RESEARCH ARTICLE

Glucuronidation of zearalenone, zeranol and four metabolites *in vitro*: Formation of glucuronides by various microsomes and human UDP-glucuronosyltransferase isoforms

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Glucuronidation constitutes an important pathway in the phase II metabolism of the mycotoxin zearalenone (ZEN) and the growth promotor α-zearalanol (α-ZAL, zeranol), but the enzymology of their formation is yet unknown. In the present study, ZEN, α-ZAL and four of their major phase I metabolites were glucuronidated *in vitro* using hepatic microsomes from steer, pig, rat and human, intestinal microsomes from humans, and eleven recombinant human UDP-glucuronosyltransferases (UGTs). After assigning chemical structures to the various glucuronides by using previously published information, the enzymatic activities of the various microsomes and UGT isoforms were determined together with the patterns of glucuronides generated. All six compounds were good substrates for all microsomes studied. With very few exceptions, glucuronidation occurred preferentially at the sterically unhindered phenolic 14-hydroxyl group. UGT1A1, 1A3 and 1A8 had the highest activities and gave rise to the phenolic glucuronide, whereas glucuronidation of the aliphatic hydroxyl group was mostly mediated by UGT2B7 with low activity. Based on these *in vitro* data, ZEN, α-ZAL and their metabolites must be expected to be readily glucuronidated both in the liver and intestine as well as in other extrahepatic organs of humans and various animal species.

Keywords:
Glucuronide / Human uridine 5′-diphosphate glucuronosyltransferases / Zearalanol / Zearalenone / Zeranol

1 Introduction

Zearalenone (ZEN, Fig. 1) is a macrocyclic β-resorcylic acid lactone (RAL) produced by *Fusarium* species [1, 2] and known for its pronounced estrogenic activity [2, 3]. Reductive metabolites of ZEN comprise zearalanone (ZAN) and the α- and β-stereoisomers of zearalenol (ZEL) and zearalanol (ZAL, Fig. 1). These congeners are fungal metabolites and therefore associated with ZEN in food items contaminated with this mycotoxin [4, 5]. α-ZEL and β-ZEL are also metabolites of ZEN formed in various animal species and in humans [2, 6–8]. In ruminants, the formation of small amounts of α-ZAL and β-ZAL has been demonstrated [9, 10]. α-ZAL is also called zeranol and used in the USA and Canada, but not in the EU, as a growth promotor in cattle under the trade name RalGro® [11, 12]. The estrogenic activity of α-ZEL and α-ZAL is even higher than that of ZEN [13].

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Abbreviations: ADH, alcohol dehydrogenase; **HSD,** hydroxysteroid dehydrogenase; **RAL,** β-resorcylic acid lactone; **UDPGA,** uridine 5′-diphosphate glucuronic acid; **UGT,** uridine 5′-diphosphate glucuronosyltransferase; **ZAL,** zearalanol; **ZAN,** zearalanone; **ZEL,** zearalenol; **ZEN,** zearalenone

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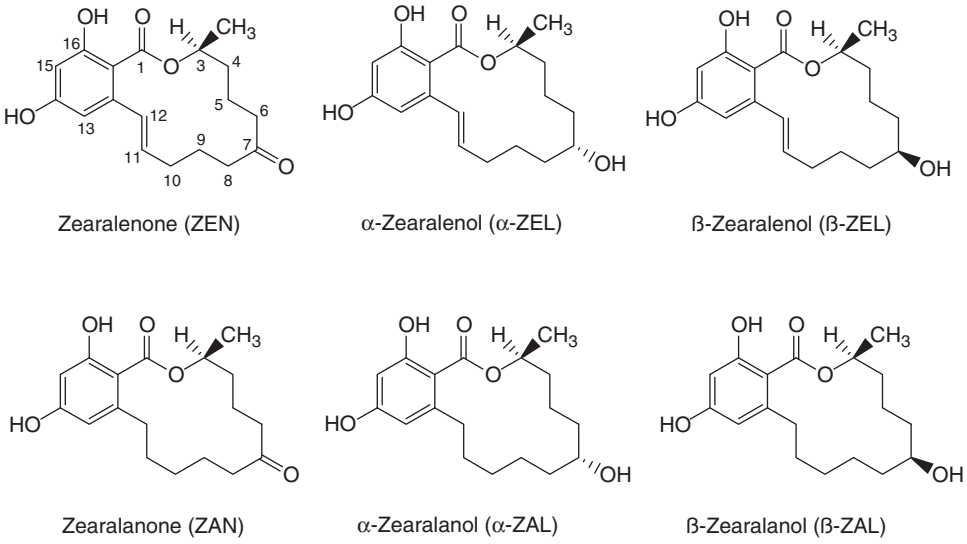

Figure 1. Chemical structures of the six RALs used in this study.

