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# The human UDP-glucuronosyltransferase UGT1A3 is highly selective towards N2 in the tetrazole ring of losartan, candesartan, and zolarsartan

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ABSTRACT

Losartan, candesartan, and zolarsartan are $AT_1$ receptor antagonists that inhibit the effect of angiotensin II. We have examined their glucuronidation by liver microsomes from several animals and by recombinant human UDP-glucuronosyltransferases (UGTs). Large differences in the production of different glucuronide regioisomers of the three sartans were observed among liver microsomes from human (HLM), rabbit, rat, pig, moose, and bovine. However, all the liver microsomes produced one or two *N*-glucuronides in which either *N*1 or *N*2 of the tetrazole ring were conjugated. *O*-Glucuronides were also detected, including acyl glucuronides of zolarsartan and candesartan. Examination of individual human UGTs of subfamilies 1A and 2B revealed that *N*-glucuronidation activity is widespread, along with variable regioselectivity with respect to the tetrazole nitrogens of these sartans. Interestingly, UGT1A3 exhibited a strong regioselectivity towards the *N*2 position of the tetrazole ring in all three sartans. Moreover, the tetrazole-*N*2 of zolarsartan was only conjugated by UGT1A3, whereas the tetrazole-*N*1 of this aglycone was accessible to other enzymes, including UGT1A5. Zolarsartan *O*-glucuronide was mainly produced by UGTs 1A10 and 2B7. UGT2B7, alongside UGT1A3, glucuronidated candesartan at the tetrazole-*N*2 position, whereas UGTs 1A7–1A10 mainly yielded candesartan *O*-glucuronide. In the case of losartan, no *O*-glucuronide was generated by any tested human enzyme. Nevertheless, UGTs 1A1, 1A3, 1A10, 2B7, and 2B17 glucuronidated losartan at the tetrazole-*N*2, while UGT1A10 also yielded the respective *N*1-glucuronide. Kinetic analyses revealed that the main contributors to losartan glucuronidation in HLM are UGT1A1 and UGT2B7. The results provide ample new data on substrate specificity in drug glucuronidation.



## 1. Introduction

Losartan, candesartan, and zolarsartan (GR117289, also called zolasartan) inhibit the effect of angiotensin II by acting as $AT_1$ receptor antagonists. Losartan and candesartan are in clinical use for the treatment of hypertension. In addition, it was recently reported that losartan might be effective in treating Marfan syndrome and perhaps other illnesses [1–4]. Zolarsartan is also an active compound both in vitro [5] and in vivo [6]. Losartan itself is not as active as its carboxylic acid metabolite, which is also longer acting. Candesartan is administered as a prodrug, candesartan cilexetil, which has better bioavailability than candesartan. Candesartan cilexetil is hydrolyzed to the active candesartan during the absorption process. The molecular structures of losartan, candesartan, and zolarsartan include a tetrazole moiety, while candesartan and

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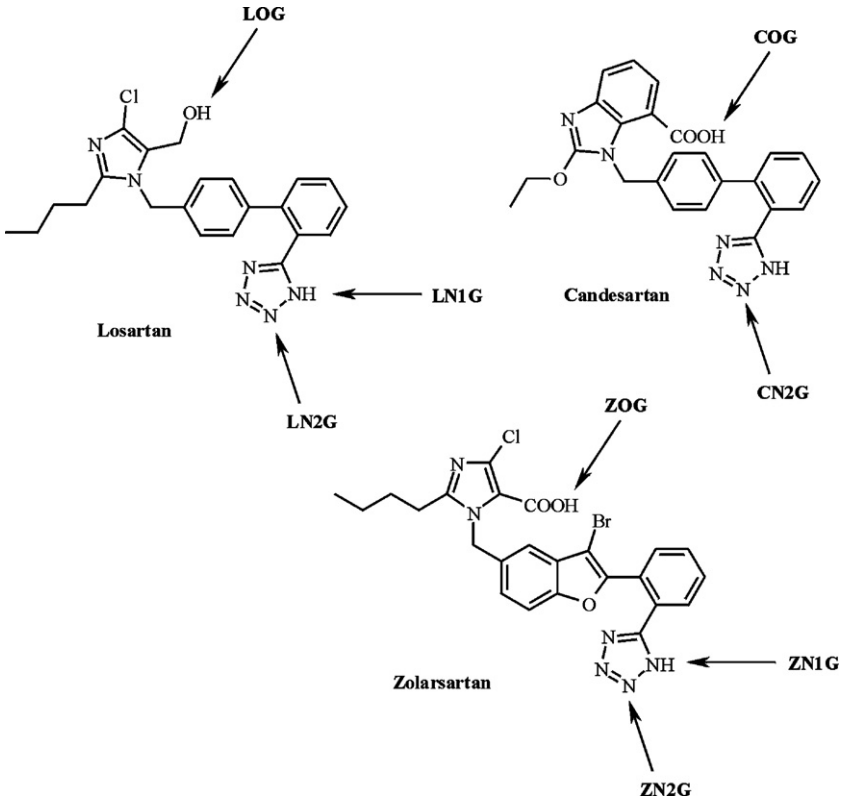


**Fig. 1 – Structures of losartan, candesartan, and zolarsartan. The arrows indicate glucuronidation sites and the abbreviation for the regioisomer.**

zolarsartan also have a carboxylic acid group (Fig. 1). Losartan is metabolized by three main routes: oxidation of the alcohol to carboxylic acid, hydroxylation, and glucuronidation [7]. Candesartan is mostly excreted unchanged [8], but glucuronide conjugates have been found from rats and dogs [9,10]. Rats and dogs also produce zolarsartan glucuronides [11].

