment	**Fraction unbound, f_u**		
	Filtrate 1	**Filtrate 2**	**Filtrate 3**
100µl of filtrate			
TW 20	0.86	1.01	N.A.
TW 80	0.87	1.01	N.A.
BAK	0.10	0.70	N.A.
50µl of filtrate			
TW 20	N.A.	0.98	1.00

4.1.2 Binding assay for 0.1% BSA

The binding of 4MU to BSA was linear in the range between 2 and 160µM of substrate (Figure 10). Even if the retained phase (feed) containing albumin was pretreated with PCA, the release of the substrate was not complete. Compared to the control, BSA was still retaining around 10% of 4MU. The f_u of the substrate in the filtrate increased from 0.7 to 0.81 of the controls filtrate or the concentration in the feed, respectively.

Since the binding experiments were incomplete at the time of writing this thesis, the f_u value for 4MU that was used in the kinetic analyses was taken from the publication of (Rowland et al., 2006), namely f_u values of 0.89, 0.49 and 0.27 in the presence of 0.1, 1 and 2% BSA, respectively. The data for Entacapone were not corrected for binding since no such data is available, although it surely binds to BSA, perhaps even to a higher degree than 4MU. Nevertheless, in the absence of determined binding values, the kinetic constantans are given in this thesis as if the f_u value is 1.0, but I would lie to stress that this is temporary and the real f_u value or values, after determining Entacapone binding to BSA, will probably be smaller, leading to smaller K_m/K_s values, too.

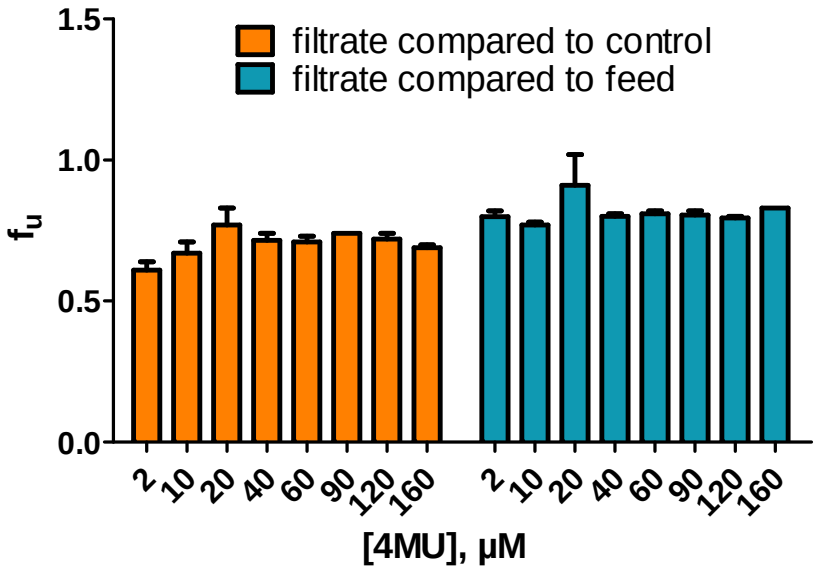


Figure 10. Unbound fractions of different concentrations of 4MU in presence of 0.1% BSA**.**

4.1.3 "Washing" membranes with 1% BSA

Contrary to hope and expectations, treatment of insect cell membranes that contain recombinant UGT1A7 with 1% BSA prior to the glucuronidation reaction incubation did not increase the glucuronidation rate. The effect of BSA was only visible when it was present in the mixture during the reaction (Figure 11, "Washed control + 0.1% BSA"). The assay was performed with Entacapone as the aglycone substrate.

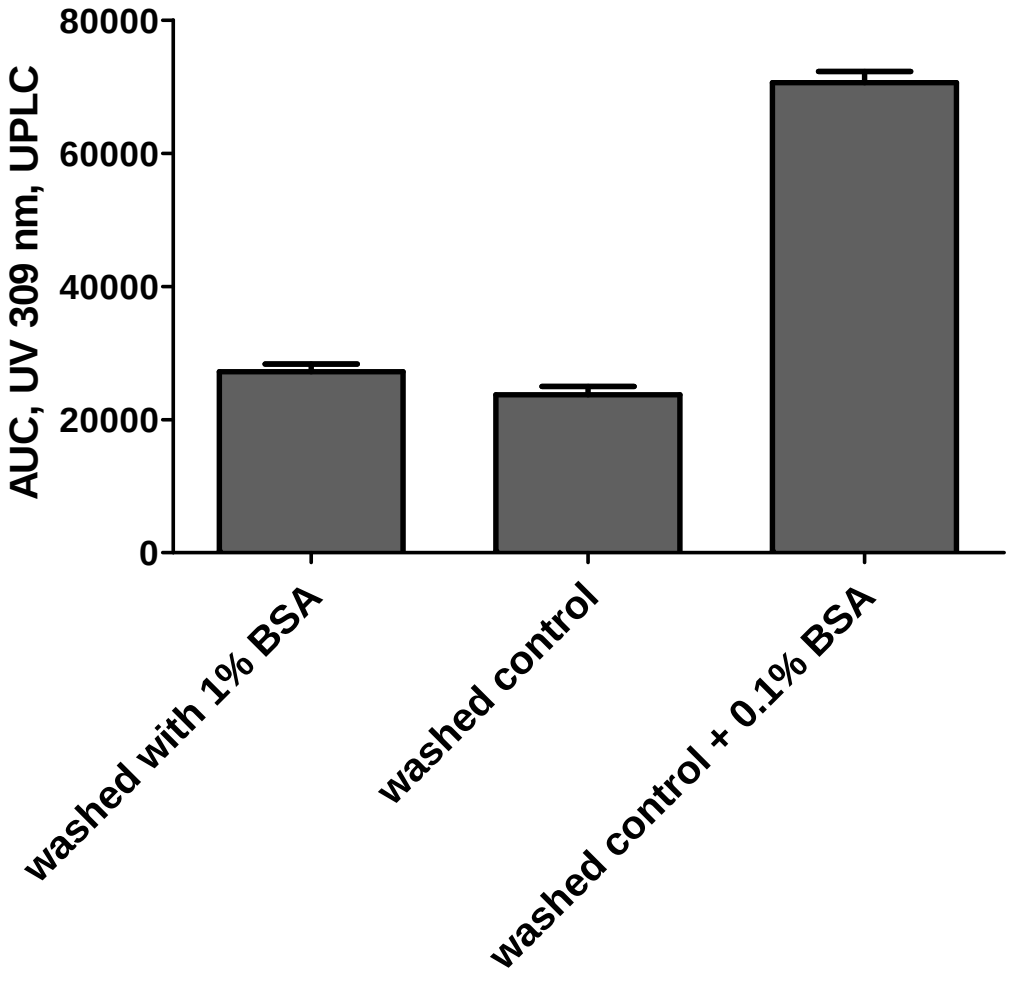


Figure 11. Washing test of UGT1A7 with Entacapone as the aglicone substrate

4.1.4 Influence of glycerol and incubation temperature on UGT2A1 activity

The test was performed on UGT2A1 with 4MU as the substrate. The addition of glycerol to the reaction mixture lowered slightly the activity of UGT2A1 (Figure 12). This decrease was also visible when glycerol was added in combination with 0.1% BSA. In the same set of incubations we have also tested the effect of incubation temperature on activity and the results show that body temperature, 37℃, is better than room temperature, 23℃ (Figure 12).

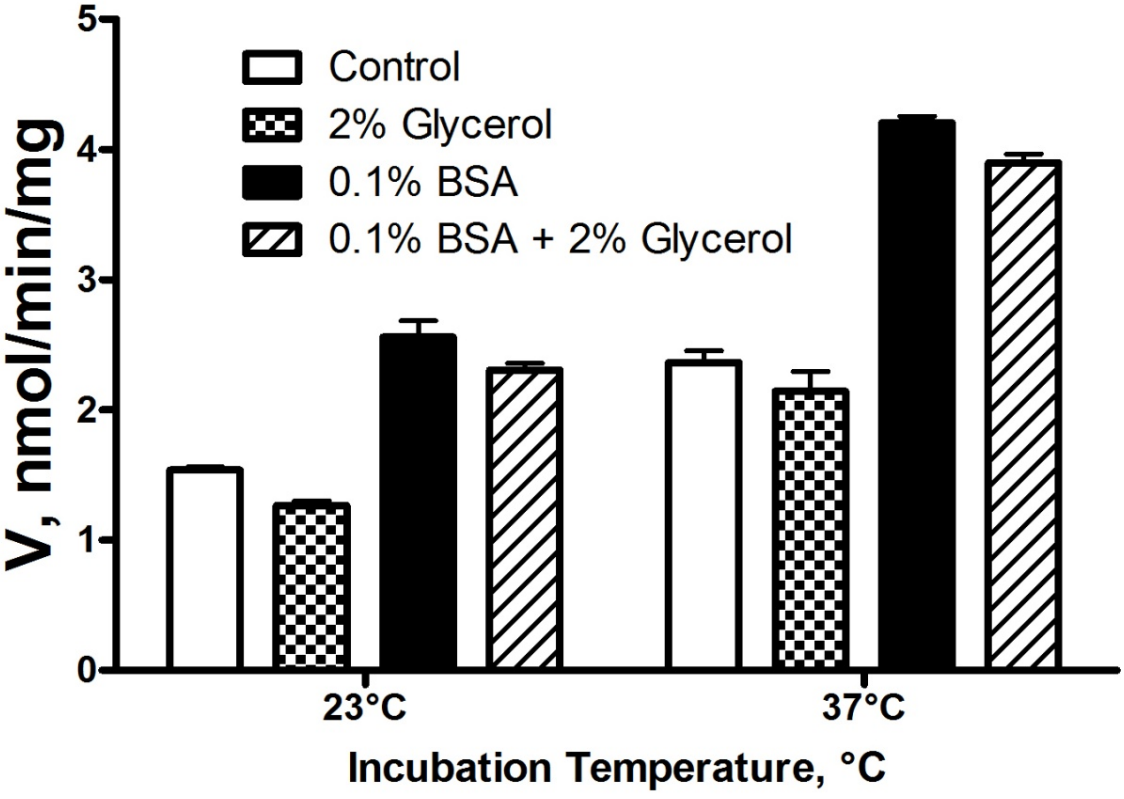


Figure 12. Influence of incubation temperature, 2% glycerol, and 0.1% BSA on 4MU (50 µM) glucuronidation by UGT2A1 (0.02 mg/ml)

4.1.5 Linearity of the reaction with respect to time with human intestinal microsomes

The glucuronidation of Entacapone by the commercial HIM was largely linear with respect to incubation time within the time range of 16 and 56 min, both in the absence and the presence of 0.1% BSA. A slight decrease of activity is, however, visible after 56 min in the sample without BSA (Figure 13).