Conjugation with glucuronic acid and sulfate are major metabolic pathways of the RALs and important for hormonal inactivation and excretion [2, 6–8]. However, because ZEN and its metabolites have two or three hydroxyl groups, more than one monoglucuronide can be formed. Indeed, a recent study by Stevenson *et al.* [14] has demonstrated that each hydroxyl group is prone to glucuronidation: When ZEN and its five reductive metabolites were incubated with uridine 5′-diphosphate glucuronosyltransferases (UGT) from bovine liver in the presence of uridine 5′-diphosphate glucuronic acid (UDPGA), 10 of the 16 possible *O*-monoglucuronides of the six RALs could be purified by preparative HPLC and their chemical structures unambiguously established by NMR and UV spectroscopy, and MS. Small amounts of the remaining six possible monoglucuronides were also observed in the incubations and their structures inferred from comparison of their mass spectrometric fragmentation patterns with those of the fully characterized monoglucuronides.

It was the purpose of the present study to determine the pattern of glucuronides of the six RALs generated by hepatic microsomes from several animal species and from humans *in vitro*, and to measure the enzymatic activities for their formation. Furthermore, the activities of eleven major human recombinant UGT isoforms for ZEN and its five metabolites and the pattern of glucuronides were determined.

2 Materials and methods

2.1 Chemicals

ZEN was purchased from Fermentek (Jerusalem, Israel); ZAN and the α- and β-isomers of ZEL and ZAL were purchased from Sigma/Aldrich/Fluka (Taufkirchen, Germany). All compounds had a purity of >98% according to HPLC analysis. UDPGA, β-glucuronidase type B-1 from bovine liver, and all other chemicals and reagents were also obtained from Sigma/Aldrich/Fluka. HPLC grade ACN was from Acros Organics (Geel, Belgium). ZEN-14-*O*-glucuronide was chemically synthesized in one of our laboratories (Hannes Mikula, manuscript in preparation) by reacting ZEN with 1-bromo-1-deoxy-2,3,4-tri-*O*-acetylglucuronic acid methyl ester and silver(I)carbonate, followed by hydrolysis with potassium hydroxide. The crude product was purified by preparative HPLC and shown by NMR spectroscopy to be identical with the compound characterized in detail by Stevenson *et al.* [14]. NMR spectra were acquired on a Bruker AVANCETM DRX-400 Fourier transform spectrometer (Bruker BioSpin, Rheinstetten, Germany) and comprised ^{1}H and ^{13}C APT spectra as well as 2-D correlated spectroscopy, heteronuclear single quantum correlation and heteronuclear multiple bond correlation.

ZEN-14-*O*-glucuronide was reduced with rat liver cytosol, containing hydroxysteroid dehydrogenase (HSD) and alcohol dehydrogenase (ADH), in the presence of NADH as described for ZEN earlier [15], to yield a mixture of α-ZEL-14-*O*-glucuronide and β-ZEL-14-*O*-glucuronide. When ZEN-14-*O*-glucuronide was dissolved in methanol and reduced with hydrogen gas at 20°C and atmospheric pressure in the presence of 10% Pd on charcoal as a catalyst, ZAN-14-*O*-glucuronide as well as the α- and β-isomers of ZAL-14-*O*-glucuronide were obtained. The α- and β-isomers had different retention times, which were obtained from the glucuronidation of the authentic stereoisomers of ZEL and ZAL (see Section 3.1).

2.2 Microsomes and human UGT isoforms

Microsomes were prepared from the fresh livers of male Wistar rats, a young steer and a female pig as described earlier [16]. Protein concentrations were measured according to Bradford [17] with bovine serum albumin as a standard.

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Human hepatic microsomes were prepared from the liver of a 63-year-old male Caucasian (kindly provided by Dr. J. Weymann, formerly of Knoll AG, Ludwigshafen, Germany). In a previous study, the activity of these microsomes for the glucuronidation of 17β-estradiol has been found to be about the average of fifteen commercially available human hepatic microsomes from donors of diverse age and both genders, indicating that our hepatic microsomes from one donor exhibit typical glucuronidation activity [18]. Pooled human intestinal microsomes, purchased from BD Gentest (Woburn, MA, USA), contained equal amounts of microsomes prepared from both the duodenum and jejunum section of each of six donors (one female and five males of Caucasian and African American race, with ages ranging from 39–64 years). Supersomes, *i.e.* microsomes from insect Sf-9 cells infected with a baculovirus strain containing the cDNA of human UGT1A1, 1A3, 1A4, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 or 2B17 were also from Gentest.