Glucuronidation is an important phase II metabolic reaction. UDP-glucuronosyltransferases (UGTs) are membrane bound enzymes that catalyze this conjugation reaction [12–16]. Glucuronidation is an $S_N2$ reaction where the nucleophilic substrate attacks the uridine-5′-diphospho-α-D-glucuronic acid (UDPGA). The most common glucuronidation products are *O*- and *N*-glucuronides formed from hydroxyl, carboxyl, or amino functional groups, but there are also reports of *S*- and *C*-glucuronides [12,16,17].

The human UGTs are divided into three subfamilies, UGT1A, UGT2A, and UGT2B, based on their amino acid sequences and gene structures [14,18]. UGTs catalyze the glucuronidation of a wide range of endogenous and exogenous compounds, including steroid hormones, dietary constituents, and drugs. The substrate specificity of most UGTs is wide and often partly overlapping. Most human UGTs catalyze the glucuronidation of hydroxyl or carboxyl groups in the form of ether or acyl *O*-glucuronides, whereas *N*-glucuronides, formed from various amines, are produced by much fewer UGTs [12,16,19].

Losartan can be glucuronidated at three different positions so that one *O*-glucuronide and two different tetrazole-*N*-glucuronides may be formed [20] (Fig. 1). The *O*-glucuronide and one of the *N*-glucuronides, tetrazole-*N*2-glucuronide, were found to be formed during biotransformation and tetrazole-*N*1-glucuronide has been synthesized both chemically [20] and enzymatically [21]. The glucuronidation of losartan has been previously studied using liver slices [7,20] and liver microsomes [22] from various species, as well as rat intestine [23]. Monkeys and rats produced both *O*-glucuronide and tetrazole-*N*2-glucuronide whereas humans and dogs produced only the tetrazole-*N*2-glucuronide of losartan. The metabolism of candesartan cilexetil has been studied in rats and dogs, yielding both the *O*-glucuronide (acyl glucuronide) and the tetrazole-*N*2-glucuronide of candesartan [9,10] (Fig. 1). Tetrazole-*N*1-glucuronides of candesartan have not thus far been found. Zolarsartan can be glucuronidated to *O*-glucuronide in rats, while dogs, in addition to acyl glucuronide, also produce tetrazole-*N*2-glucuronide [11] (Fig. 1). Zolarsartan *N*1-glucuronide has been synthesized enzymatically using rat liver microsomes as a catalyst [21].

The aim of this study was to examine the glucuronidation of the aglycones losartan, candesartan, and zolarsartan by the human UGTs of subfamilies 1A and 2B. We have selected the three substrates on the basis of similarity of structure, i.e. all contain a tetrazole, and in addition both *O*- and *N*-glucuronides can be formed without a phase I metabolism reaction. Among other things, we wanted to find out if the tetrazole group, common to all three substrates, was glucuronidated by the same UGT isoform(s). In addition, liver microsomes from

human, bovine, moose, pig, rabbit, and rat were studied to explore species differences in glucuronidation of sartans.

## 2. Materials and methods

### 2.1. Reagents

Losartan potassium (98% purity), candesartan (purity not reported), and zolarsartan (purity not reported) were obtained from Merck (Rahway, NJ), AstraZeneca (Mölndal, Sweden), and GlaxoSmithKline (Hertfordshire, UK), respectively. Glucuronide regioisomers of losartan, candesartan, and zolarsartan were synthesized previously in our laboratory and their structures were characterized by nuclear magnetic resonance spectroscopy (NMR) [21]. Saccharic acid 1,4-lactone and UDPGA (trisodium salt, 99.8% purity) were purchased from Sigma (St. Louis, MO). Analytical or high-performance liquid chromatography (HPLC) grade reagents and solvents were used and they were purchased from J.T. Baker (Deventer, Holland; acetic acid), Fluka (Steinheim, Germany; disodium hydrogen phosphate dihydrate), Merck (Darmstadt, Germany; magnesium chloride hexahydrate and perchloric acid), Rathburn (Walkerburn, UK; acetonitrile, ACN), Riedel-de Haën (Seelze, Germany; ammonium acetate, dimethyl sulfoxide, formic acid, and potassium dihydrogen phosphate). Water was purified with a Milli-Q water purification system (Millipore, Molsheim, France). The human recombinant UGTs 1A1, 1A3–1A10, 2B4, 2B7, 2B10, 2B15, 2B17, and 2B28 were expressed in baculovirus-infected insect cells [24]. Pooled human liver microsomes (HLMs) and male New Zealand White rabbit liver microsomes were purchased from BD Gentest (Woburn, MA) and In Vitro Technologies (Baltimore, MD), respectively. Rat liver microsomes were prepared from Aroclor 1254-induced male Sprague–Dawley rats, and pig, moose, and bovine liver microsomes were prepared from untreated animals, as previously described [25]. A BCA Protein Assay Kit (Pierce Chemical, Rockford, IL) was used to measure the protein concentrations of UGTs and microsomes. The relative expression level of each recombinant UGT was determined by dot-blot analyses using anti-His-tag antibodies, as detailed elsewhere [24].

### 2.2. Glucuronidation assays

Screening assays of recombinant UGTs and liver microsomes were carried out in 250 μL reactions containing 1 mM substrate (losartan, candesartan, or zolarsartan), 5 mM saccharic acid 1,4-lactone, 5 mM UDPGA, 5 mM $MgCl_2$, 50 mM phosphate buffer (pH 7.4), and 625 μg protein (recombinant UGT membrane or microsome). Substrates were added as dimethyl sulfoxide solutions so that the final solvent concentration was 2% of the reaction volume. All samples were prepared in duplicate except respective controls without UDPGA. Samples were incubated at +37 °C for 4 h.