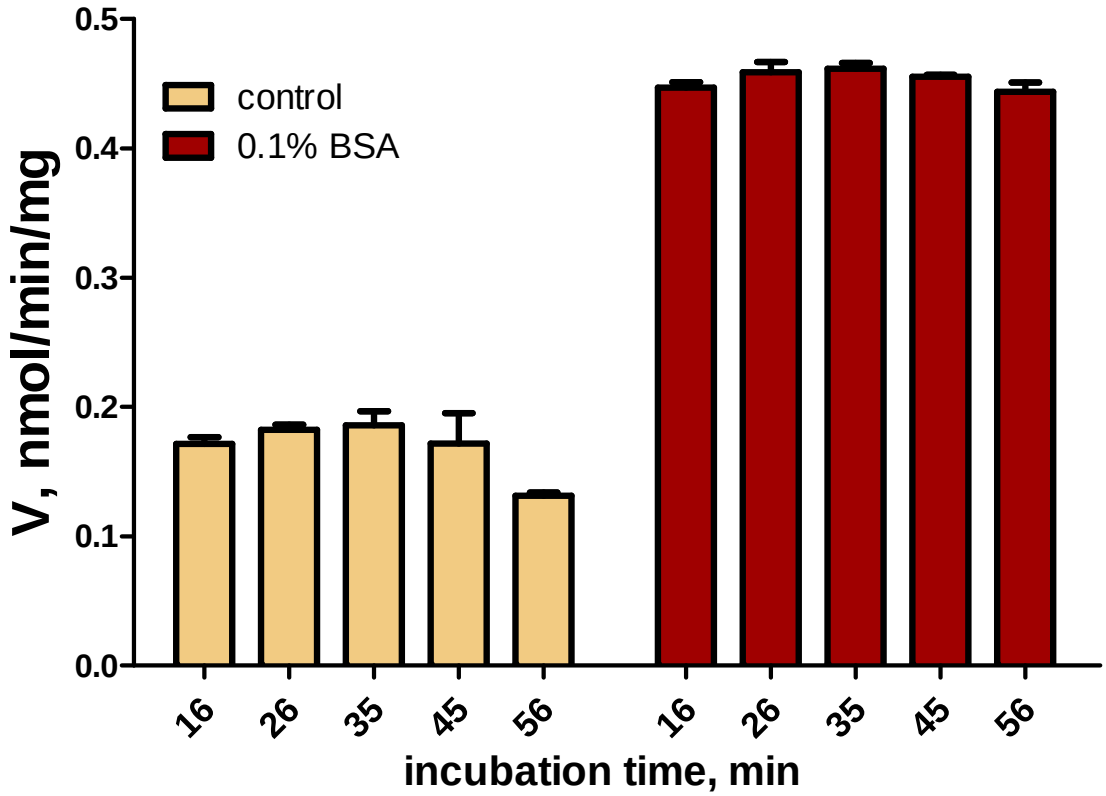


Figure 13. Preliminary test for the linearity with time of HIM (5 µg) with Entacapone (500 µM) as the aglycone substrate.

4.1.6 Linearity of the glucuronidation rate with respect to enzyme concentration and selection of BSA concentration

The results showed that the effects of 0.1% BSA and 1% BSA were similar when the enzyme concentration, UGT2A1-containing membranes in this case, was between 2 and 50µg of UGT2A1, and that the glucuronidation rate, calculated per mg (total) membrane protein was essentially the same for all the tested concentrations (Figure 14). The BSA concentration of 0.05% stimulated the activity of the enzyme like higher BSA concentrations only up to 20µg of UGT2A1. At higher enzyme concentrations, like 50 µg, 0.05% BSA seemed to be too low for a proper activation (Figure 14). The concentration of 0.1% BSA was therefore used through all the experiments. Other concentrations were anyway still tested in other experiments to analyze the variability between different systems.

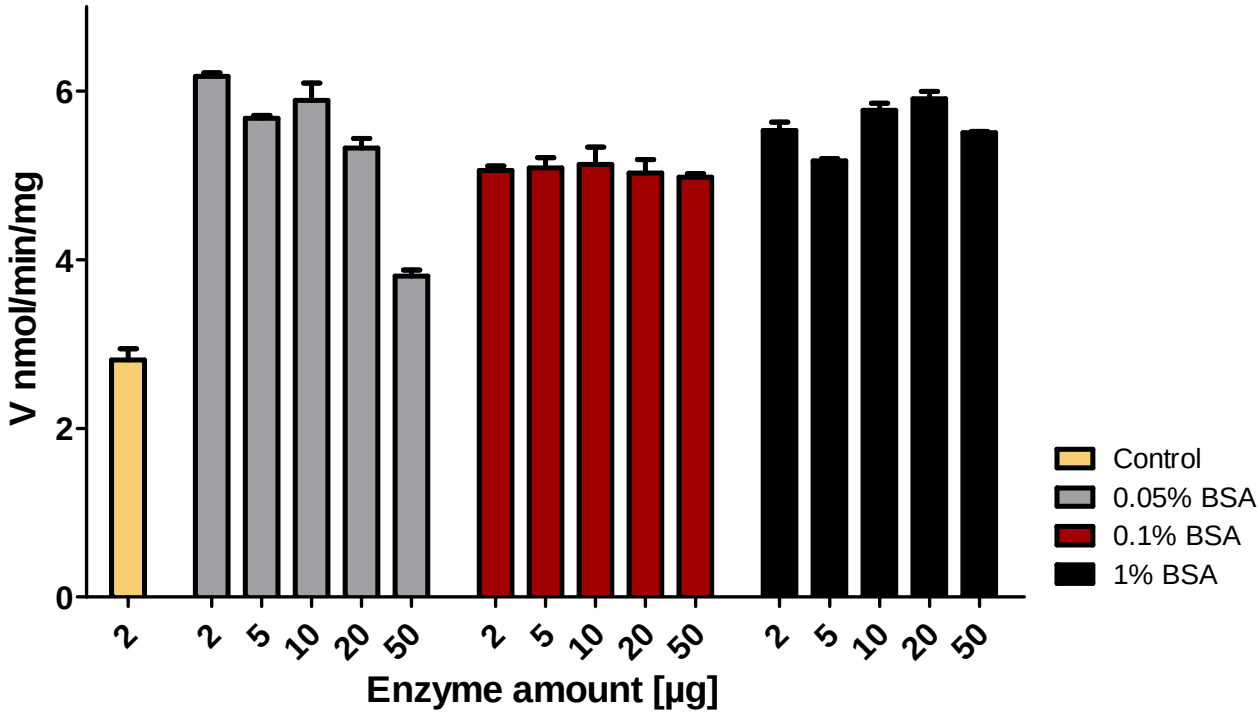


Figure 14. Glucuronidation of 4MU (50µM) by UGT2A1 in presence of 0.05%, 0.1%, 1% BSA

4.1.7 4MU screening

We have assayed all the human UGTs for the glucuronidation rate of 4MU, with and without BSA. The highest 4MU glucuronidation rates were found with UGTs 1A7, 1A9, 1A10, 2A1 and 2B15 (Figure 15). Very low rates were found for UGTs 1A1, 1A3, 1A6, 1A8, 2A2 and 2B7. The activation of the enzymes by the addition of 2% BSA was observed in almost all the isoforms, with the exception of UGT1A8 and UGT2B7, where the albumin seemed even to lower the activity. The human liver and intestine microsomes (HLM and HIM, respectively) and the commercially available recombinant UGT2B15, also showed an increased activity in presence of BSA. The data of 2% BSA are corrected for substrate binding, by using the data of (Rowland et al., 2007).

