ORIGINAL ARTICLE

Interspecies comparison of the glucuronidation processes in the man, monkey, pig, dog and rat

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Abstract

OBJECTIVES: The study of interspecies differences in glucuronidation processes in the man, monkey, pig, dog and rat using liver microsomal fraction. The study is focused on determination of the enzyme activity of UGT1A6 (having also a toxicological importance) in microsomes of different species.
METHODS: For determination of glucuronides formed, an HPLC method with UV detection and LC-MS characterization was used. p-Nitrophenol and 4-methylumbelliferon and silybin were chosen as model substrates.
RESULTS: The data presented in this paper show an overall similarity in kinetic parameters of the UGT1A6 with p-nitrophenol and 4-methylumbelliferon for man, pig and monkey. The pattern of silybin glucuronides formed in monkey and dog samples are relatively close to this of the man.
CONCLUSIONS: For studies of glucuronidation of xenobiotics where the role UGT1A6 is expected, the use of pig and monkey microsomes should be considered. As an optimal model for study of silybin glucuronidation, both the rhesus monkey and dog (Beagle) seem to be the best models. To elucidate the role of the UGT forms involved in metabolism of silybin, the experiments with recombinant UGT enzymes are needed.

Abbreviations

UGT	- UDP-glucuronosyltransferase
CYP	- Cytochrome P450
AhR	- arylhydrocarbon receptor
4-MU	- 4-methylumbelliferon
p-NP	- p-nitrophenol
UDPGA	- uridine 5′-diphosphoglucuronic acid

INTRODUCTION

In mammals, glucuronidation is a major conjugation reaction providing for metabolic elimination of exogenous compounds (e.g. drugs, environmental chemicals and dietary constituents) and endogenous compounds (e.g. bile acids, bilirubin and steroids). Glucuronide formation is catalyzed by a family of UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17). In human, UGT-catalyzed glucuronidation reactions are responsible for approximately 35% of all drugs metabolized by phase II enzymes [15]. To date, 18 human UGTs are known [7]. UGTs are mainly located in the endoplasmic reticulum and its activities *in vitro* are only detected after the use of detergent, phospholipase or sonication. Most, but not all, of these enzymes are expressed in liver. Organs and tissues where the UGTs have also been detected include e.g. the gastrointestinal tract, lungs, kidney, prostate, brain, placenta and nasal epithelium [1,6,16,23]. Hitherto known mammalian UGTs have been identified from various species by protein purification or cDNA cloning and classified into two families of protein, designated UGT1 and UGT2 according to their amino acid sequence analogy and gene structure [17]. In humans, UGT1 family enzymes are encoded by one gene which has multiple unique exons located upstream of four common exons. On the other hand, UGT2 family enzymes are encoded by a separate gene comprised of six exons [10,18,22]. In last years, the focus on developing tools to evaluate CYP-mediated drug metabolism and interactions has resulted in the lag of knowledge of other important drug metabolizing enzymes, namely the UGTs in the areas of *in vitro* methods, enzymology and substrate specificity. Unfortunately, data about activity of UGTs in other mammals are rare, although the activity of UGTs in liver is an inseparable part of toxicological research for xenobiotic chemicals.

The UGT1A6 form is one of two most expressed forms in mammals and is also co-induced with CYP1A forms via aryl hydrocarbon receptor (AhR) [10]. 4-methylumbelliferon (4-MU) and p-nitrophenol (p-NP) have been reported to be prototypic substrates for UGT1A6 in mammals and UGT activities toward these substrates have been often used as toxicological markers for environmental chemicals [4, 19]. UGT1A6 substrates, namely 4-MU and p-NP, also inhibit formation of silybin glucuronides and the role of UGT1A6 in formation of silybin glucuronides is considered [8, Jancova and Matal, unpublished results]. The aim of this work is to study in more detail interspecies differences in the enzymatic properties of UGT1A6 as one of the major forms in the human liver. The enzymatic properties were examined by kinetic analysis using p-nitrophenol and 4-methylumbelliferon as substrates in the liver microsomal fraction of man, pig, monkey (*Maccaca rhesus*), rat, and dog (Beagle). Another part of this work was to determine whether these species are able to form the silybin glucuronides similar to man and subsequently, if they can be used as a model for glucuronide metabolism of this compound *in vitro*. Silybin is a flavolignan and an active component of silymarin, an extract from *Silybum marianum* (milk thistle) seeds. Silybin consist of two diastereomers (A and B) and forms the glucuronides in positions 7 and 20 (See Figure 1) [12].