Kinetic analyses were carried out, in triplicates, with HLM and the three most active UGTs in the formation of losartan tetrazole-*N*2-glucuronide, UGT1A1, UGT1A3, and UGT2B7. The samples contained 2–600 μM losartan. The protein concentration and incubation time were within the linear range with respect to both parameters. Otherwise the reactions were carried out similarly to the screening assays described above. The kinetic parameters were obtained by fitting the observed velocities to kinetic models using GraphPad Prism 4.02 for Windows (GraphPad Software Inc., San Diego, CA). The kinetic models were Michaelis–Menten ($v = V_{max}[S]/(K_m + [S])$) and substrate inhibition equation ($v = V_{max}/(1 + (K_m/[S]) + ([S]/K_{si}))$).

### 2.3. Analytics

Reactions containing losartan as the aglycone were terminated by adding 25 μL cold 4 M perchloric acid and transferring the samples to ice for 10 min. The denatured proteins were then removed by centrifugation (10 min, 16,100 × *g*). The reactions with candesartan and zolarsartan were terminated by transfer to ice for 10 min (perchloric acid was not used since the compounds would have precipitated under acidic conditions) followed by centrifugation (10 min, 16,100 × *g*) and subsequent ultrafiltration of 200 μL of the supernatants (Ultrafree-MC 30,000 NMWL, Millipore, Bedford, MA; 20 min, 5000 × *g*) to remove residual proteins. The supernatants of the losartan reactions and filtrates of candesartan and zolarsartan reactions were analyzed by an Agilent 1100 Series HPLC equipped with an autosampler and the Agilent 1100 Series fluorescence or UV multiwavelength detector (Waldbronn, Germany). Details of the analytical HPLC conditions are given in Table 1.

The HPLC methods were validated with respect to specificity and repeatability. Method specificity was determined by comparing incubation samples to three different controls prepared either without UDPGA, protein, or aglycone. Repeatability of the HPLC was assessed by injecting incubation samples, which were diluted to two different concentrations, four times for both concentrations. The detection limit in the screening assays and the quantitation limit in the kinetic analyses were based on a signal-to-noise ratio of 3 and 9, respectively. In kinetic studies the quantitation was performed using an authentic standard, losartan *N*2-glucuronide. The retention times of the different glucuronide regioisomers were identified with glucuronide standards that were recently synthesized in our laboratory [21], as well as by LC–MS. The mass spectrometric analysis of losartan glucuronides was carried out on a PerkinElmerSciex API 3000 triple quadrupole mass spectrometer (MDS Sciex, Toronto, ON, Canada). Glucuronides of candesartan and zolarsartan were analyzed by a quadrupole time-of-flight mass spectrometer (Q-Tof Micro, Waters/Micromass, Manchester, UK). Both apparatuses were equipped with an ionspray source. Analyses were performed in positive ion mode with a capillary voltage of 5000 V for losartan glucuronides and 3000 V for candesartan and zolarsartan glucuronides.

## 3. Results

We have recently biosynthesized and characterized by NMR the different glucuronides of the three sartans that are included in the current study [21]. The glucuronidation assays used here were carried out using a validated HPLC method.

**Table 1 – HPLC methods for analysis of losartan, candesartan, zolarsartan, and their glucuronides[a]**

| | Losartan | Candesartan | Zolarsartan |
|---|---|---|---|
| Column | HP Hypersil BDS C18 5 μm 150 mm × 4.6 mm | HP Hypersil BDS C18 5 μm 250 mm × 4.0 mm | HP Hypersil BDS C18 5 μm 250 mm × 4.0 mm |
| Eluents A and B | 20 mM $NH_4Ac$ pH 4.5—ACN | 1% $CH_3COOH/H_2O$—ACN | 0.1% $HCOOH/H_2O$—ACN |
| Eluent A:B | 73:27 | Gradient (% B)<br>0–7 min 28%, 7–25 min 28–55%<br>25–30 min 55%<br>30–31 min 55–28%<br>31–46 min 28% | Gradient (% B)<br>0–9 min 36%, 9–14 min 36–40%<br>14–21 min 40%, 21–23 min 40–80%<br>23–26 min 80%, 26–27 min 80–36%<br>27–42 min 36% |
| Flow rate (mL/min) | 1 (40 °C) | 1 (25 °C) | 1 (40 °C) |
| Injection volume (μL) | 40 | 30 | 40 |
| Detector | UV 256 nm | Fluorescence Ex 260 nm, Em 395 nm | Fluorescence Ex 306 nm, Em 428 nm |
| Retention time of aglycone | 12.0 min | 17.4 min | 24.7 min |
| Retention time of glucuronides | 3.6 min (LOG),<br>4.1 min (LN1G),<br>8.5 min (LN2G) | 10.4 min (COG), 15.2 min (CN2G) | 10.6 min (ZN1G), 12.9 min (ZOG),<br>19.1 min (ZN2G) |

[a] LOG, losartan *O*-glucuronide; LN1G, losartan *N*1-glucuronide; LN2G, losartan *N*2-glucuronide; COG, candesartan *O*-glucuronide; CN2G, candesartan *N*2-glucuronide; ZN1G, zolarsartan *N*1-glucuronide; ZOG, zolarsartan *O*-glucuronide; ZN2G, zolarsartan *N*2-glucuronide.

The results of the repeatability experiments are presented in Table 2, and they demonstrated that this method gave reliable analyses of the samples. In particular, the losartan *N*2-glucuronide standard curve that was used for quantitation in the kinetic assays, was linear between 0.5 and 20 μM ($R = 0.99971$). The LC–MS analyses confirmed the analyte peaks to be sartan glucuronides, since the *m*/*z* was 176 Da higher than for the corresponding aglycones. The identification of glucuronide regioisomers was based on similar retention times to the glucuronide standards that were earlier characterized by NMR [21]. Chromatograms of losartan, candesartan, and their glucuronides are shown in Fig. 2.