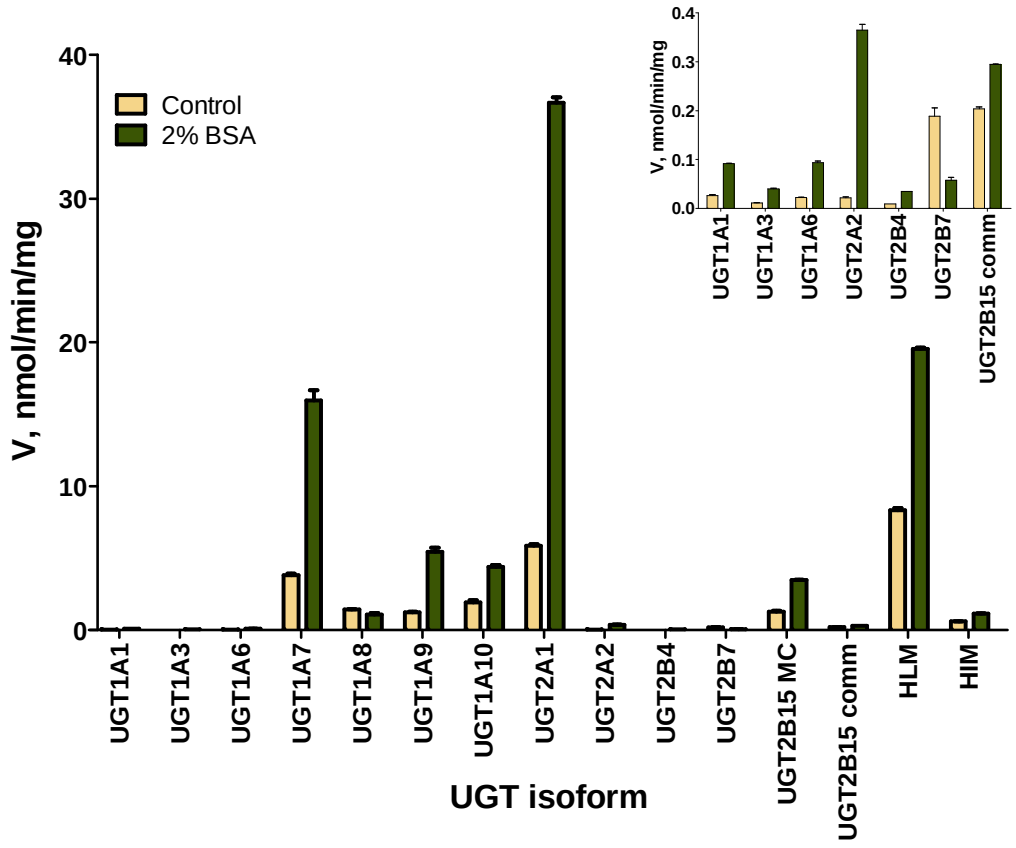


Figure 15. Screening of UGT selectivity towards 4MU
The substrate concentrations used were different for different isoforms, as described in Table 3. The commercial UGT2B15 has no His-tag and, therefore, its glucuronidation rate could not be normalized for expression level. The same holds for the different UGTs in HLM and HIM.

4.1.8 Entacapone screening

The main UGTs responsible for the glucuronidation of Entacapone were found to be 1A7, 1A8, 1A9, 1A10, whereas UGTs 1A1, 1A3, and 2A1 showed a minimal or almost no activity (Figure 16). The activity of all the tested isoforms increased in presence of 0.1% BSA. Data for the BSA concentration of 0.05% are only available for the UGTs 1A8, 1A9, 1A10 and 2A1. The visible increase of the activity in the presence of 0.05% compared to 0.1% BSA is given by the lower binding of the substrate to BSA. Comparisons between the two concentrations might, therefore, be misleading at this stage. It is anticipated that once the exact degree of substrate binding to each concentration of BSA (to be achieved soon), the effect on activity will be similar. An increase of activity compared to the control was however significant for both.

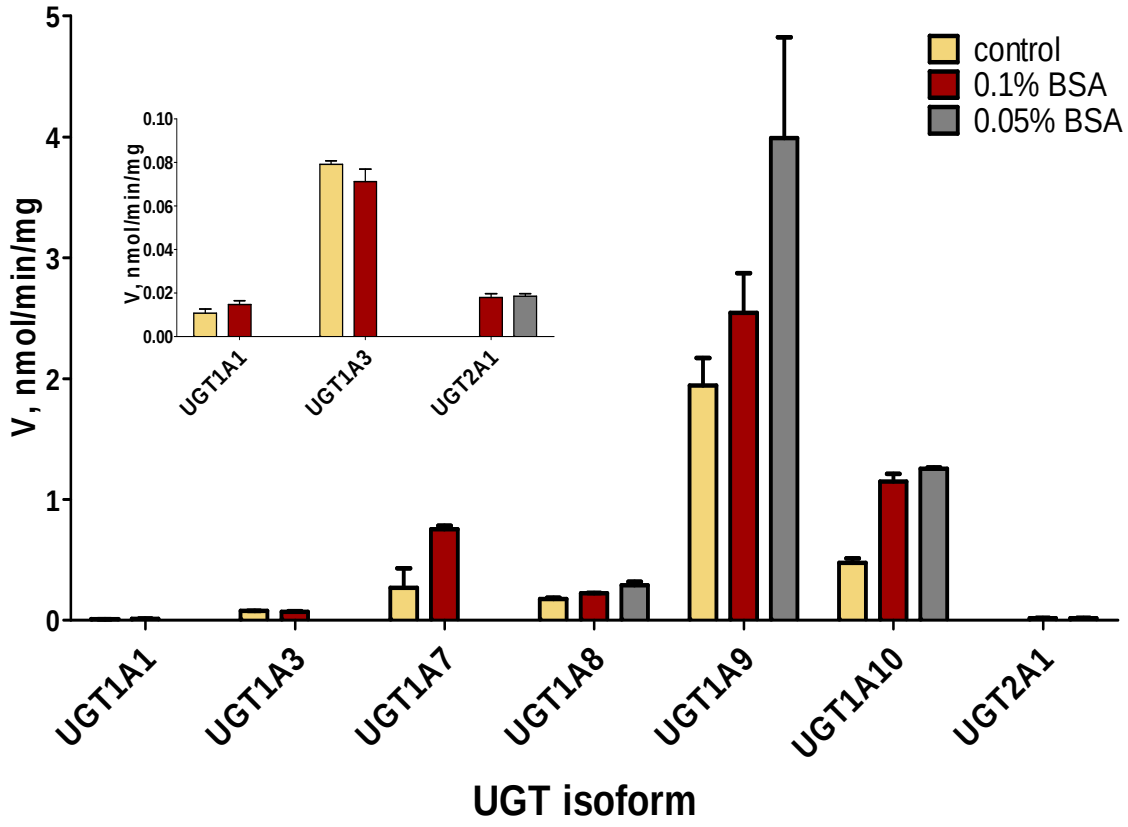


Figure 16. Screening of UGTs activity towards Entacapone
The substrate concentrations used are different in each isoform (Table 3). It is not possible to accomplish direct comparisons between the different isoforms.

4.2 Enzyme kinetics

4.2.1 4- Methylumbeliferone

4.2.1.1 Substrate kinetics

Based on the AICc and the r^2 values, the two-site model equations were found to best describe the 4MU kinetics of UGTs 1A7, 1A9, 1A10, 2A1 and 2B15 in presence of BSA. The controls (no added BSA) for these isoforms were not clearly demonstrating a two-site model, but for better comparisons between the kinetics, the two-site model equations were used to fit all the kinetics. The two-site model equation I was , therefore, used to fit the kinetics of the UGTs 1A7 and 2B15, whereas equation II was used for the UGTs 1A9, 1A10 and 2A1.

All the isoforms mentioned increased or even unmasked the evidence of the presence of a second binding site for 4MU when BSA was added. A look to the graphs where the rate of product formation was plotted versus the concentration of substrate, it seemed that 4MU was exhibiting a partial inhibition of the enzyme (Figure 17). A two site model autoactivation, however, was not visible in the Eadie- Hofstee plots for any isoform (Figure 18)

The addition of BSA to the samples increased Vmax by 2 to 5 fold in all the isoforms. Given by the presence of atypical kinetics, a comparison of the Ks (all data reported in Table 8) will not be very meaningful.