Silymarin is known for his hepatoprotective effect for hundreds of years. Recently, it was also shown to exhibit an antioxidative and radical scavenging activity [24] as well as e.g. an anticancer effect [21]. Recently, the CYP form responsible for silybin biotransformation *in vitro* was found [14] and as a next step, the enzymes involved in phase II metabolism should be examined.

MATERIAL AND METHODS

Material

All chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic), if not stated otherwise and were in purity of best available. Silybin was a gift from Ivax-CR a.s. (Opava, Czech Republic). Human liver microsomes were purchased from Biopredic (Rennes, France) and were obtained in accordance with ethical regulations of the country of origin (France). Pooled pig liver microsomes were prepared according to established methods [20] from experimental material obtained in a local slaughterhouse. Pooled liver microsomes from dog, monkey and rat were prepared from livers obtained earlier in accordance with the respective ethical regulations for the use of experimental animals.

Enzyme kinetics of UGT1A6 activity in liver microsomes

All incubations were performed in glass test tubes. The incubation mixture for kinetic analyses of formation 4-MU and p-NP glucuronides contained 4-MU or p-NP (0–3000 μM) as substrates, liver microsomal proteins (10 μg), 10 mM $MgCl_2$ and 3 mM UDPGA (uridine 5'-diphosphoglucuronic acid) in a final volume of 400 μl of 50 mM Tris–HCl buffer (pH 7.4). After preincubation at 37 °C for 1 min, the reaction was started by the addition of UDPGA. The mixture was incubated at 37°C for 15 min and the reaction was termi-

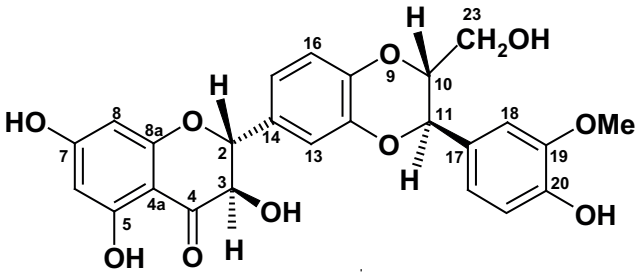

Figure 1. Structure of silybin

nated with 50 µl of icecold 15% (w:v) perchloric acid. The reaction mixture was centrifuged at 12 000 rpm for 10 min at 4 °C. Then the supernatant was analyzed by HPLC according to an HPLC method with UV detection described in original paper of Hanioka *et al*. [13].

Comparison of formation of silybin glucuronides in liver microsomes

Reaction conditions for determination of silybin glucuronides were as follows: 350 µg of microsomal protein, 3 mM UDPGA, 5 mM $MgCl_2$, 10 mM D-saccharic acid 1,4-lactone, 50 µM silybin and 100 mM K/PO_4 buffer (pH 7.4) in total volume of 100 µl. After the incubation (30 min, 37 °C) the reaction was stopped by addition of 200 µl of icecoled methanol. The reaction mixture was centrifuged at 5 000 rpm for 5 min. Supernatant was dried under the flow nitrogen and dissolved in 100 µl of mobile phase. For determination of silybin glucuronides, HPLC method with UV detection based on method of Gunaratna and Zhang [11] was used; glucuronides were characterized by LC-MS method (LCQ Fleet ion-trap mass spectrometer, Thermo Fisher Scientific, Waltham, MA, USA) as 7 and 20-glucuronides (according to method of Han *et al*. [12]). For analyses, an HPLC system (Class VP; Shimadzu, Kyoto, Japan) with UV detection was used. The stock solution of silybin was 25 mM in 60% (v/v) dimethyl sulfoxide. Liver microsomes were sonicated 3×10 s (with 10 s breaks) on ice. Parameters of enzyme kinetics (K_m, V_{max}) were obtained using the Sigma Plot 8.0.2 scientific graphing software (SPSS, Chicago, IL, USA).