2.3 Glucuronidation assays

Hepatic and intestinal microsomes as well as individual human UGTs were incubated with the RALs in a total volume of 0.2 mL of 100 mM phosphate buffer pH 7.4 (mammalian microsomes) or 50 mM tris buffer pH 7.5 (UGTs). The same UGT isoforms were used as in a previous study, in which their activities were determined with diverse model substrates, *e.g.* 17β-estradiol, 4-(trifluoromethyl)umbelliferone and trifluoperazine [19]. Typical incubations contained 0.02–0.2 mg of the microsomal protein, which was first mixed with 5 μg of alamethicin in 40 μL buffer and placed on ice for 10 min. Alamethicin improves the accessability of the membrane-bound UGTs by forming pores in the microsomal membranes [20]. Subsequently, magnesium chloride (final concentration 10 mM), the β-glucuronidase inhibitor saccharolactone (10 mM), UDPGA (2 mM) and finally the substrate (50–100 μM) dissolved in DMSO (final concentration 2%) were added and the mixture incubated at 37°C for various lengths of time up to 1 h. Incubations were then placed on ice and the microsomal protein precipitated by adding 20 μL of 20% aqueous trichloroacetic acid. After neutralization with 20 μL of 0.1 M aqueous sodium hydroxide, protein was sedimented by centrifugation and glucuronides were analyzed in the supernatant by HPLC. Pilot experiments had shown that the formation of glucuronides was linear under these conditions for 2 h with all microsomes and UGT isoforms. Control incubations were conducted with microsomes in the absence

Table 1. Characterization of the monoglucuronides (G) of ZEN and the other five RALs

		M-H	MS^2 of M-H (*m/z*, rel. intens.)					
RAL	RT[a] (min)	(*m/z*)	M-H-CO_2	M-H-Gluc	Gluc[b]	UV maxima (nm)		
ZEN	21.8	317	273 (100)			236	274	316
14-*O*-G	11.3	493	n.d.[c]	317 (100)	175 (29)	235	269	317
16-*O*-G	8.1	493	449 (45)	317 (100)	175 (23)	226	257	299
α-ZEL	18.1	319	275 (100)			239	277	316
7-*O*-G	10.0	495	451 (100)	319 (4)	n.d.	235	277	316
14-*O*-G	8.2	495	n.d.	319 (100)	175 (31)	234	271	318
16-*O*-G	5.2	495	451 (11)	319 (100)	175 (23)	222	257	298
β-ZEL	15.7	319	275 (100)			240	275	314
7-*O*-G	8.3	495	451 (100)	319 (7)	n.d.	240	268	313
14-*O*-G	6.4	495	n.d.	319 (100)	175 (35)	224	261	306
16-*O*-G	5.1	495	451 (11)	319 (100)	175 (22)	223	257	298
ZAN	21.7	319	275 (100)			220	265	304
14-*O*-G	11.2	495	n.d.	319 (100)	175 (34)	215	260	305
16-*O*-G	8.0	495	451 (8)	319 (100)	175 (7)	n.d.[d]		
α-ZAL	17.9	321	277 (100)			221	265	303
7-*O*-G	10.6	497	453 (100)	321 (5)	n.d.	219	265	305
14-*O*-G	8.3	497	n.d.	321 (100)	175 (40)	203	258	305
16-*O*-G	7.3	497	453 (20)	321 (100)	175 (24)	226	264	308
β-ZAL	15.0	321	277 (100)			231	263	298
7-*O*-G	8.8	497	453 (100)	321 (4)	n.d.	204	262	303
14-*O*-G	7.1	497	n.d.	321 (100)	175 (35)	200	253	285
16-*O*-G	6.3	497	453 (7)	321 (100)	n.d.	204	245	277

a) RT, retention time in HPLC.
b) Gluc, glucuronic acid.
c) n.d., not detected (<0.5% relative intensity).
d) n.d., not detected (Insufficient amount)

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of UDPGA and with supersomes lacking a UGT isoform in the presence of UDPGA.

The amounts of glucuronides formed in the incubations were calculated from the area of the HPLC peak of the glucuronide determined at the wavelength of the second UV maximum (Table 1), assuming that the glucuronide had the same extinction coefficient as the respective aglycone. The scan range of the DAD detector was from 200 to 600 nm, and the peak area was obtained by manual integration after subtracting the baseline before and after the peak. The peak area corresponding to 0.1 pmol and 0.5–1 pmol was the limit of detection and quantitation, respectively.

From each RAL, a portion of an incubation sample containing significant amounts of glucuronides was hydrolyzed by incubation with β-glucuronidase as reported previously [19]. The HPLC and LC-MS profiles of equivalent volumes of the sample with and without enzymatic hydrolysis were then determined.

2.4 HPLC analysis

A Hewlett Packard 1100 system equipped with a binary pump, a DAD detector and HP ChemStation Rev. A.07.01 software for data collection and analysis were used. Separation was carried out on a 250 × 4.6 mm id, 5 μm, reversed-phase Luna C18 column (Phenomenex, Torrance, CA, USA). Solvent A was deionized water containing 0.1% formic acid, and solvent B was ACN with 0.1% formic acid. For the separation of the RALs and their glucuronides, a linear solvent gradient was used, changing from 30% B to 50% B in 15 min, then to 70% B in 5 min, then to 100% B in 3 min. After 3 min of eluting the column with 100% B, the initial 30% B were reached in 1 min. The flow rate was 1 mL/min.