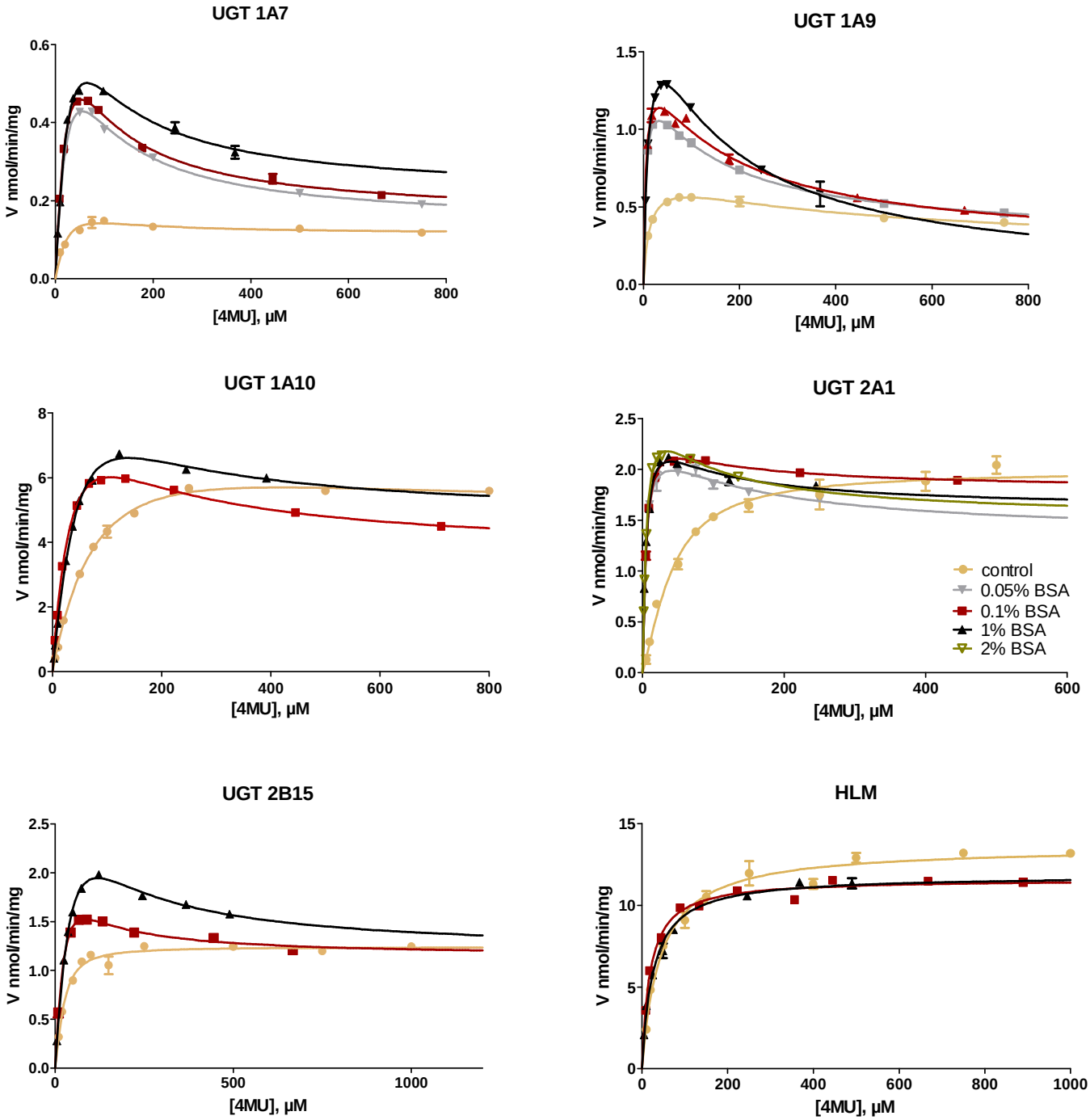


Figure 17. Kinetics of 4MU glucuronidation in the presence of different BSA concentrations

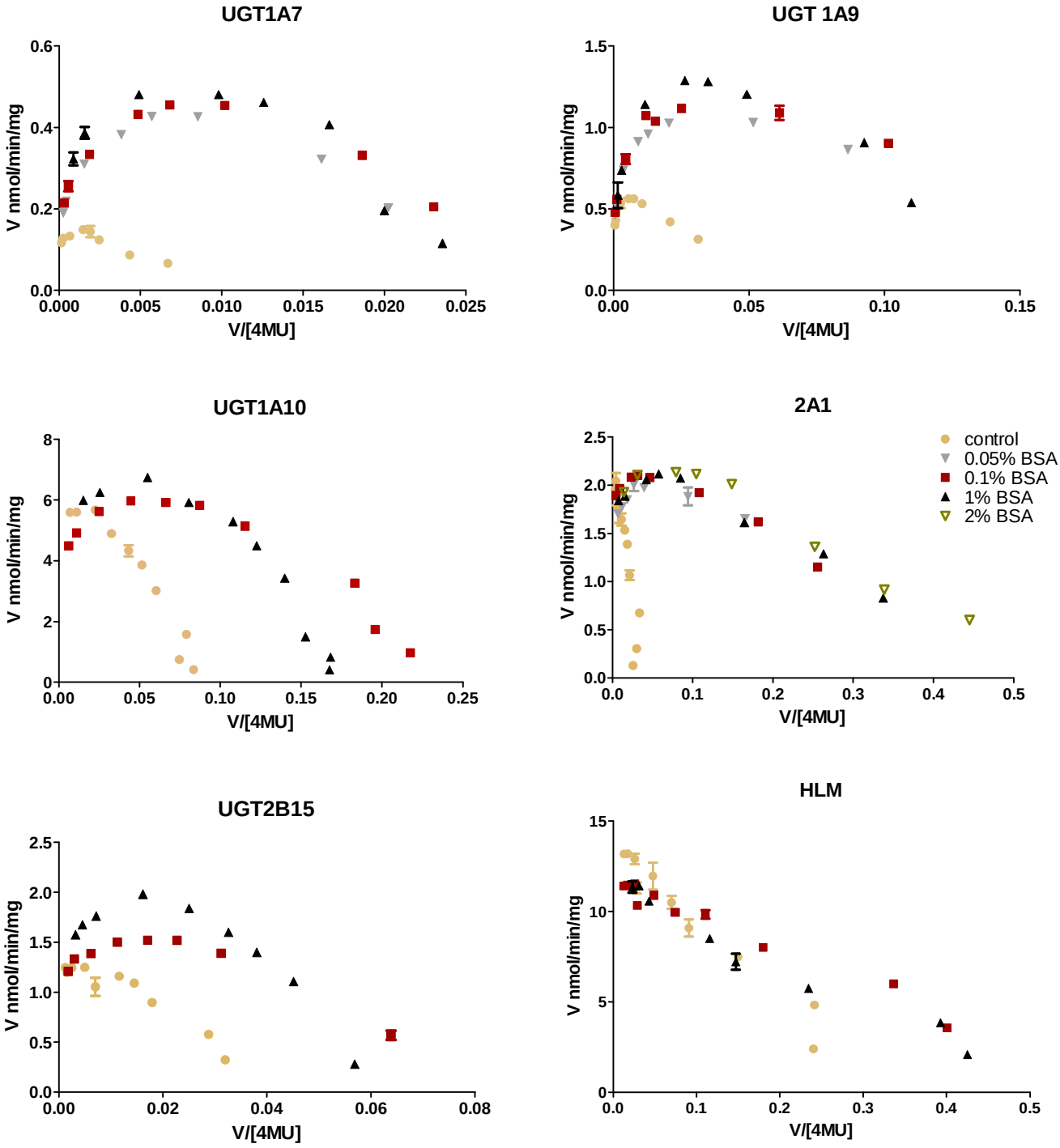


Figure 18. Eadie-Hofstee plots of 4MU kinetics

Table 8. Parameters of the substrate kinetics with 4MU as the main substrate

Parameter	control	Concentration of BSA			
		0.05%	**0.1%**	**1%**	**2%**
1A7[1]					
V_{max}	0.269 ± 0.03	1.138 ± 0.02	1.203 ± 0.04	1.262 ± 0.01	N.A.
K_S	41.0 ± 6.5	45.36 ± 1.1	42.74 ± 1.2	49.13 ± 2.8	N.A.
b	0.421 ± 0.06	0.119 ± 0.01	0.1302 ± 0.01	0.169 ± 0.02	N.A.
1A9[2]					
V_{max}	1.44 ± 0.08	2.902 ± 0.13	3.041 ± 0.21	4.148 ± 0.22	N.A.
K_s	26.74 ± 4.06	12.31 ± 1.67	11.34 ± 2.39	25.73 ± 2.63	N.A.
a	7.474 ± 5.44	5.109 ± 1.51	7.08 ± 3.49	2.667 ± 0.89	N.A.
b	0.156 ± 0.06	0.109 ± 0.01	0.073 ± 0.02	0.006 ± 0.03	N.A.
1A10[2]					
V_{max}	16.28 ± 0.95	N.A.	22.52 ± 1.27	29.71 ± 3.02	N.A.
K_s	179.9 ± 20.0	N.A.	92.67 ± 8.47	173.1 ± 26.85	N.A.
a	0.847 ± 0.66	N.A.	0.517 ± 0.15	0.181 ± 0.09	N.A.
b	0.296 ± 0.049	N.A.	0.162 ± 0.007	0.160 ± 0.015	N.A.
2A1[2]					
V_{max}	2.172 ± 0.059	4.863 ± 0.547	5.106 ± 0.150	5.358 ± 0.272	5.83 ± 0.208
K_s	7.375 ± 39.30	9.277 ± 3.78	11.01 ± 0.93	11.37 ± 1.36	11.68 ± 0.95
a	N.A.	6.468 ± 9.627	3.169 ± 1.29	2.686 ± 1.77	2.663 ± 1.533
b	N.A.	0.276 ± 0.046	0.352 ± 0.010	0.300 ± 0.018	0.259 ± 0.031
2B15[1]					
V_{max}	1.001 ± 0.460	N.A.	3.046 ± 0.085	4.394 ± 0.089	N.A.
K_s	30.57 ± 11.65	N.A.	42.47 ± 2.42	75.22 ± 2.36	N.A.
b	1.241 ± 0.598	N.A.	0.374 ± 0.019	0.267 ± 0.013	N.A.
HLM[3]					
V_{max}	13.58 ± 0,24	N.A.	11.6 ± 0,11	11.83 ± 0,193	N.A.
K_m	42.4 ± 3,8	N.A.	18.5 ± 1,06	25.91 ± 1,77	N.A.

[1] Two-site model equation I; [2] Two site model equation II; [3] Michaelis Menten equation

4.2.1.2 Substrate kinetics for human liver microsomes

Although HLMs contain a mixture of different 4MU glucuronidating UGT isoforms, the curves of the performed kinetics appeared linear in the Eadie-Hofstee plot (Figure 18). The Michaelis-Menten equation was therefore used to give an approximate estimation of the parameters (Table 8). The addition of 0.1 and 1% BSA to the incubation mixture seemed to slightly lower both the V_{max} and the K_m.

4.2.1.3 UDPGA kinetics

The kinetic analyses with respect to UDPGA, using 4MU as the aglycone substrate, are shown in Fig. 18. The analyses for UGTs 1A7 and 1A9 were consistent with the Michaelis-Menten model, while UGT2A1 presented a biphasic model with respect to UDPGA. The addition of BSA seemed to lower the biphasic effect in the latter enzyme. Due to this, the Michaelis Menten equation was also used to fit the kinetics of UGT2A1.

As shown in Table 9, the V_{max} was increased while K_m decreased in all the isoforms, when 0.1% BSA was added to the incubation mixture.