RESULTS

Enzyme kinetics of UGT1A6 activity in liver microsomes

Kinetic parameters of UGT1A6 in pooled liver microsomes of selected species were determined, using 4-MU (Table 1A, Figure 2A) and p-NP (Table 1B, Figure 2B) as the substrates. Concentration of the substrates in both experiments was 0, 25, 50, 100, 250, 500, 1 000 µM, and the K_m and V_{max} values were determined from respective double reciprocal plots. At 3 000 µM substrate concentration, an inhibition of the reaction by the substrate was occured. Hence, for K_m and V_{max} calculations this value was omitted. All experiments were performed in triplicate. The data show that the difference in K_m and

Table 1A. Interspecies comparison of formation 4-MU glucuronides

	4-MU glucuronides	
	K_m (µM) ±S.D.	V_{max} (nmol/min/mg prot.) ±S.D.
Man	120.7 ±12.3	312.5 ±13.8
Pig	127.7 ± 9.9	384.6 ±7.3
Monkey	70.9 ± 6.2	208.3 ± 15.1
Rat	218.7 ± 22.7	592.7 ± 22.6
Dog	92.2 ± 5.8	263.2 ± 17.6

Table 1B. Interspecies comparison of formation p-NP glucuronides

	p-NP glucuronides	
	K_m (µM) ±S.D.	V_{max} (nmol/min/mg prot.) ±S.D.
Man	98.4 ± 8.1	53.8 ± 6.2
Pig	85.9 ± 7.4	39.7 ± 4.1
Monkey	106.5 ± 10.5	185.2 ± 12.9
Rat	132.1 ± 16.7	56.5 ± 6.2
Dog	147.4 ± 15.2	188.7 ± 19.2

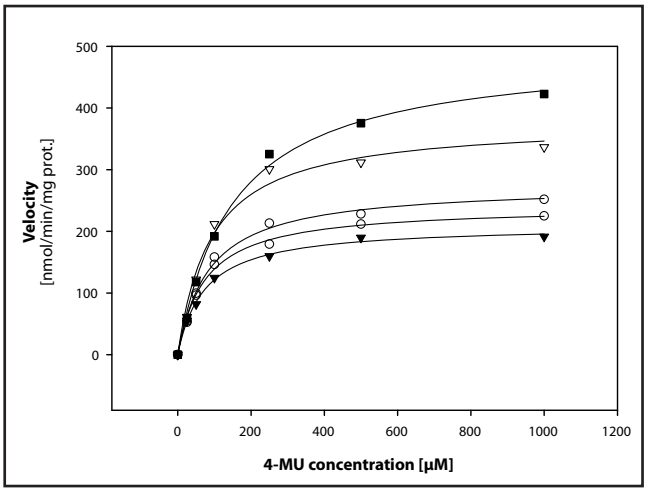

Figure 2A. Enzyme kinetic of formation of 4-MU glucuronides in microsomes of selected species. Man (empty circles), dog (full circles), pig (empty triangles), monkey (full triangles) and rat (full squares).

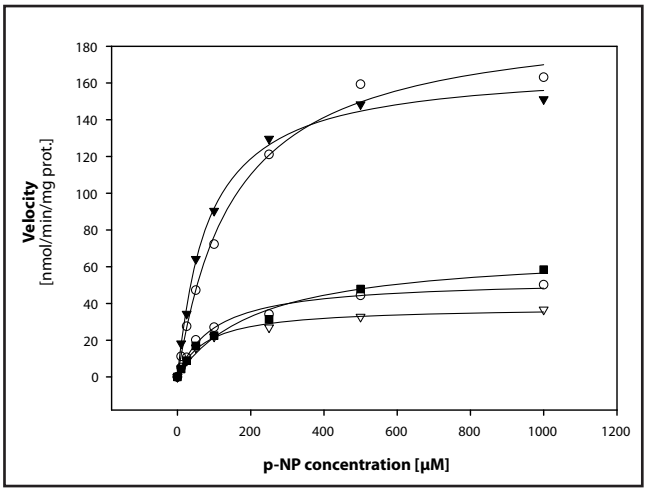

Figure 2B. Enzyme kinetic of formation of p-NP glucuronides in microsomes of selected species. Man (empty circles), dog (full circles), pig (empty triangles), monkey (full triangles) and rat (full squares).