2.5 LC-MS analysis

A LXQ Linear Ion Trap MSn system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Finnigan Surveyor Autosampler Plus and a Finnigan Surveyor PDA Plus detector was used. This allowed on-line detection of UV absorption and MS. The column and solvents were the same as with HPLC. A linear solvent gradient was used, changing from 30% B to 100% B in 30 min. After 1 min of eluting the column with 100% B, the initial 30% B were reached in 1 min and kept for 4 min before the next injection. The flow rate was 0.5 mL/min. The mass spectrometer was operated in the negative ESI mode. Nitrogen was used as sheath gas and auxiliary gas with flow rates of 30.0 and 15.0 L/min, respectively. Spray voltage was 4.5 kV and capillary temperature was 350°C. Ion optics were automatically tuned with a 10 μM solution of ZEN in methanol. MS^n were conducted at CID 35 (35% of 5 V).

3 Results and discussion

Each of the six RALs used in this study (Fig. 1) has two or three hydroxyl groups and gives rise to two or three different monoglucuronides, as recently shown by Stevenson *et al.* [14] using UGT from bovine liver. To determine the enzymatic activities of hepatic microsomes from various species and of human UGT isoforms for glucuronidation of the six RALs, the individual glucuronides were first prepared by incubating each RAL with bovine hepatic microsomes and UDPGA, separated by analytical HPLC, and characterized by comparing their mass spectra, UV spectra and LC retention times with those reported by Stevenson *et al.* [14]. For some of the 14-*O*-glucuronides, reference compounds were prepared by chemical or enzymatic modification of ZEN-14-*O*-glucuronide, which was available as an authentic standard.

3.1 Structure confirmation of the RAL glucuronides

When ZEN was incubated with hepatic bovine microsomes in the presence of UDPGA and the incubation mixture analyzed by reversed-phase HPLC, two products more polar than ZEN were observed (Fig. 2A, upper chart), which were not present in control incubations without UDPGA. The larger peak cochromatographed with authentic

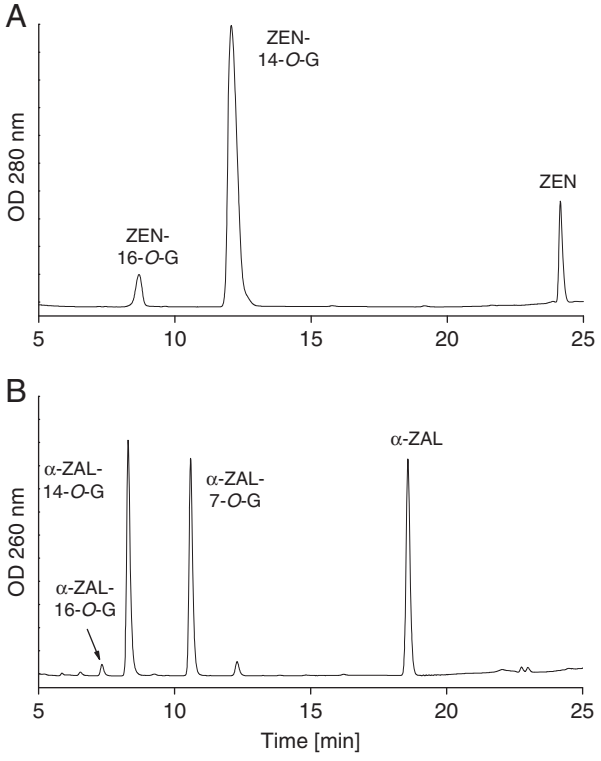

Figure 2. HPLC profiles of the glucuronides of ZEN (A) and α-ZAL (B) generated by UDPGA-fortified bovine liver microsomes.

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ZEN-14-*O*-glucuronide. In LC-MS analysis operated in the negative ESI mode, it had a quasimolecular ion of *m/z* 493, which gave rise to fragment ions of *m/z* 317 and 175 in MS^2 (Table 1). The more polar small peak had the same M-H ion and fragment ions, and was therefore assumed to represent ZEN-16-*O*-glucuronide. In agreement with these structures, the two glucuronide peaks disappeared and the aglycone was the only peak present with increased peak area after treatment with β-glucuronidase.