Table 9. Parameter for the UDPGA kinetics with 4MU as the aglycone substrate; the parameter are evaluated by fitting the Michaelis Menten equation to the kinetics

Parameter	**control**	**0.1% BSA**
1A7		
V_{max} (nmol/min/mg)	0.207± 0.008	0.450 ±0.008
K_m (µM)	906.2 ± 86.11	750.2 ±36.83
1A9		
V_{max} (nmol/min/mg)	0.477 ±0.0133	0.946 ±0.018
K_m (µM)	180.1 ±21.62	104.5 ±9.85
2A1		
V_{max} (nmol/min/mg)	0.64 1 ±0.020	2.225 ±0.031
K_m (µg)	285.5 ±33.78	75.75 ±5.66

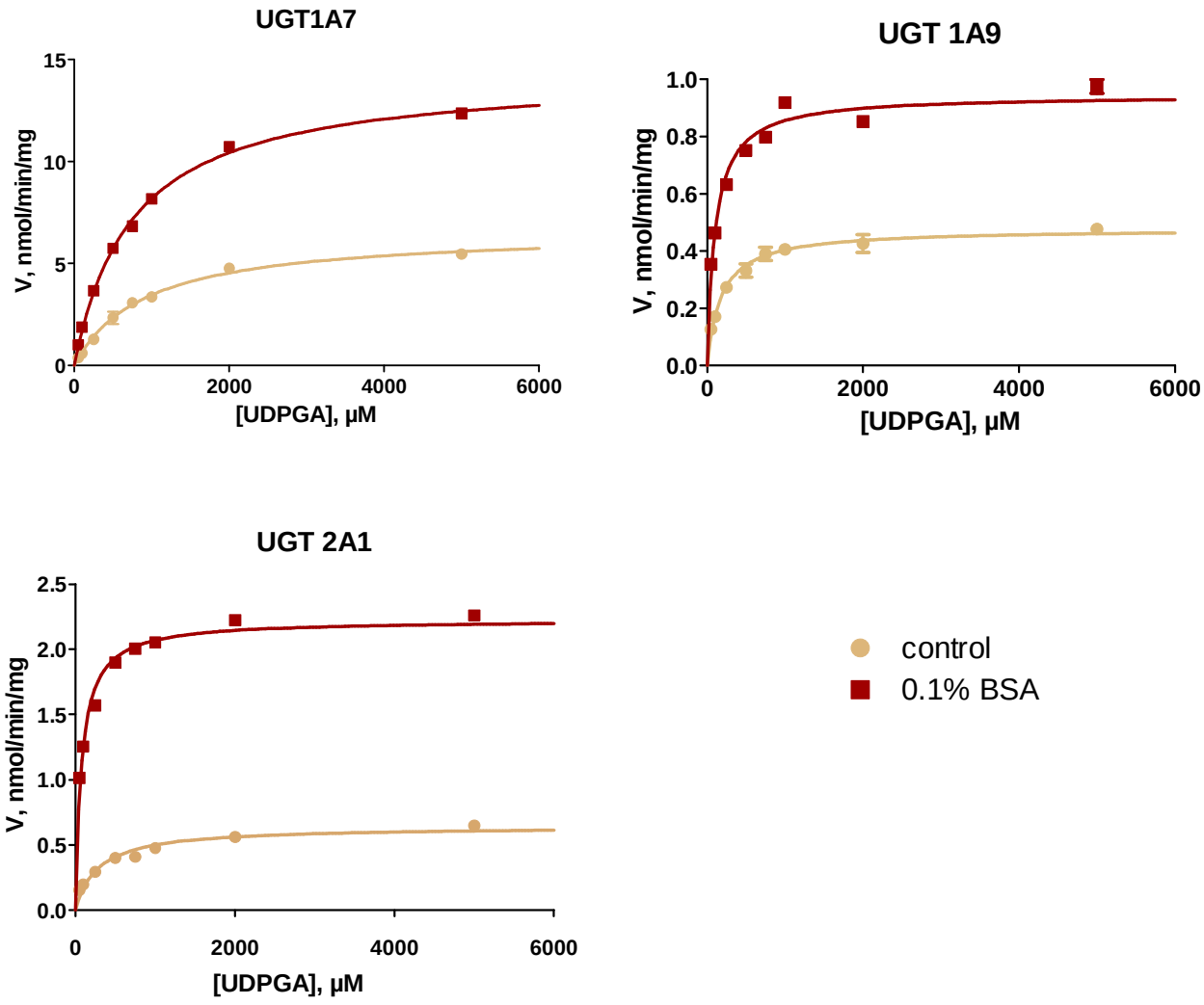


Figure 19. UDPGA kinetics for the glucuronidation of 4MU

4.2.2 Entacapone

4.2.2.1 Substrate kinetics

The kinetics of Entacapone glucuronidation were analyzed for UGTs 1A7 - 1A10, as well as for HIM and HLM (Figure 20). Eadie-Hofstee plots of the results for UGTs 1A7- 1A10 (Figure 21) suggest atypical kinetics both in the absence and the presence of BSA for most of these UGTs. UGT1A7 was the only enzyme that was linear in the Eadie-Hofstee plot and was best described by the Michaelis-Menten equation. Inhibition by the substrate was visible in UGT1A9, whereas the substrate inhibition model and the two-site model no. I were consistent with the control and the kinetics in presence of BSA for this enzyme, respectively. Comparisons between latter two kinetics were accomplished by using the two-site model equation I for both. In UGT1A10, the addition of BSA increased the already present autoactivation. The Hill equation was, therefore, applied for these kinetics.

The description of the kinetics of the UGT1A8 turned out to be more complicated, given by the three phases visible in the Eadie-Hofstee plot. The enzyme exhibited autoactivation at lower concentrations of Entacapone, but substrate inhibition at higher concentrations. According to the AICc, the substrate inhibition equation and the two-site model equation II were best describing the control and the kinetics of UGT1A8 in the presence of BSA, respectively. Due to the difficulty to compare between these two kinetics, the concentrations in the range of inhibition were excluded and the Hill equation was fitted for both. In this case, the autoactivation was considered and the S_{50} values could be used to determine the inhibition pattern present in absence of BSA.

The V_{max} of the UGTs 1A8, 1A9 and 1A10 were 2-3 fold higher in presence of BSA than in its absence. Moreover, the V_{max} increase in UGT1A7 was even 7 fold. In presence of BSA K_m or K_s increased in UGTs 1A7 and 1A9, respectively. In contrast, the S_{50} decreased in UGTs 1A8 and 1A1