V_{max} values obtained with different species are generally below one order of magnitude indicating a gross similarity in the properties of these enzymes.

The data presented show an overall similarity in kinetic parameters of the UGT1A6 namely for man and pig. Interestingly, the other species seem to exhibit activities differing from these of the man (cf. Figure 2B, substrate p-NP).

Comparison of formation of silybin glucuronides in liver microsomes

Silybin is a more complex molecule than previously used substrates and more UGT forms are involved in formation of respective glucuronides. Previously, it was published that silybin forms *O*-glucuronides in positions 7 and 20 [5,12], but interspecies comparison, using the widely used experimental models, hasn't been performed yet. The results of chromatographic analyses are documented in Figure 3. The total peak area was summed together and a ratio of respective peak to total peak area was counted. The results of determination of silybin glucuronides formed in samples from liver microsomes of different animals are in Table 2. Here, the differences in the relative amounts of individual silybine glucuronides can be found with the results with the monkey and dog samples, being closer to this of the man.

DISCUSSION

UGT1A6, a member of the UGT superfamily plays a key role in the glucuronidation of drugs and environmental chemicals. UGT1A6 has also been shown to be regulated by aryl hydrocarbon receptor agonists [2]. Although monkey, rat, pig, and dog are the most widely used experimental models for drug metabolism, little is known about the enzymatic properties of theirs UGT enzymes. The functional evaluation of UGTs of species mentioned should provide important information