Glucuronidation of losartan, candesartan, and zolarsartan was studied using recombinant human UGTs and liver microsomes from various species. Analyses of losartan glucuronidation revealed that of the three potential glucuronides of the drug, human UGTs produced only two, tetrazole-*N*1-glucuronide and tetrazole-*N*2-glucuronide. The main metabolite was tetrazole-*N*2-glucuronide of losartan and the most active human UGT isoforms were 1A3, 2B7, 1A10, 1A1, and 2B17 (Fig. 3). UGT1A10 also catalyzed the formation of tetrazole-*N*1-glucuronide at significant rates. Very small amounts of tetrazole-*N*1-glucuronide were detected in the reactions of UGTs 1A1, 1A3, 1A7, and 1A8, whereas very small amounts of tetrazole-*N*2-glucuronide were found in the reactions of UGTs 1A4, 1A6–1A9, and 2B4 (Fig. 3).

Glucuronidation of candesartan by human UGTs yielded two metabolites, identified as *O*-glucuronide and tetrazole-*N*2-glucuronide. The main candesartan glucuronide produced by UGTs 1A7–1A10 was the *O*-glucuronide, while 1A3 and 2B7 produced primarily tetrazole-*N*2-glucuronide (Fig. 3). In addition, the *O*-glucuronide of candesartan was produced by 1A1, 1A3, 1A4, and 2B7, as was tetrazole-*N*2-glucuronide by 1A1, 1A7–1A10, 2B4, and 2B17.

In the case of zolarsartan, human UGTs produced three different glucuronides: *O*-glucuronide, tetrazole-*N*1-glucuronide, and tetrazole-*N*2-glucuronide. UGT1A3 mainly catalyzed the formation of tetrazole-*N*2-glucuronide, as well as smaller amounts of *O*-glucuronide (Fig. 3). UGT1A10 produced mainly *O*-glucuronide plus tetrazole-*N*1-glucuronide. Interestingly,

**Table 2 – Repeatability of the HPLC methods for the analysis of sartan glucuronides[a]**

| | Repeatability (%R.S.D.) | | | |
|---|---|---|---|---|
| | Concentration 1 ($n = 4$) | | Concentration 2 ($n = 4$) | |
| | Area | Retention time | Area | Retention time |
| LOG | 0.7 | 0.1 | 0.4 | 0.1 |
| LN1G | 0.6 | 0.1 | 1.3 | 0.1 |
| LN2G | 0.1 | 0.03 | 0.1 | 0.04 |
| COG | 2.5 | 0.4 | 1.2 | 0.1 |
| CN2G | 2.3 | 0.1 | 0.8 | 0.04 |
| ZOG | 2.8 | 0.2 | 0.7 | 0.1 |
| ZN1G | 0.3 | 0.2 | 0.8 | 0.1 |
| ZN2G | 2.8 | 0.1 | 1.8 | 0.1 |

[a] LOG, losartan *O*-glucuronide; LN1G, losartan *N*1-glucuronide; LN2G, losartan *N*2-glucuronide; COG, candesartan *O*-glucuronide; CN2G, candesartan *N*2-glucuronide; ZOG, zolarsartan *O*-glucuronide; ZN1G, zolarsartan *N*1-glucuronide; ZN2G, zolarsartan *N*2-glucuronide.