When α- and β-ZEL, ZAN, and α- and β-ZAL were incubated with bovine hepatic microsomes, the glucuronides listed in Table 1 were obtained. The assigned structures are predominantly based on ESI-MS and MS^2, HPLC retention times and UV spectra, as reported by Stevenson *et al.* [14]. In particular, MS^2 fragmentation has been shown useful for defining the position of the glucuronide at the RAL molecule [14]. Fragmentation may involve the loss of carbon dioxide (44 amu), the loss of glucuronic acid (176 amu) or the loss of the aglycone from the quasimolecular M-H, the latter fragmentation accounting for the daughter ion at *m/z* 175. None of the 14-*O*-glucuronides releases carbon dioxide upon MS^2 (Table 1). Instead, fragmentation is dominated by the loss of glucuronic acid and, to a minor extent, loss of the aglycone. In contrast, all the 16-*O*-glucuronides exhibit some loss of carbon dioxide upon MS^2, in addition to the other fragmentations. In 7-*O*-glucuronides, the loss of carbon dioxide represents the dominating fragmentation, with little loss of glucuronic acid and no loss of the aglycone (Table 1).

As a further example of the microsomal incubations, the HPLC profile of the incubation of α-ZAL is depicted in Fig. 2B (lower chart). Three of the four new peaks had the ESI-MS of an α-ZAL-monoglucuronide with a M-H ion of *m/z* 497 and, in part, MS^2 fragment ions of *m/z* 453, 321 and 175 (Table 1), and released the equivalent amount of α-ZAL upon treatment with β-glucuronidase. According to their MS^2 spectra, the largest peak, lacking the M-H-CO_2 fragment, was α-ZAL-14-*O*-glucuronide, the small and more polar peak was α-ZAL-16-*O*-glucuronide, and the large and less polar peak was α-ZAL-7-*O*-glucuronide, as indicated in Fig. 2B. Like with α-ZAL, three monoglucuronides were also detected in the bovine microsomal incubations of α-ZEL, ß-ZEL and β-ZAL. In analogy to ZEN, only two monoglucuronides were obtained with ZAN (Table 1).

The structures of the 14-*O*-glucuronides of the RALs could be confirmed through enzymatic and chemical modification of authentic ZEN-14-*O*-glucuronide. It has long been known that hepatic cytosol, containing HSD and ADH, reduces the keto group of ZEN [21, 22]. When a mixture of ZEN and ZEN-14-*O*-glucuronide was treated with rat hepatic cytosol in the presence of NADH, the products depicted in Fig. 3A (upper chart) were obtained. In addition to α-ZEL and β-ZEL, identified by comparison of their retention times and ESI-MS with that of authentic standards, two peaks with the ESI-MS of ZEL-monoglucuronides were observed. They were more polar than ZEN-14-*O*-glucuronide and were assigned the structures of α- and β-ZEL-14-*O*-glucuronide, based on their retention times

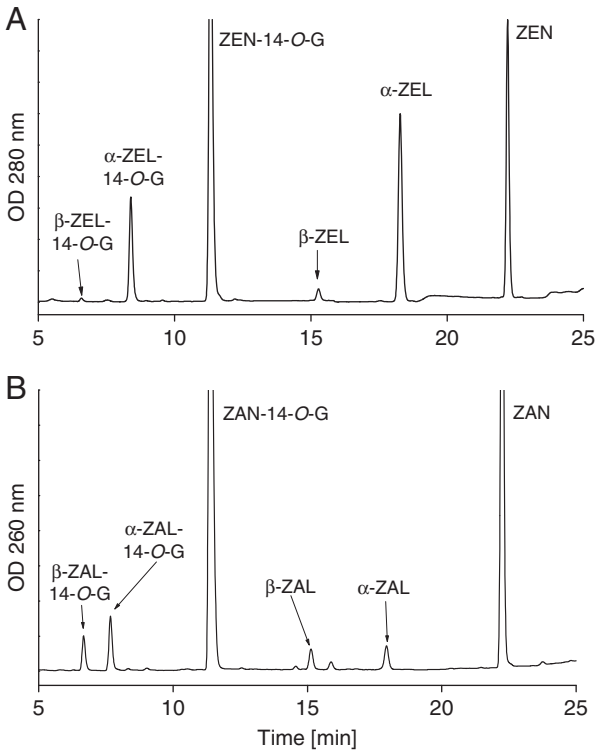

Figure 3. HPLC profiles of the compounds obtained when a mixture of ZEN and ZEN-14-*O*-glucuronide was reduced enzymatically with NADH-fortified rat liver cytosol (A) or chemically with hydrogen/Pd/C (B).

which were identical with those of the respective products obtained by the glucuronidation of α-ZEL and β-ZEL. The assignment of α-ZEL-14-*O*-glucuronide to the larger and less polar peak was also consistent with the fact that the α-stereoisomer was preferentially formed under these enzymatic conditions and that the α-isomers were always less polar than the β-isomers under these HPLC conditions (Table 1).

Although HSD and ADH selectively reduce the keto group of ZEN at C-7 to yield α- and β-ZEL, catalytic hydrogenation preferentially reduces the olefinic double bond but also to some extent the keto group. Thus, when a mixture of ZEN and ZEN-14-*O*-glucuronide in methanol was hydrogenated in the presence of Pd/C, the products depicted in Fig. 3B (lower chart) were obtained. ZAN, α-ZAL and β-ZAL were again identified by ESI-MS and retention time comparison with authentic standards. Based on their ESI-MS and retention times, the structures of ZAN-14-*O*-glucuronide and of α- and β-ZAL-14-*O*-glucuronide were assigned to the more polar peaks.