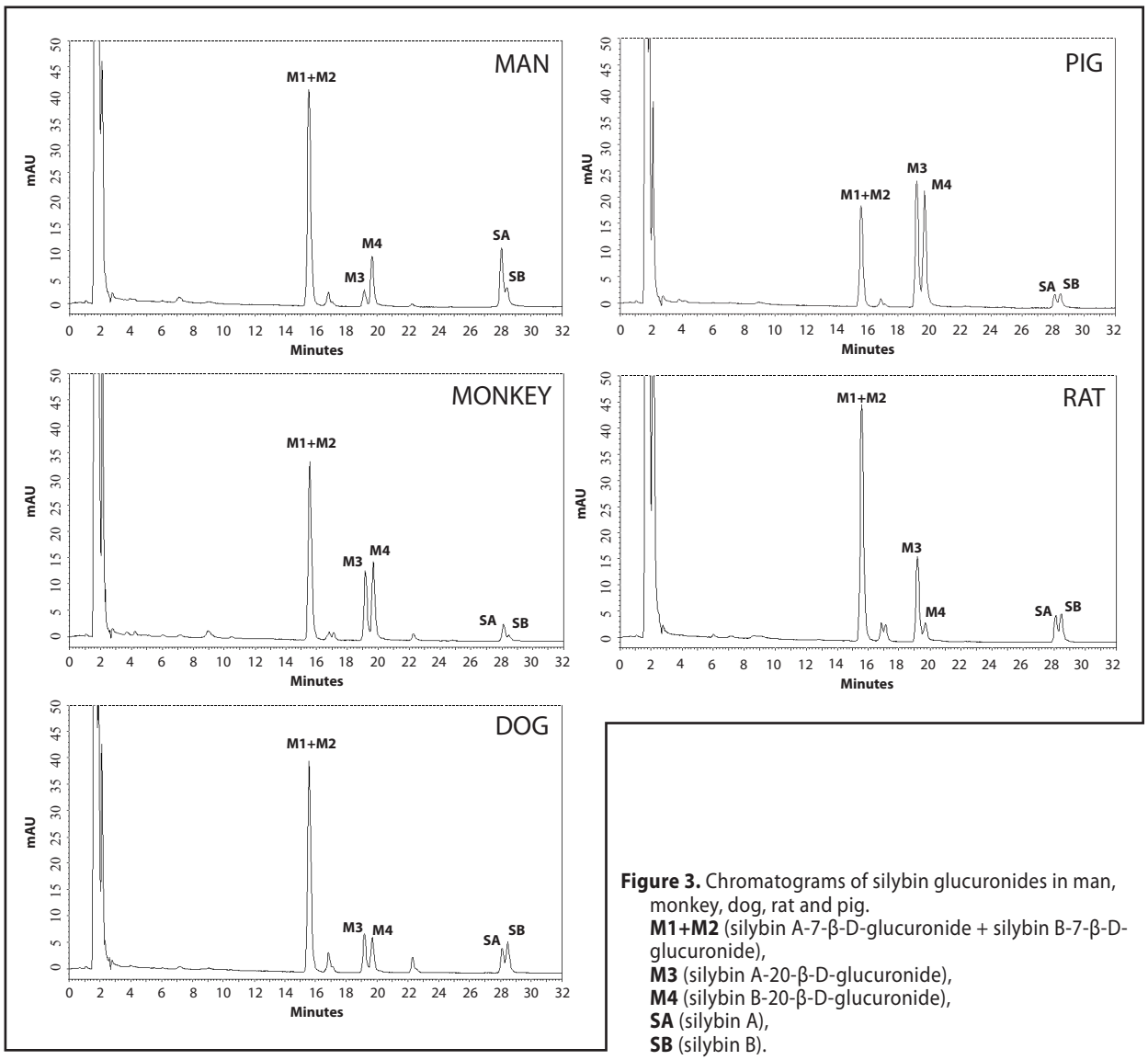

Figure 3. Chromatograms of silybin glucuronides in man, monkey, dog, rat and pig.
M1+M2 (silybin A-7-β-D-glucuronide + silybin B-7-β-D-glucuronide),
M3 (silybin A-20-β-D-glucuronide),
M4 (silybin B-20-β-D-glucuronide),
SA (silybin A),
SB (silybin B).

Table 2. Comparison of formation of silybin glucuronides in selected species.

	Ratio of the total silybin glucuronides (%)		
	A+B-7-β-D-glucuronide	A-20-β-D-glucuronide	B-20-β-D-glucuronide
Man	77.1	5	17.9
Pig	30	34.8	35.2
Rat	69.6	23.9	6.5
Monkey	55.8	19	25.2
Dog	69.4	11.6	19

Results were calculated as stated in the text, a mean of three experiments which not differed more than 5% was taken.

for the prediction of metabolism of drugs and other xenobiotics. Data presented in this paper indicate that man and pig (at least according to the K_m values, Table 1A, 1B) show similar kinetic properties towards the prototypic UGT1A6 substrates in liver microsomal fraction. An inspection of the data presented in the Tables 1A, 1B and Figure 2A, 2B show that the monkey may be also an acceptable model for xenobiotic metabolism in the man, even when the kinetic profile of the p-NP glucuronidation is showing more efficient enzyme reaction in this species. The results show that similarity as to the first phase of xenobiotics metabolism (mediated mostly by the CYP enzymes) [3,9], the selection of the experimental model depends on the substrate and on the particular enzyme form. To give a more complex view on hepatic glucuronidation processes the further experiments with UGT forms and theirs prototypic substrates are needed, although not all of the UGTs have theirs prototypic substrates determined yet [15].

To study the glucuronidation processes in microsomes of selected species with a model compound, the silybin was chosen as a substrate as it is known that more UGT forms are involved in its glucuronidation. Silybin forms *O*-glucuronides in positions 7 and 20; as the UGTs are known for their stereospecificity, the formation of four different glucuronides has been previously published [5,12]. Interestingly, different amounts of respective silybin glucuronides were formed in every species. With exception of the pig, formation of 7-β-D-glucuronides was predominant. In pig, the formation of all three metabolites was almost equal. Different formation of glucuronides indicates distinct activity and stereospecificity of UGTs towards silybin substrate in liver microsomes of species used. With this respect, as an optimal model for study of silybin glucuronides, both the rhesus monkey and dog (Beagle) seem to be the best models. To elucidate the role of the UGT forms involved in metabolism of silybin, the experiments with recombinant UGT enzymes are needed.

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