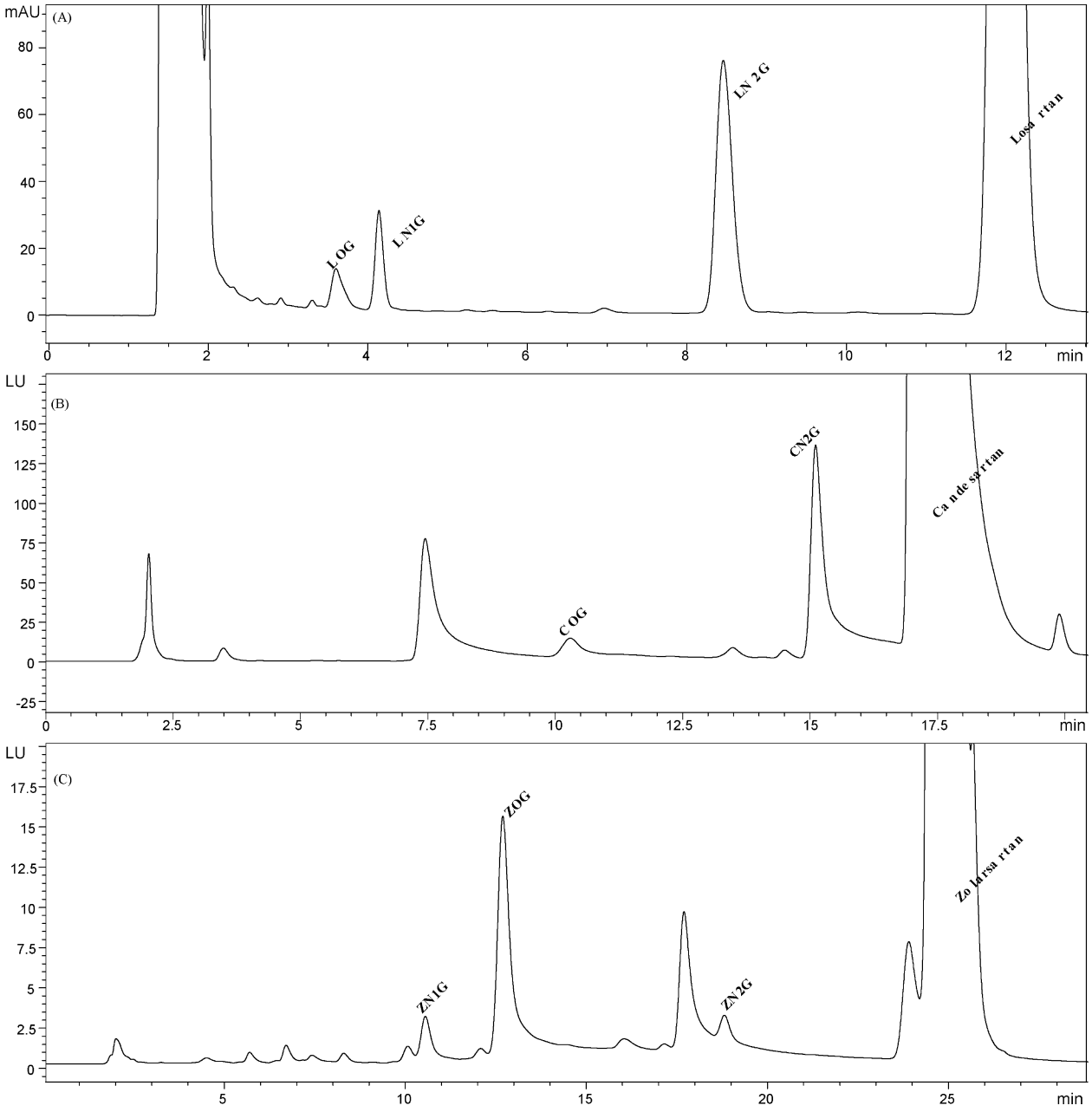


**Fig. 2 – Chromatographic separation of losartan glucuronides (A), candesartan glucuronides (B), and zolarsartan glucuronides (C). The chromatograms are from screening assays in which the substrate concentration was 1 mM and the protein concentration was 2.5 mg/mL. The enzyme sources were rat liver microsomes in (A), recombinant human UGT1A3 in (B), and pig liver microsomes in (C) zolarsartan.**

UGTs 1A5 and 2B4 selectively and fairly effectively produced tetrazole-*N*1-glucuronide of zolarsartan, whereas UGT2B7 did the same for *O*-glucuronide. Traces of tetrazole-*N*1-glucuronide of zolarsartan were detected in the reactions of 1A1, 1A6, 1A7, and 1A8. UGTs 1A1, 1A3, 1A7, and 1A8 formed small amounts of zolarsartan *O*-glucuronide.

Liver microsomes from human, bovine, moose, pig, rabbit, and rat produced three glucuronide regioisomers from losartan, two from candesartan, and three from zolarsartan (Fig. 4). All the tested liver microsomes glucuronidated losartan to tetrazole-*N*2-glucuronide. In addition, significant amounts of losartan *O*-glucuronide were produced by rabbit and bovine liver microsomes. Moose liver microsomes were exceptionally active, yielding mainly tetrazole-*N*2-glucuronide from losartan, as well as selectively producing *O*-glucuronide from zolarsartan. In the case of candesartan, moose liver microsomes catalyzed the formation of both glucuronide regioisomers almost equally. Candesartan glucuronides were produced almost equally by all of the tested liver microsomes, except rat liver microsomes that generated the *O*-glucuronide of candesartan at much higher rates. Rat liver microsomes were also exceptional in the glucuronidation