The 14-*O*-glucuronides of α-ZEL, β-ZEL, ZAN, α-ZAL and β-ZAL thus derived cochromatographed with the respective microsomal metabolites of the RALs and had identical mass and UV spectra, thereby confirming the structure assignment in Table 1.

A comparison of the retention times of the monoglucuronides of α-ZAL and also of the other RALs shows that

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the site of glucuronidation has a rather profound effect on the chromatographic behavior, as has already been observed by Stevenson *et al.* [14]. The aromatic glucuronides, carrying the glucuronic acid moiety at C-14 or C-16, are more polar than the aliphatic glucuronide at C-7. There is also a marked difference between the 14-*O*- and the 16-*O*-glucuronides of α-ZAL and of ZEN, the 16-*O*-glucuronides having the higher polarity. This may be explained by the fact that the remaining aromatic hydroxyl group is hydrogen-bonded with the neighboring carbonyl group of C-1 in the 14-*O*-glucuronides but not in the 16-*O*-glucuronides [14]. A similar difference was observed in the polarity of the two aromatic glucuronides of the mycotoxins alternariol and alternariol-9-methyl ether, which are also RALs [19].

3.2 Activities of microsomes from various species for the glucuronidation of RALs

Knowing the chemical structures of all monoglucuronides of the six RALs allowed us to determine the pattern of glucuronides generated by different microsomes. Because these patterns were obtained under conditions of linear glucuronide formation, the activities of the microsomes for glucuronidation could be calculated. Table 2 summarizes these data obtained with hepatic microsomes from steer, female pig, male Wistar rat and male human, as well as from intestinal human microsomes.

An obvious limitation of the method of quantifying the glucuronides through their absorbance is the absence of authentic standards. Therefore, it had to be assumed that the

Table 2. Activities[a)] of various microsomes for the glucuronidation of ZEN and its five metabolites, and pattern[b)] of isomeric glucuronides formed

RAL	Bovine liver (male)	Porcine liver (female)	Rat liver (male)	Human liver (male)	Human intestine (both genders)
ZEN	13±2[a)] (91/9/–)[b)]	34±10 (99/1/–)	10±1 (96/4/–)	6±1 (96/4/–)	5±1 (91/9/–)
α-ZEL	14±1 (76/5/19)	29±4 (86/0/14)	9±1 (87/1/12)	6±1 (92/0/8)	4±0 (90/5/5)
β-ZEL	11±4 (74/19/7)	29±2 (77/4/19)	11±1 (74/11/15)	5±1 (52/33/15)	4±1 (42/55/3)
ZAN	13±1 (99/1/–)	38±3 (99/1/–)	8±1 (100/0/–)	7±2 (99/1/–)	2±1 (89/11/–)
α-ZAL	11±1 (57/2/41)	28±4 (85/0/15)	10±1 (86/0/14)	4±1 (77/0/23)	3±1 (39/18/43)
β-ZAL	15±1 (64/6/30)	36±6 (95/0/5)	9±1 (43/3/54)	4±1 (75/4/21)	1±0 (57/24/19)

Data represent the mean value±standard deviation of three independent experiments.
a) nmol min^{-1} mg $protein^{-1}$ for the sum of all glucuronides.
b) Percent of 14-*O*-glucuronide/16-*O*-glucuronide/7-*O*-glucuronide; –, formation not possible.

Table 3. Activities[a)] of various human UGTs for the glucuronidation of model substrates and of six RALs to their isomeric monoglucuronides

UGT	Activity[b)] for	ZEN	α-ZEL	β-ZEL	ZAN	α-ZAL	β-ZAL
1A1	17β-Estradiol: 870	4200±400	5200±300	3000±200	3600±600	2900±100	1200±100
1A3	17β-Estradiol: 250	400±40	1800±500	2400±200	1400±300	1100 ±200	1100±300
1A4	Trifluoperazine: 1100	<5	40±0	60±0	<5	100±0	30±10
1A7	TFMU: 9000	240±30	50±10	80±20	<5	<5	40±0
1A8	TFMU: 880	2900±200	700±50	3400±100	1500±410	300±20	500±30
1A9	TFMU: 7200	600±10	200±0	700±30	500±70	500±20	400±20
1A10	TFMU: 90	100±20	70±0	100±10	50±20	<5	50±10
2B4	TFMU: 300	<5	30 ±10	100±20	600±40	200±10	200±0
2B7	TFMU: 2300	20±10	300±80	300±20	<5	200±10	500±50
2B15	TFMU: 2600	150±10	50±10	20±0	800±300	<5	<5
2B17	Eugenol: 1400	50±20	20±10	30±0	<5	200±30	50±10

Data represent the mean value±range of two independent experiments. TMFU, 4-(trifluoromethyl)umbelliferone.
a) Expressed as pmol min^{-1} mg $protein^{-1}$ for the model substrate or for the sum of all glucuronides.
b) Determined as reported in [18].