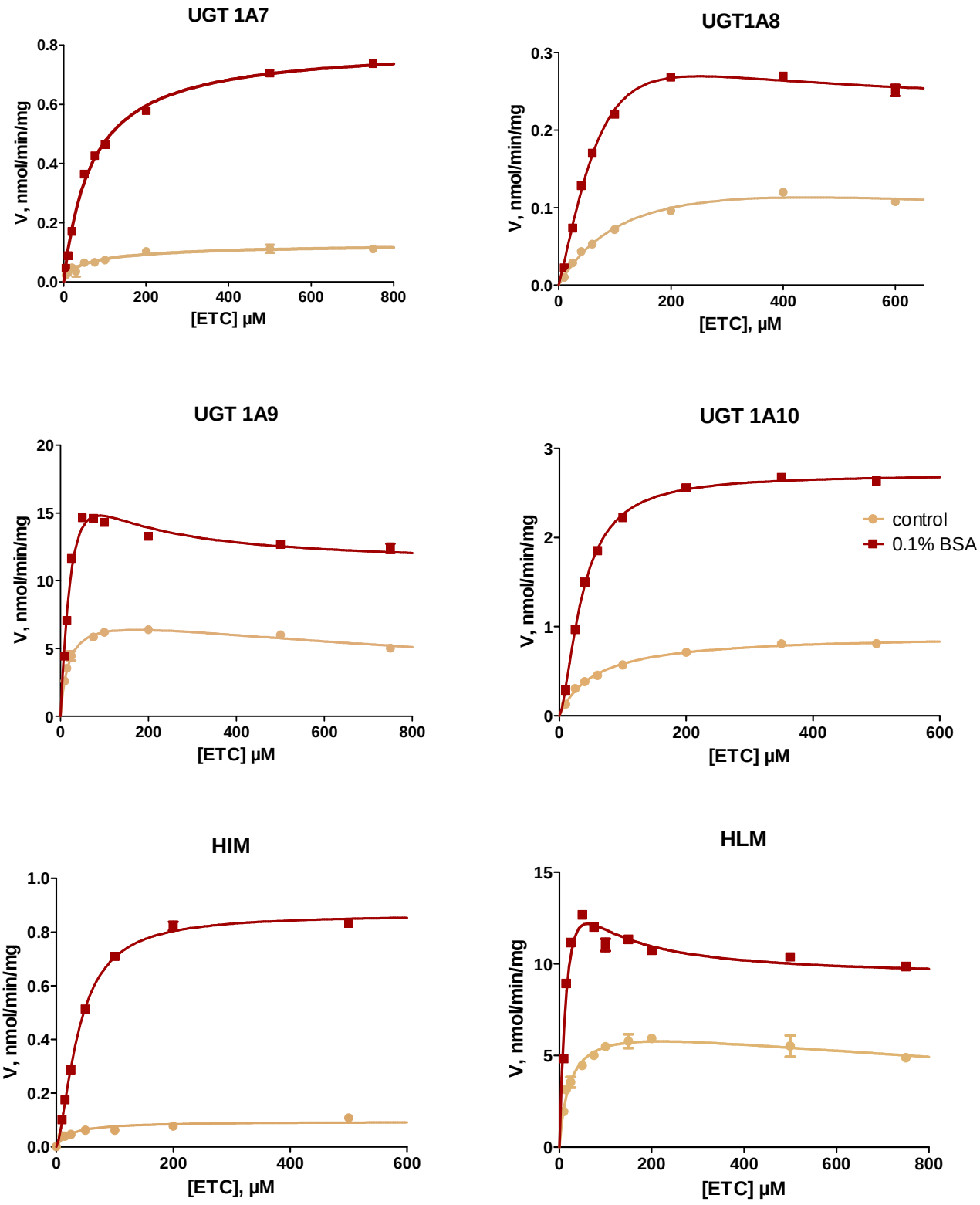


Figure 20. Kinetics of Entacapone in presence or absence of BSA

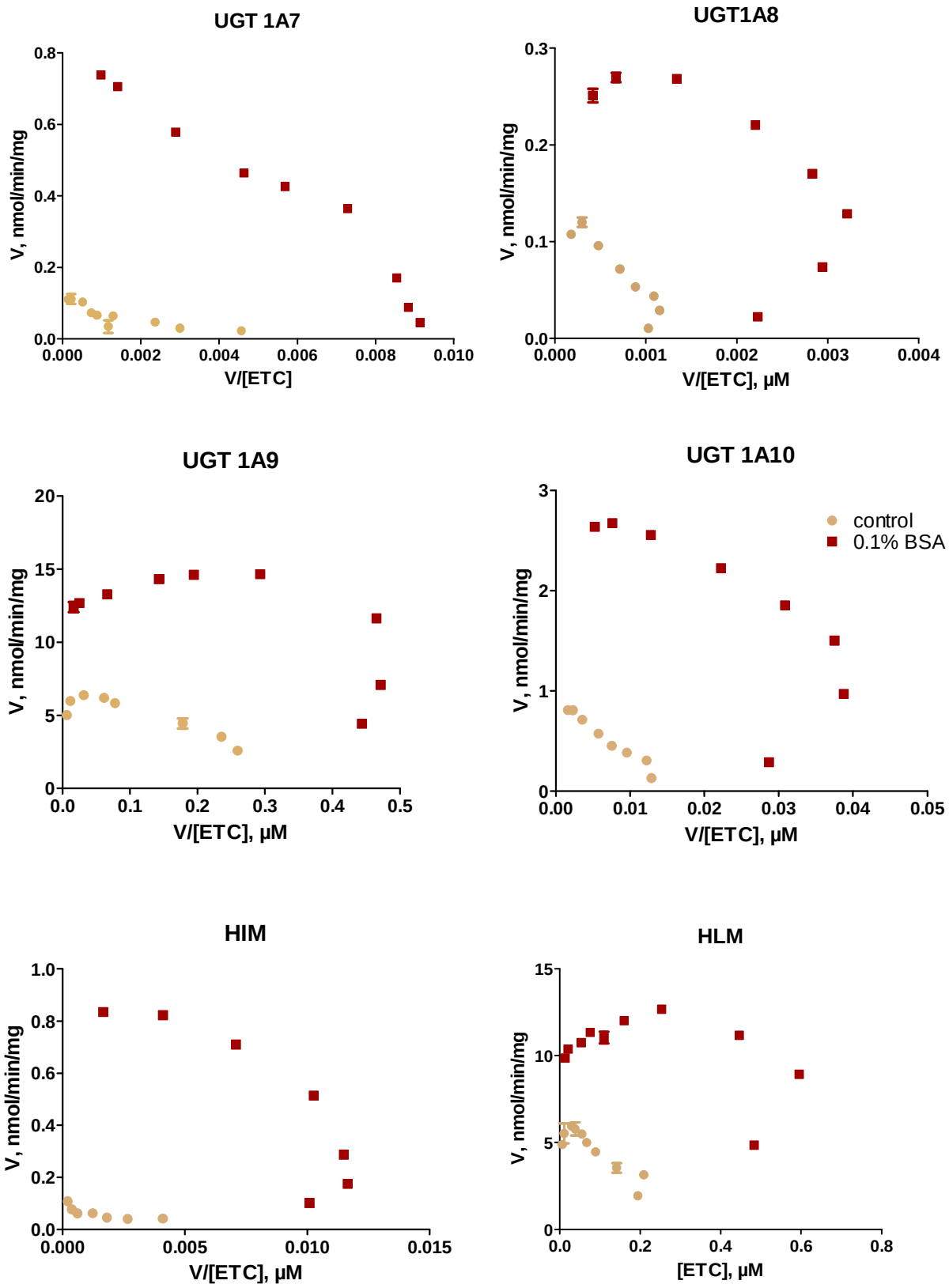


Figure 21. Eadie-Hofstee plots of Entacapone kinetics

Table 10. Parameters of the substrate kinetics with Entacapone as the aglycone substrate

Parameter	control	0.1% BSA	Used equation
1A7			
V_{max}	0.119 ± 0.119	0.794 ± 0.800	Michaelis Menten
K_m	45.73 ± 45.73	65.93 ± 68.55	
1A8			
V_{max}	0.156 ± 0.0166	0.282 ± 0.005	Hill equation
S_{50}	117.4 ± 22.25	45.66 ± 1.517	
h	0.964 ± 0.132	1.68 ± 0.053	
1A9			
V_{max}	10.85 ±1.001	29.58 ±1.684	Two-site model
K_s	36.6 ± 4.640	45.01 ± 3.976	equation I
b	0.488 ± 0.064	0.3756 ± 0.036	
1A10			
V_{max}	0.933 ± 0.026	2.708 ± 0.018	Hill equation
S_{50}	59.76 ± 4.585	36.14 ± 0.5812	
h	0.954 ± 0.051	1.587 ± 0.041	
HIM			
V_{max}	0.094 ± 0.005	0.990 ± 0.035	Michaelis Menten
K_m	23.6 ± 4.633	52.53 ± 5.628	
HLM			
V_{max}	7.757 ± 1.504	24.29 ± 1.300	Two-site model
K_s	34.75 ± 8.341	30.41 ± 2.736	equation I
b	0.677 ± 0.164	0.378 ± 0.034	

4.2.2.2 Entacapone glucuronidation kinetics of HLM and HIM

The kinetics of HLM in the absence of BSA were best described by the substrate inhibition model, whereas the addition of BSA revealed a two-site model. Entacapone seemed to act as a partial inhibitor by attaching a second binding site. The two site model equation I was, therefore, used to evaluate the parameters of both kinetics.

Given the presence of different isoforms in HIM, it was difficult to find an appropriate equation for its kinetics. The simple Michaelis-Menten equation was fitted to the control and to the kinetics in presence of BSA to give an approximate estimation of the changes in V_{max}.

BSA increased significantly the V_{max} of both microsomes. Comparisons between the K_s and in this case also the K_m are not meaningful (Table 10).

4.2.2.3 UDPGA Kinetics in Entacapone glucuronidation

The evaluation of the results of the UDPGA kinetics with Entacapone as the aglycone substrate (Figure 22) showed a decrease in activity at higher concentrations of UDPGA in most cases. Only UGT1A9 in the presence of BSA, and the UGT1A10 control did not exhibit any inhibition by UDPGA below 5mM. The substrate inhibition equation was fitted for all the kinetics, namely with UGTs 1A7 - 1A10.

The addition of BSA to the incubation mixture increased the V_{max} of all the isoforms and the K_m of UGTs 1A7 and 1A8. The K_m of UGTs 1A9 and 1A10, however, were decreased in the presence of BSA (Table 11).

Table 11. Kinetic parameter for UDPGA kinetics with Entacapone as the main substrate; the parameters were evaluated by fitting the substrate inhibition equation to the kinetics

Parameter	**control**	**0.1% BSA**
1A7		
V_{max} (nmol/min/mg)	0.3306 ±0.02816	1.389 ±0.09792
K_m (µM)	781.9 ±115.5	1’884 ±189.1
K_i (µM)	5’690 ±1169	11’145 ±2’406
1A8		
V_{max} (nmol/min/mg)	0.1221 ±0.01444	0.3026 ±0.02236
K_m (µM)	1’180 ±237.8	1’680 ±195.4
K_i (µM)	8’133 ±2’599	22’851 ±8’974
1A9		
V_{max} (nmol/min/mg)	2.605 ±0.1223	5.359 ±0.07346
K_m (µM)	416.0 ±43.24	247.4 ±13.22
K_i (µM)	13’556 ±2’852	N.A.
1A10		
V_{max} (nmol/min/mg)	0.3 ±0.003468	1.534 ±0.05616
K_m (µM)	704.2 ±22.67	592.0 ±43.06
K_i (µM)	N.A.	15’044 ±2’445