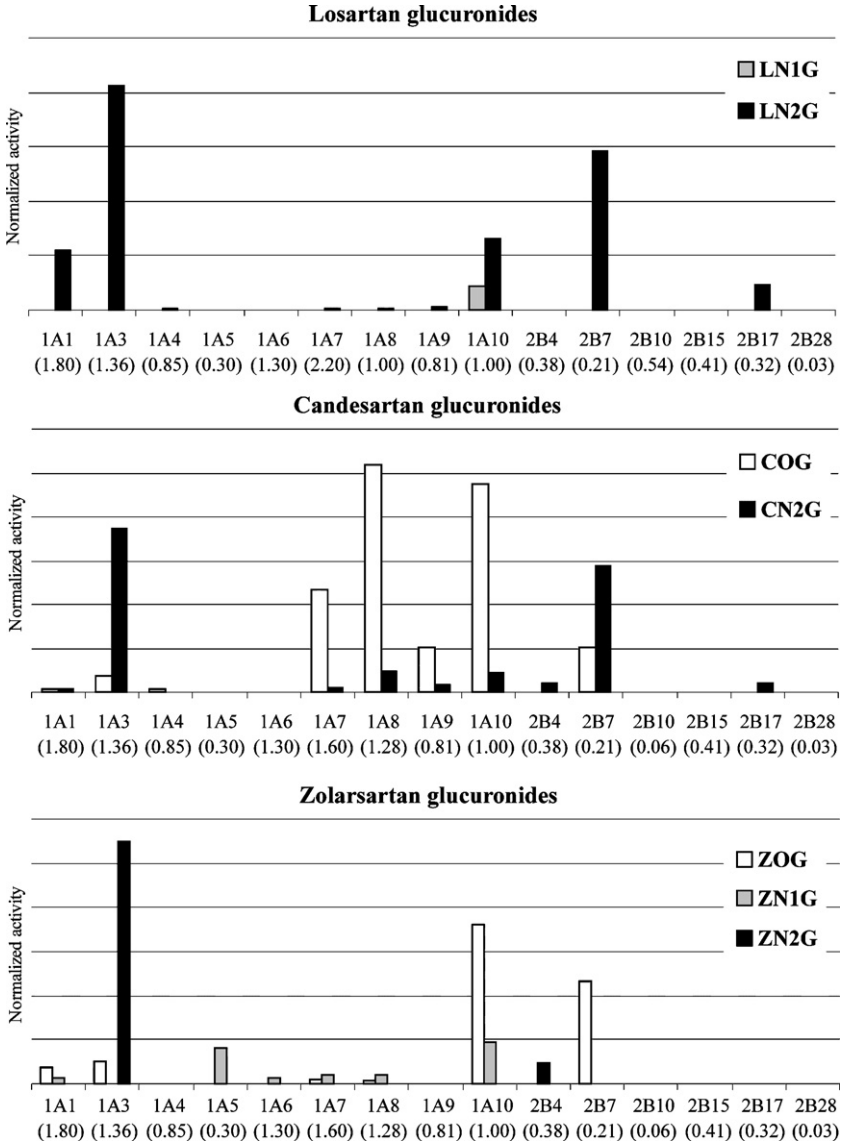


**Fig. 3 – Screening the glucuronidation activity of recombinant human UGTs towards losartan, candesartan, and zolarsartan. The normalized activity (activity corrected for expression level of individual UGTs) is presented and relative expression levels of the different recombinant UGTs are given in parenthesis under the enzyme name. The different glucuronide regioisomers are indicated by the column colour, *O*-glucuronide (white bar), tetrazole-*N*1-glucuronide (gray bar), and tetrazole-*N*2-glucuronide (black bar). The values are averages of duplicate samples in which the substrate and protein concentrations were 1 mM and 2.5 mg/mL, respectively, and the incubation time was 4 h.**

of zolarsartan, since their main product was tetrazole-*N*1-glucuronide rather than the *O*-glucuronide. Rabbit liver microsomes were highly selective, producing only *O*-glucuronide of zolarsartan. Tetrazole-*N*2-glucuronide of zolarsartan was only obtained at low rates in the reactions catalyzed by human and pig liver microsomes.

Losartan is one of the oldest $AT_1$ receptor antagonists and is thus probably the most widely used drug of the three sartans that were included in this study. Glucuronidation is an important metabolic pathway for losartan and to get deeper insight the pharmacokinetics of this drug, we have examined the kinetics of its glucuronidation by HLM and by the three hepatic human UGTs that exhibited the highest activity towards this compound, UGT1A3, UGT1A1, and UGT2B7 (Fig. 3). The results of the kinetic analyses are presented in Fig. 5. Losartan glucuronidation by human liver microsomes followed Michaelis–Menten kinetics, as did the reactions catalyzed by UGTs 1A1 and 2B7 (Fig. 5B and D). In contrast, the catalysis of losartan *N*2-glucuronide by UGT1A3 is governed by substrate inhibition kinetics (Fig. 5C). The kinetic parameters for HLM and the three UGTs are presented in Table 3.

## 4. Discussion

The glucuronidation of losartan, candesartan, and zolarsartan by different animal liver microsomes and a large set of

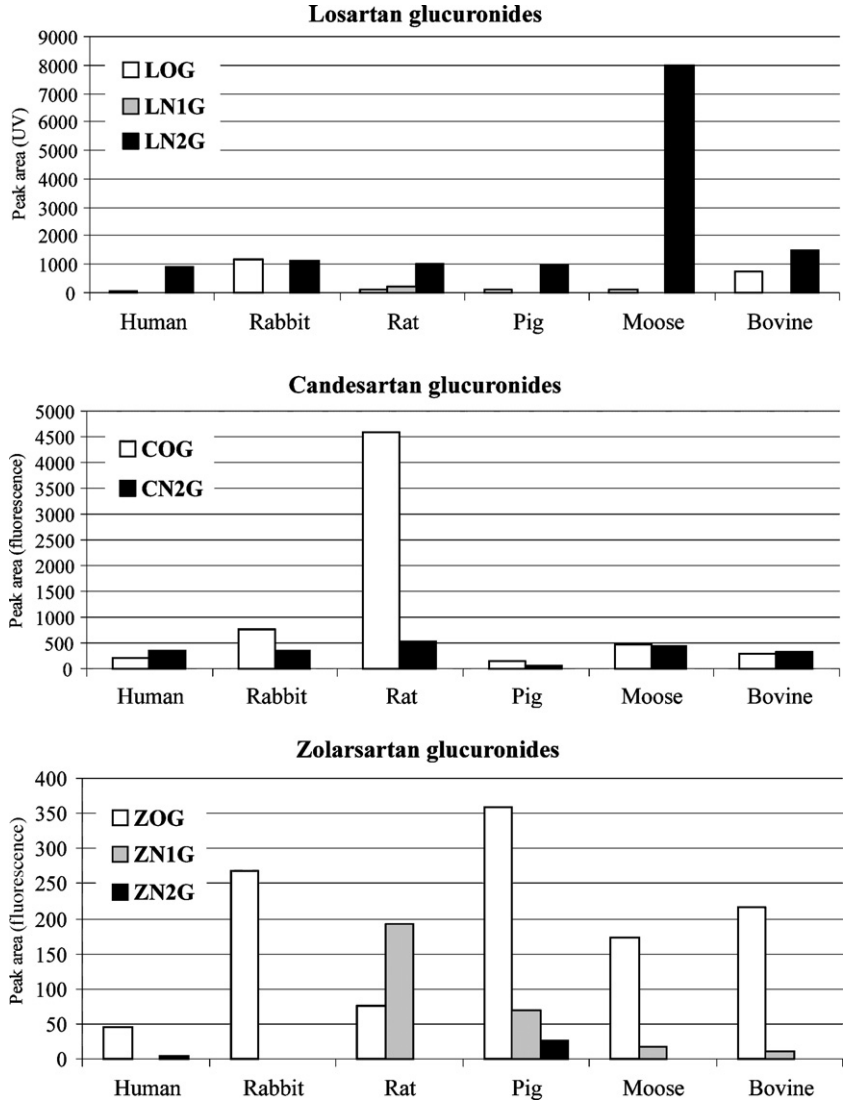


**Fig. 4 – Screening the activity of liver microsomes from various species towards losartan, candesartan, and zolarsartan. The type of glucuronides and the incubation conditions are the same as described for the recombinant UGTs in the legend to Fig. 3.**