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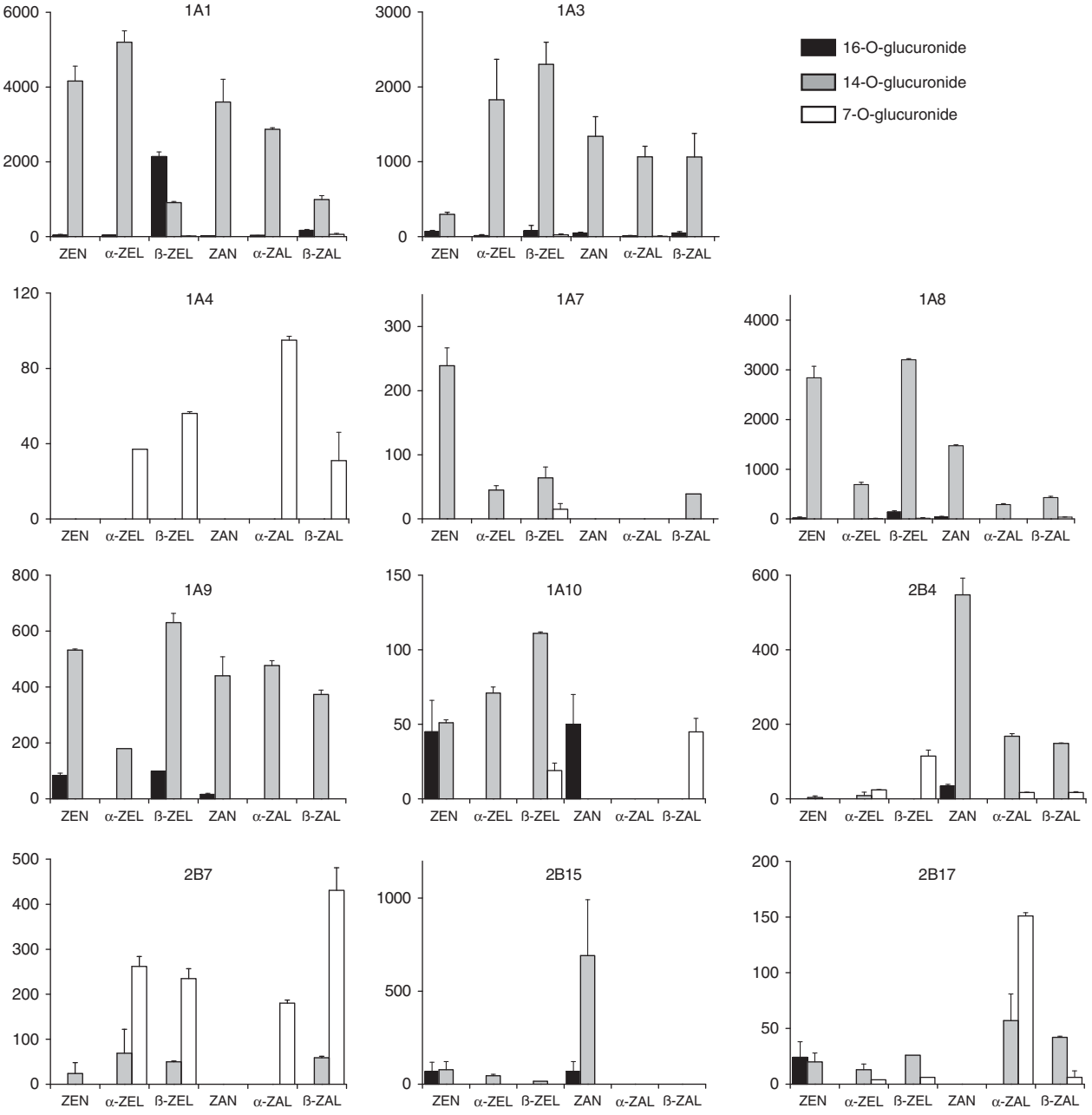

Figure 4. Pattern of glucuronides of the six RALs generated by UDPGA-fortified human UGT isoforms. Data represent the mean value ± range of two independent experiments.

extinction coefficients of the glucuronides are close to that of their aglycones, which were used for calibration. During the experiments conducted to assign the glucuronide structures (see Section 3.1), the mixture of glucuronides obtained from each RAL was hydrolyzed to release the aglycone. As the sum of the peak areas of the glucuronides approximately matched the peak area of the aglycone, it can be assumed that the differences in extinction coefficients are small and do not significantly influence quantitation.