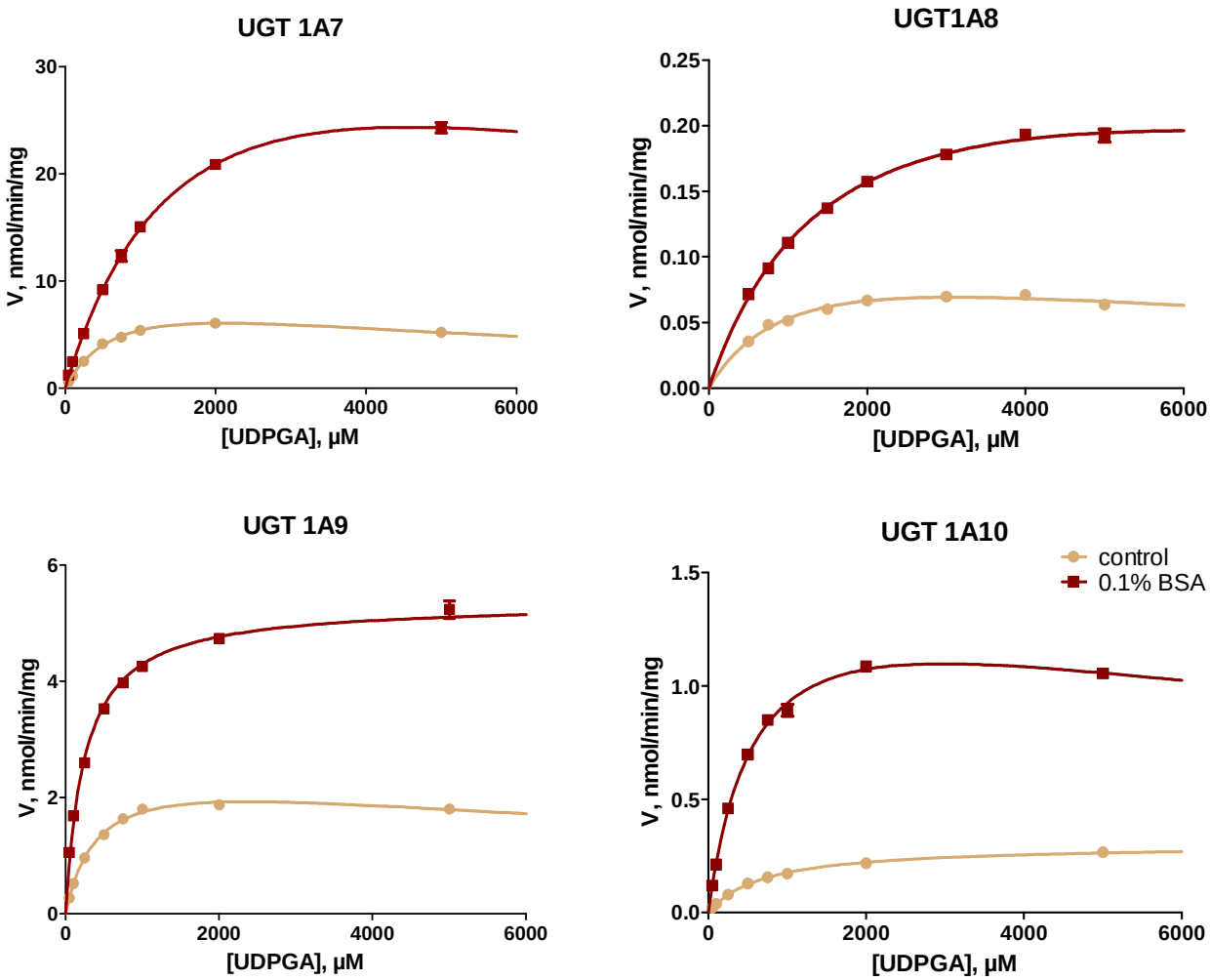


Figure 22. UDPGA kinetics for the glucuronidation of Entacapone

5 Discussion

5.1 Preliminary assays

5.1.1 Unspecific binding to the filter membrane

It was previously reported that the nonspecific binding of the neutral substance 4MU to the filter membrane decreases, when the filter is pretreated with Tween 80 (Lee et al 2003). This effect was also visible in our hands when the slightly different detergent Tween 20 was used (Figure 11).

5.1.2 Binding of 4MU to BSA

About 10 to 20% of the effective concentrations of 4MU were removed by binding to 0.1% BSA. This percentage remained stable in the range between 2 to 160 µM of substrate. The linearity of f_u for different concentrations of substrate is, therefore, in agreement with the data reported in (Rowland et al., 2008).

At the stage of completing my work, it was not yet possible to perform a full and accurate estimation of the f_u, since there was still an open question of whether or not some 4MU is trapped with the BSA when it is precipitated by the addition of PCA. Comparing the concentration of 4MU in the feed to the original concentration, it seemed that 10% were still 'trapped' in albumin. The comparison between the original unfiltered concentration and the filtrate resulted in a f_u of 0.7.

In the previous article (Rowland et al. 2008), it was claimed that the f_u for 4MU in presence of BSA was 0.89. This value could be almost achieved by us as well, if we only compared the filtrate with the feed in our system (f_u = 0.81) and thereby overlook the residual binding of 4MU to albumin. Further studies have to be performed to establish the correct f_u.

5.1.3 Washing insect cell membranes with BSA

The results concerning membranes pretreatment with BSA revealed that the removal of the inhibitory factor can not be achieved in this way. One may suggest that if the inhibitor is a fatty acid, then following the removal of BSA there is further release of inhibitory fatty acids from the membranes. A pretreatment is therefore difficult, given by the impossibility of preventing the release of additional FA without affecting the activity of the UGTs. As seen in the experiment, a proper removal of all the inhibitor molecules that could be removed by BSA requires the presence of BSA during the reaction, namely the incubation of the membranes with the substrates.

5.2 Kinetics

5.2.1 Characterization of the UGTs inhibition

According to previous studies (Rowland et al., 2008; Uchaipichat et al., 2006; Raungrut et al., 2010; Rowland et al., 2006), the addition of BSA increases the activity of all the tested UGTs. As reported in these publications, the effect was caused by removing a mixture of inhibitors (FAs) by albumin. The parameters V_{max} and K_m were, therefore, used to analyze the type of the inhibitors affecting our recombinant UGTs.

All the substrate kinetics performed showed a significant increase of V_{max} when BSA was added to the incubation mixture. This observation led to the assumption that the inhibitors removed by albumin were binding to another binding site than 4MU and Entacapone. UDPGA kinetics were therefore performed to test, whether or not the inhibitor was competing for the same binding site as UDPGA. Interestingly, also the V_{max} of these kinetics were increased as a consequence of BSA addition. The inhibitors were therefore modulating the activity of the enzyme mainly through a third binding site.

Since most of the isoforms expressed atypical kinetics at least in presence of BSA, it was not possible to compare the K_m. This lack of information hindered

to establish whether the inhibitor was binding with prevalence to the free enzyme or to the ES complex.

A significant use of this parameter was only possible for the substrate kinetics of UGT1A7 with Entacapone as the aglycone substrate and the UPDGA kinetics of the UGTs 1A7, 1A9 and 2A1 with 4MU. The increase of the K_m in the presence of BSA suggested that in the glucuronidation of Entacapone by UGT1A7, the inhibitor was prevalently binding to the ES complex. In contrast, the data of the UDPGA kinetics showed that the inhibition was occurring prior to the binding of UDPGA. These results are, however, incomplete at this stage.

Concluding, the data concerning the experiments with our recombinant UGTs 1A7, 1A8, 1A9, 1A10, 2A1 and 2B15 indicated that BSA was removing a mixture of noncompetitive and/or mixed type inhibitors.

Although this result seemed to be coherent through all our experiments, it was not fully consistent with the reported results regarding the effect of BSA on the activity of UGTs *in vitro* (Table 2). According to these publications, the mixture of inhibitors was acting competitively towards several substrates glucuronidated by the UGTs 1A9, 2B4 and 2B7. Only the glucuronidation of lamotrigine by the UGT1A4 seemed to be affected by a mixed type inhibitor (Rowland et al., 2006).

The most interesting data for the purpose of this thesis was the glucuronidation of 4MU by UGT1A9 reported in Rowland et al., 2008 (Table 12). In this publication the authors claimed, based on the observed decrease in K_m and unchanged V_{max} that BSA was removing a competitive inhibitor. They also analyzed the inhibitory effect of a mixture of fatty acids (oleic acid, arachidonic acid, linoleic acid) on the same system and got similar results. It is also reported by Tsoutsikos et al. 2004 that arachidonic and linoleic acid inhibited separately the glucuronidation of 4MU by UGT1A9 in a competitive manner. This inhibitory effect was also visible in human kidney cortical microsomes.

Table 12. Effect of BSA and fatty acids on UGT1A9, HLM and HKCM

Source	Tested system	Type of inhibition
Our data		
4MU kinetics in the presence of BSA	Recombinant UGT1A9 expressed in Sf9 cells	Mixed type
	Human liver microsomes	Slightly competitive
Entacapone kinetics in the presence of BSA	Recombinant UGT1A9 expressed in Sf9 cells	Mixed type
	Human liver microsomes	Mixed type
Rowland et al., 2008		
4MU kinetics in the presence of BSA	recombinant UGT1A9 expressed in HEK293 cells	Competitive
Rowland et al., 2007		
4MU kinetics inhibited by a mixture of oleic acid, linoleic acid and arachidonic acid	HLM	Competitive (but with slight decrease of V_{max})
Tsoutsikos et al., 2004		
4MU kinetics inhibited by arachidonic or linoleic acid	recombinant UGT1A9 expressed in HEK293 cells	Competitive
	human kidney cortical microsomes	Competitive

In contrast to the previously published reports, the glucuronidation of 4MU by our recombinant UGTs seemed to be affected by a mixed type inhibitor, visible as a significant increase of V_{max}. This effect was, however, not present in the glucuronidation of 4MU by HLM (Table 8 and Figure 17). The slightly lowered V_{max} and K_m that we have detected could be explained by the contribution of different isoforms.

Nevertheless, the effect of BSA towards UGT1A9 in human liver microsomes could be evaluated by measuring the glucuronidation of Entacapone by HLM. This substance is glucuronidated in HLM almost exclusively by UGT1A9 (Lautala et al., 2000).

Our data showed that BSA was removing a mixed type inhibitor from the system, when the activity of recombinant UGT1A9 was tested towards Entacapone. Interestingly, this effect was also present in HLM and HIM when entacapone was the aglycone substrate, confirming the presence of mixed type inhibitors also in the more native systems.