recombinant human UGTs was studied. An interesting aspect of these sartans is the presence of several potential sites for both *N*- and *O*-glucuronidations within each aglycone (Fig. 1). We have recently characterized the structures of the *N*- and *O*-glucuronides that are produced from these sartans by NMR [21], providing a tool to study the regioselectivity of individual UGTs in such reactions.

Many of the human UGTs were active in the glucuronidation of one or more of the three sartans, at one site or more (Fig. 3). Among them, the activity of UGT1A3 was perhaps the main finding of this study, particularly due to its high regioselectivity towards the tetrazole-*N*2 of the three aglycones (Fig. 3). These results agree with the previously published study in which UGT1A3 catalyzed the formation

**Table 3 – Enzyme kinetic parameters (±S.E.) for the formation of losartan *N*2-glucuronide**

| | HLM[a] | UGT1A1 | UGT1A3 | UGT2B7 |
|---|---|---|---|---|
| $K_m$ (μM) | 100.2 ± 11.7 | 21.5 ± 1.7 | 35.9 ± 3.9[b] | 162.3 ± 20.7 |
| $V_{max}$ (pmol/min/mg) | 85.7 ± 3.0 | 32.2 ± 0.6 | 225.7 ± 12.2 | 16.1 ± 0.8 |

[a] Human liver microsomes.
[b] Substrate inhibition interaction $K_{si}$ = 400.4μM ± 48.6.

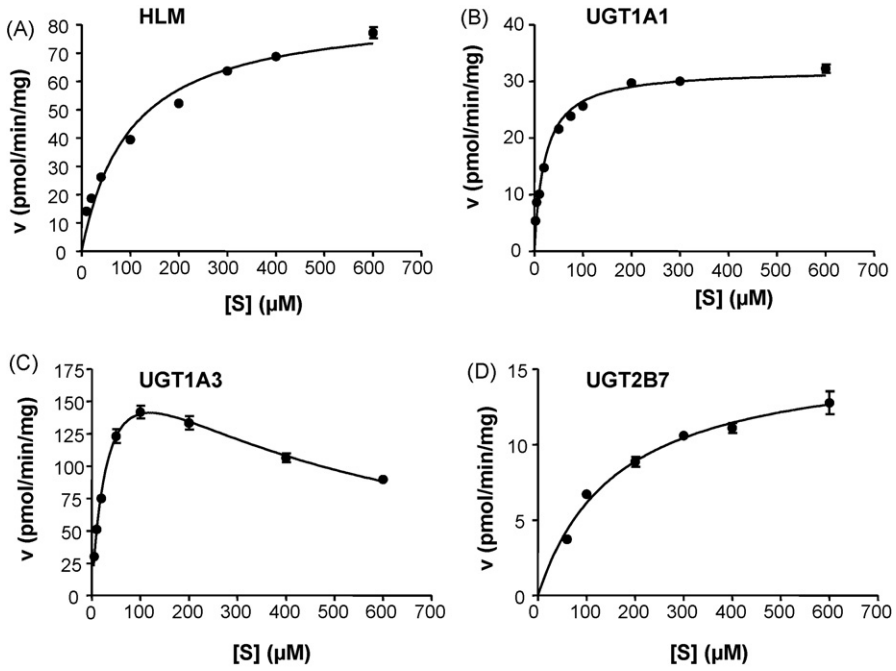


**Fig. 5 – Kinetics of losartan glucuronidation by human liver microsomes (HLM, A), and recombinant human UGTs 1A1 (B), 1A3 (C), and 2B7 (D). The data points, averages of triplicate measurements ± S.E.M., were fitted to the Michaelis–Menten (A, B, and D) or the substrate inhibition (C) equations.**

of tetrazole-*N*2-glucuronide of RG 12525, a chemical entity evaluated for the treatment of type II diabetes [26]. UGT1A3 was previously shown to be active in several *N*-glucuronidation reactions, not merely with tetrazoles, but in those cases it was often less active than UGT1A4 [12,16,19,27–29].

The results of the present study revealed that many UGTs are able to catalyze *N*-glucuronidation reactions, even if the enzyme most frequently associated with *N*-glucuronidation, UGT1A4 [12,16,19,28–32], exhibited no significant activity towards any of the three sartans studied here (Fig. 3). A possible reason for this is that tetrazole is acidic, with a $pK_a$ value of 4.9. The $pK_a$ value is similar to those of carboxylic acids and therefore tetrazoles have been used as non-classical isosteres for carboxylic acid moieties in biologically active molecules [33,34]. In comparison to carboxylic acids, tetrazoles are more lipophilic, which could increase their affinity to the UGTs.

Zolarsartan was an interesting aglycone for several different reasons. Since only UGT1A3 was the only human UGT that catalyzed the formation of zolarsartan *N*2-glucuronide (Fig. 3), this reaction could be used as a specific probe for the presence of active UGT1A3 in human tissue samples. In contrast to *N*2, the conjugation of zolarsartan *N*1-tetrazole, at least at low rates, was catalyzed by several different UGTs, including 1A5, 1A10, 2B4, and perhaps also 1A6–1A8 (Fig. 3). This is somewhat surprising since none of these human UGTs is known for catalyzing *N*-glucuronidation. Incidentally, zolarsartan appears to be a reasonably good substrate for recombinant UGT1A5, an enzyme that is highly homologous to both UGT1A3 and UGT1A4, but which was thus far exhibited only marginal activities [35].