All six RALs were good substrates for glucuronidation by all hepatic microsomes studied, and the activities of any one type of microsomes for the six congeners differed by less than a factor of two in most cases. In contrast, larger differences – up to a factor of 10 – were observed for the same RAL between microsomes from different species. In general, porcine hepatic microsomes had the highest activity, followed by bovine, rat and human liver microsomes. An even lower activity than obtained with human hepatic microsomes was

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determined for human intestinal microsomes. Extrapolated to the *in vivo* situation, this may suggest that glucuronidation of ZEN and its congeners is faster in cattle and pigs than in rats and humans, and the human liver contributes more to glucuronide formation than the intestine.

With regard to the types of glucuronides formed, hepatic microsomes clearly preferred the conjugation of the six RALs at C-14 over C-16, as has also been observed before with bovine UGT [14]. This is probably due to the strong hydrogen bonding of the hydroxyl group at C-16 with the carbonyl group at C-1. A similar preference was recently reported for the RALs alternariol and alternariol-9-methyl ether [19]. An aliphatic hydroxyl group at C-7, as present in the epimers of ZEL and ZAL, is readily conjugated by hepatic microsomes, but glucuronidation at C-14 is still preferred, except by rat liver microsomes for β-ZAL. When the extent of glucuronidation at C-7 and C-16 is compared, C-7 is preferred except for β-ZEL with bovine and human hepatic microsomes. Human intestinal microsomes appear to have a somewhat higher tendency than hepatic microsomes to form 16-*O*-glucuronides of all RALs (Table 2).

3.3 Activities of recombinant human UGTs for six RALs

To clarify which human UGT isoforms contribute to the glucuronidation of ZEN and its congeners, the enzymatic activity of ten human UGT isoforms available as recombinant enzymes and the pattern of glucuronides were determined. These isoforms are not pure enzymes but microsomes from insect cells expressing the respective human UGT gene. As the level of expression differs between isoforms, the amounts of UGTs vary considerably, and activities can only be compared when model substrates for the individual isoforms are used.

The enzymatic activities of all UGT isoforms for their model substrates and for the six RALs are listed in Table 3. Among the 1A isoforms, the highest activities for most RALs resided in UGT1A1, 1A3 and 1A8. In most cases, the activities of these three isoforms for the six RALs were higher than for their model substrates measured at comparable concentrations. In contrast, UGT1A4, 1A7, 1A9, 1A10 and most of the 2B isoforms had much lower activities for the RALs than for their model substrates. Exceptions are UGT1A10 for ZEN and β-ZEL, and UGT2B4 for ZAN. As UGT1A1 and 1A3 are strongly expressed in the human liver [23–25], these isoforms must be assumed to contribute markedly to the activity of human hepatic microsomes (Table 2). Likewise, the activity of human intestinal microsomes is probably due to UGT1A1 and 1A8. Thus, the activities of the human UGT isoforms for the six RALs (Table 3) are consistent with the activities determined with human hepatic and intestinal microsomes (Table 2).

The pattern of RAL glucuronides generated by the eleven human UGT isoforms is depicted in Fig. 4. The UGTs with the highest activity, *i.e.* UGT1A1, 1A3 and 1A8, preferred the aromatic hydroxyl group at C-14 for glucuronidation over the hydroxyl group at C-16 and C-7. Notable exceptions were the incubation of UGT1A1 with β-ZEL and UGT1A10 with ZAN, which gave rise to more 16-*O*-glucuronide than 14-*O*-glucuronide. This is reflected by the high proportion of β-ZEL-16-*O*-glucuronide observed with human hepatic and intestinal microsomes, and of ZAN-16-*O*-glucuronide with human intestinal microsomes (Table 2). Glucuronidation of the epimers of ZEL and ZAL at the aliphatic hydroxyl group at C-7 was only catalyzed with low activity by UGT2B7 and 1A4 (Fig. 4), explaining the relatively low proportion of the aliphatic glucuronides of α- and β-ZEL and α- and β-ZAL observed with human microsomes (Table 2).

4 Concluding remarks

In summary, our *in vitro* study has determined the activities of hepatic microsomes from various farm and experimental animals as well as of human hepatic and intestinal microsomes and eleven human UGT isoforms for the glucuronidation of the six RALs. A consistent picture was obtained, showing little difference in the activity of any one type of microsomes for the six RALs, but larger differences for each RAL between species. The highest activity among the human UGTs resided in UGT1A1, 1A3 and 1A8, explaining the efficient glucuronidation by both hepatic and intestinal human microsomes. Extrapolated to the *in vivo* situation, these data imply that the RALs are readily glucuronidated both in the human liver and intestine, and probably also in extrahepatic tissues. The major glucuronide to be expected *in vivo* is the aromatic 14-*O*-glucuronide, which was more abundant than the other aromatic 16-*O*-glucuronide and, in the case of the ZELs and ZALs, the aliphatic 7-*O*-glucuronide with most microsomes and UGT isoforms.

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