A possible reason for the apparent differences between our and the previously published results could be the use of a different recombinant UGT system. According to Rowland et al., 2007 and Marheineke et al., 1998, the amounts and percentages of fatty acids differ between HEK293, HLM and Sf9 cells. The types of inhibition have only been tested for arachidonic acid and linoleic acid separately (Tsoutsikos et al., 2004) and for a mixture of oleic acid, arachidonic acid and linoleic acid (Rowland et al., 2007). Kinetics in the presence of palmitoleic acid, for example, an inhibitor of at least UGT1A9 and 2B7 (Tsoutsikos et al., 2004), have not been performed yet. Interestingly, palmitoleic acid is the second most abundant FA in Sf9 cells, but its presence in HLM and HEK293 cells is minor in comparison to other FAs.

Another explanation for the differences between the current and previous studies could be that the type of inhibition is isoform and/or substrate dependent. The effect of BSA on UGTs 1A7, 1A8, 1A10, 2A1 and 2B15 has not been studied or published so far. Also the kinetics of UGT1A9 with Entacapone has not been performed previously. The published data for the glucuronidation of lamotrigine by UGT1A4 (Rowland et al., 2006), where BSA seems to remove a mixed type inhibitor, is in line with our results, but this is not an evidence since a different UGT and a different substrate were examined. Still, it shows that not all inhibitions are the same, even when inhibitors removal by BSA is tested.

5.2.2 Atypical kinetics

As previously observed (Table 2) the addition of BSA seems to increase or even unmask the presence of two site model kinetics in almost all the tested isoforms (Table 13). By removing the inhibitor, the substrate can bind also to a second site, affecting the glucuronidation of another molecule of the same substrate. The binding of 4MU to a second site seemed to inhibit the activity of all the tested isoforms, while Entacapone was inducing an autoactivation. An additional (partial) substrate inhibition occurred also in the glucuronidation of Entacapone by UGT1A8 and 1A9.

Table 13. Changes of the kinetics as a consequence of BSA addition
Kinetic models: MM= Michaelis Menten ; SI= Substrate Inhibition; PI= Partial Inhibition; AA= Autoactivation

Isoform	4MU		ENTACAPONE	
	Control	0.1% BSA	Control	0.1% BSA
1A7	Slight PI	Strong PI	MM	MM
1A8	N.A.	N.A.	AA + SI	AA + SI
1A9	Slight PI	Strong PI	SI	AA + PI
1A10	MM	PI	Slight AA	AA
2A1	MM	PI	N.A.	N.A.
2B15	MM	PI	N.A.	N.A.
HIM	N.A.	N.A.	Slight AA	AA
HLM	MM	MM	SI	AA + PI

Interestingly, the kinetics of Entacapone glucuronidation by HLM was similar to the kinetics of this reaction by recombinant UGT1A9 (Figure 20). In both systems BSA was increasing or unmasking an autoactivation with subsequent partial inhibition. As already described, Entacapone is glucuronidated in HLM almost exclusively by UGT1A9 (Lautala et al., 2000). Hence, this result proved that our system is comparable to the commercial available HLM.

The removal of the inhibitors is therefore not only increasing the activity of the enzyme, it is also releasing a second binding site for the substrate. Based on the published data, it can be assumed that the inhibitors in our system are FAs. Since FAs can act as competitive inhibitors (Rowland et al., 2006; Rowland et al., 2008a; Rowland et al., 2009; Uchaipichat et al., 2006; Raungrut et al., 2010), while they are also substrates for the UGTs (Little et al., 2002; Turgeon et al., 2003; Little et al., 2004), it can be concluded that both, the catalytic and the inhibitory sites are capable of binding both the substrate and the FAs. This leads to the assumption that both binding sites are therefore similar to each other.

It was previously discussed extensively that UGTs may be oligomeric enzymes, and that the occurrence of homo- and hetero- dimers is likely (Finel and Kurkela, 2008). According to that, the above mentioned inhibitory site could be the catalytic site of the second half of the dimer (Figure 23). This would implicate a reciprocal influence between the two halves of the dimer. In this case, the inhibitor would act as competitive and mixed type inhibitor towards the glucuronidation of the same substrate and the same enzyme.

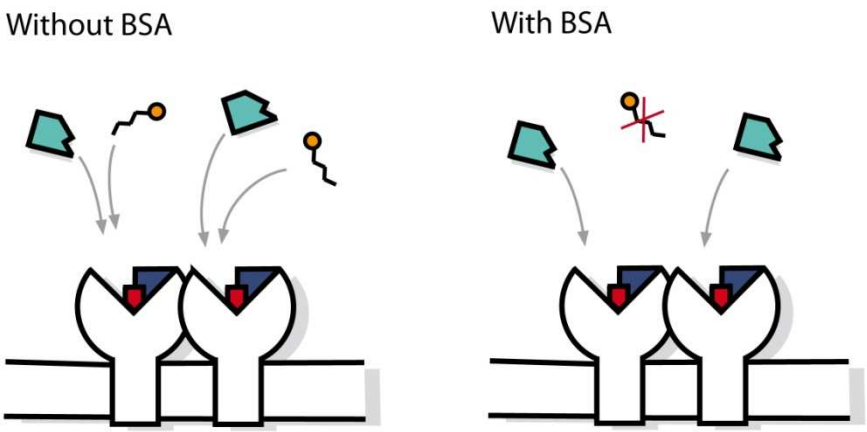


Figure 23. Scheme of a dimer, where substrate and FAs compete for the same binding sites. The addition of BSA removes the FAs and facilitates the binding of substrate.

As visible in Figure 24, it is not possible to ascertain a competitive inhibition if a mixed type inhibitor is affecting the system at the same time. The additional competitive inhibition in our system could be therefore masked by the presence of a mixed type inhibition.

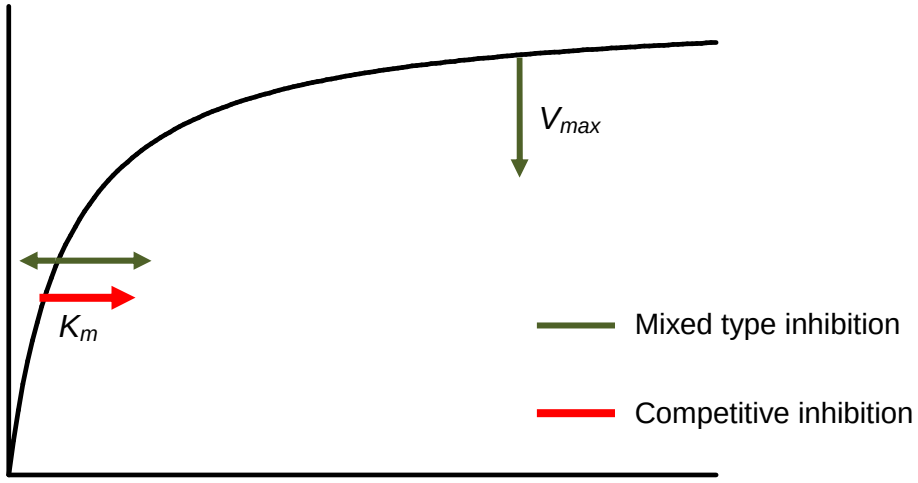


Figure 24. Influence of inhibitors on K_m and V_{max}

This hypothesis could be also used to explain the particular results obtained for the glucuronidation of 4MU by HLM. The difference between the isoforms present in the microsomes and the recombinant isoforms is that while the latter can only form homo- dimers, hetero- dimers could occur in HLM as well. It might be that 4MU acts as a stronger inhibitor towards its own glucuronidation than the FA, when two different isoforms are linked to a dimer. In this case, BSA is increasing the chance for 4MU to bind also to the second site and is hence decreasing the activity of the enzyme. The lowered V_{max} would be given by the mixed type inhibition by 4MU and the decreased K_m would indicate the removal of the competitively inhibiting FA. Further experiments are, however, needed to confirm this assumption.

Since it is not fully confirmed that UGTs form dimers, it is not possible to exclude the presence of a second binding site for the inhibitor and the substrate on the same enzyme/monomer.

6 Conclusion

Our results showed that, in contrast to previously published findings, a mixed type inhibitor was affecting our recombinant UGTs 1A7, 1A8, 1A9, 1A10, 2A1 and 2B15, when the glucuronidation of both 4MU and Entacapone was tested. This outcome was also visible in the glucuronidation of Entacapone by the commercial available HLM and HIM. The glucuronidation of 4MU by HLM, however, demonstrated a particular influence of BSA. While K_m was decreased (suggesting the removal of a competitive inhibitor), V_{max} was also lower, indicating the appearance of a mixed type inhibitor. We tried to explain this outcome by supposing that 4MU was in this case acting as a stronger mixed type inhibitor towards its own glucuronidation, than FAs.

Another interesting result was the appearance of two-site model kinetics by the addition of BSA to almost all the tested UGT isoforms. We observed that this effect was also visible in previous publications (Table 2). Noticing, that the substrates and the FAs were both binding to two sites in this model, we suggest that these sites were the identical catalytic sites in a UGT dimer (Finel and Kurkela, 2008). This would implicate that both halves of the dimer are influencing the activity of each other.

Our suggestions to explain the differences between our results and the previous ones are based on the use of different expression systems for producing recombinant UGTs, something that might lead to:

- different inhibitors are present in our system (different concentrations of FAs or additional presence of other inhibitors)
- the inhibition pattern is substrate and/or isoform dependent
- a different distribution of monomers and dimers can occur

Nevertheless, these hypotheses have still to be proven by the quantification of FAs in our system and following kinetics in presence of each FA.

We confirmed with our results that BSA increases the activity of UGTs 1A7, 1A8, 1A9, 1A10, 2A1 and 2B15. This reinforces the recommendation to consider the inhibitors present in the systems when *in vitro* tests are performed with UGTs.

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Curriculum Vitae

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