Losartan, like zolarsartan, turned out to be a useful aglycone for exposing *N*-glucuronidation activity of unexpected enzymes, in this case UGTs 1A1, 1A10, 2B7, and 2B17. In contrast to zolarsartan, however, nearly all the enzymes that were active towards losartan, glucuronidated it at the same site, the tetrazole-*N*2. The only exception was UGT1A10 that also catalyzed losartan conjugation at the tetrazole-*N*1, even if at a lower rate (Fig. 3). The versatility of UGT1A10 in this case may reflect its activity in dobutamine glucuronidation, where it was found to be the only human UGT that catalyzed high glucuronidation of both catechol hydroxyls [36].

Candesartan glucuronidation may be considered in terms of both pharmacokinetics and human UGT substrate specificity. Candesartan differed from the two other sartans, particularly from losartan, in being readily conjugated to *O*-glucuronide, giving rise to candesartan acyl glucuronide (Fig. 3). Interestingly, the three UGTs that exhibited the highest *O*-glucuronidation of candesartan, 1A7, 1A8, and 1A10, are mainly extrahepatic enzymes and could account for the low bioavailability of candesartan.

Comparing the glucuronides produced from zolarsartan by human liver microsomes and individual UGTs (Figs. 3 and 4) may be useful for assessing the contribution of UGT1A3 to hepatic glucuronidation in humans. While UGT1A3 readily and specifically produced zolarsartan tetrazole-*N*2-glucuronide (Fig. 3), the fraction of zolarsartan tetrazole-*N*2-glucuronide in the HLM reaction, although detectable, was small (Fig. 4). The main product in the latter reaction was the zolarsartan *O*-glucuronide, even though the enzyme most efficient in this reaction, UGT1A10 (Fig. 3), is extrahepatic [14]. Nevertheless, UGT2B7, an important liver enzyme, also

catalyzed relatively high rates of zolarsartan *O*-glucuronidation (Fig. 3), probably accounting for most of the glucuronide produced by the liver microsome sample (Fig. 4). UGT1A1, another important liver enzyme, was also found to catalyze zolarsartan *O*-glucuronidation, but at a low rate (Fig. 3). Nevertheless, if the contribution of UGT1A1 for zolarsartan was significant, one would have also expected to detect zolarsartan tetrazole-*N*1-glucuronide in the liver microsome reaction (Fig. 4). Since the latter metabolite was not detected in this case, UGT1A1 was probably not significantly involved. It may thus be suggested that mutations that lower the expression level or activity of UGT1A1 should not significantly affect the pharmacokinetics of zolarsartan.

The kinetics of losartan glucuronidation was examined in order to obtain a deeper insight into its metabolism within the human liver. Losartan glucuronidation by HLM followed Michaelis–Menten kinetics, even if two enzyme kinetics could not be excluded in this case (Fig. 5A). In trying to assess the contribution of UGTs 1A1, 1A3, and 2B7 to the losartan glucuronidation activity of HLM, we have examined their reactions as well. UGT1A3 exhibited the highest losartan glucuronidation activity in the screening assays (Fig. 3) and its apparent affinity for this compound is high (Table 3). Interestingly, UGT1A3 exhibited clear substrate inhibition kinetics (Fig. 5C), a phenomenon not seen in HLM (Fig. 5A). It can thus be concluded that the contribution of UGT1A3 to the losartan glucuronidation activity of HLM is low, regardless the high activity of this enzyme. This finding is in full agreement with our conclusion about the minor involvement of UGT1A3 in the zolarsartan glucuronidation activity of HLM and it strongly implies that the concentration of UGT1A3 in HLM is low.

The kinetic analyses of UGT1A1 and UGT2B7, two enzymes that play major roles in hepatic glucuronidation, suggest that both of them contribute to losartan glucuronidation by HLM. UGT1A1, as well as UGT2B7, exhibited Michaelis–Menten kinetics without significant substrate inhibition (Fig. 5B and D). Nevertheless, there is a clear difference in the $K_m$ values of these UGTs for losartan with UGT1A1 having a significantly lower $K_m$ for this substrate (Table 3). Interestingly, the $K_m$ of HLM for losartan, about 100 μM, is higher than the respective value in UGT1A1, about 22 μM, but lower than the $K_m$ of UGT2B7, about 162 μM (Table 3). These findings, together with the shape of the kinetic curve and the above conclusion on the only minor contribution of UGT1A3, suggest that both UGTs 1A1 and 2B7 contribute significantly, and rather similarly, to the losartan glucuronidation activity of human liver microsomes.

The analyses of the glucuronidation activity of liver microsomes from different animals revealed, once again, that there are major differences in glucuronidation activity between different species (Fig. 4). The differences are both qualitative, in the type of metabolites formed, and quantitative. Since acyl glucuronides may be toxic [37–40], it is important to note that rat liver microsomes catalyze the formation of candesartan acyl glucuronides faster than any other microsomes sample, certainly much faster than the human liver microsomes (Fig. 4).

In conclusion, this study of losartan, candesartan, and zolarsartan glucuronidation produced interesting findings regarding *N*-glucuronidation, provided a model substrate for UGT1A3, and demonstrated large differences in glucuronidation activity between liver microsomes of different animals. The new knowledge should be highly useful for understanding substrate specificity of the UGTs, the formation of acyl glucuronides and the suitability or unsuitability of using animal liver microsomes for metabolic studies on drugs for humans.

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