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Research Article

Glucuronidation of curcuminoids by human microsomal and recombinant UDP-glucuronosyltransferases

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Glucuronidation is an important pathway in the metabolism of curcumin, but the isoforms of uridine-5′-diphosphoglucuronosyltransferase (UGT) involved are not known. Here, we report on the glucuronidation of the three natural curcuminoids and their major phase I metabolites with microsomes from human liver and intestine as well as with human recombinant UGTs. Microsomes from human liver generated predominantly the phenolic and small amounts of the alcoholic glucuronide of each curcuminoid, whereas intestinal microsomes formed only the phenolic conjugates but with higher activities. The phenolic glucuronidation of the curcuminoids was predominantly catalyzed by hepatic UGT1A1 and intestinal UGT1A8 and 1A10, whereas UGT1A9, 2B7, and 1A8 exhibited high activities for hexahydro-curcuminoids. UGT1A9 was able to form the alcoholic glucuronide of each curcuminoid in addition to the phenolic conjugate. These data suggest that the gastrointestinal tract contributes substantially to the glucuronidation of curcuminoids in humans, which may have important implications for their pharmacokinetic fate *in vivo*.

Keywords: Curcumin / Glucuronidation / Microsomes / UGTs

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1 Introduction

Curcumin, demethoxy-curcumin, and bisdemethoxy-curcumin (Fig. 1) are polyphenolic compounds isolated from the powdered rhizomes of the plant *Curcuma longa*, commonly called turmeric. These curcuminoids are widely used as food coloring agent and spice [1], and have been found to exhibit anti-inflammatory, antioxidant, and anticarcinogenic activities [2–4]. Recently, evidence has been provided for the chemoprevention of human colorectal cancer by curcumin [5–8]. In contrast to the broad interest in the biological effects of curcuminoids, only few studies have so far focused on their metabolism in the mammalian organism [9, 10]. The present state of knowledge can be summarized as follows: in phase I metabolism, curcumin and its two demethoxy congeners undergo successive reduction to their dihydro-, tetrahydro-, hexahydro-, and octahydro-metabolites in the liver as well as in the intestinal mucosa. The hexahydro curcuminoid (Fig. 1) usually represents the major metabolite. Both the parent curcuminoids and their reductive metabolites are extensively conjugated with glucuronic acid *in vivo* [11–13]. We have recently reported that curcuminoids give rise to two different monoglucuronides in human hepatic microsomes, *i.e.*, a major phenolic and a minor alcoholic glucuronide [14]. In contrast, only phenolic glucuronides are formed from the hexahydro-metabolites [14].

Glucuronidation is a major phase II conjugation reaction of numerous xenobiotic as well as endobiotic compounds [15]. By adding the glycosyl group of uridine-5′-diphosphoglucuronic acid (UDPGA), such compounds become more hydrophilic and are therefore more readily excreted. The reaction is catalyzed by a superfamily of uridine-5′-diphosphoglucuronosyltransferases (UGTs), which are membrane-bound enzymes localized at the luminal side of the ER. Currently, 17 functional human UGT isoforms have been identified and are classified into the two subfamilies, UGT1 and UGT2, based on sequence homologies [16, 17]. Isoforms belonging to the UGT1A subfamily primarily conjugate bilirubin and a variety of drugs and xenobiotics

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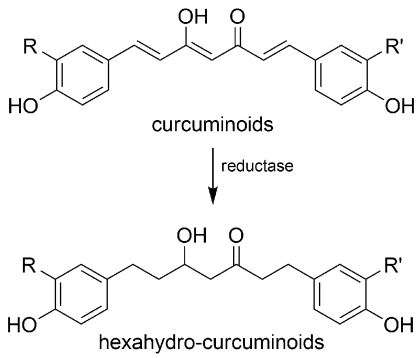
Abbreviations: E2, 17β-estradiol; **HIM,** human intestinal microsomes; **HLM,** human liver microsomes; **TFMU,** 4-(trifluoromethyl)-umbelliferone; **UDPGA,** uridine-5′-diphosphoglucuronic acid; **UGT,** uridine-5′-diphosphoglucuronosyltransferase



Curcuminoid	R	R'
Curcumin	$-OCH_3$	$-OCH_3$
Demethoxy-curcumin	$-OCH_3$	-H
Bisdemethoxy-curcumin	-H	-H

Figure 1. Chemical structures of curcuminoids and hexahydro-curcuminoids.

[16, 18], whereas UGT2B isoforms preferentially catalyze the conjugation of endogenous substrates, such as steroid hormones, bile and fatty acids, as well as various drugs [19]. In general, the UGTs have broad and overlapping substrate specificities, but a few specific reactions have been described for certain UGT isoforms, *e.g.*, the formation of the phenolic 3-glucuronide of 17β-estradiol (E2) by UGT1A1 [20]. UGTs are not limited to the liver, and some extrahepatic tissues are known to exhibit significant activities. For example, UGT1A7, 1A8, and 1A10 are specifically expressed in the gastrointestinal tract with little or no activity in the liver [21, 22].

Although glucuronidation is the major pathway of curcuminoid conjugation in humans, no information is available to date about the isoforms of UGTs involved in this reaction. In the present study, we have therefore determined the activities of human hepatic and intestinal microsomes as well as nine human recombinant UGT isoforms for the three curcuminoids and their hexahydro-metabolites. The nine UGT isoforms included 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7. The isoforms 2B4, 2B11, and 2B17, which are also expressed in human liver and intestine, could not be tested because they are not commercially available. UGT2B15 was omitted because its expression is very low in liver and absent in intestine, and also because its substrate specificity is generally very similar to that of UGT2B7 [15].

2 Materials and methods

2.1 Chemicals

Curcumin and bisdemethoxy-curcumin were chemically synthesized as described earlier [11], using the method of Pabon [23]. Briefly, acetylacetone was reacted with the appropriate substituted benzaldehyde and boric acid anhydride as a catalyst. The final products obtained after crystallization from methanol were >99% pure according to HPLC analysis and exhibited the correct molecular ions in LC-MS analysis. Demethoxy-curcumin was isolated from turmeric by extraction, column chromatography, and crystallization at the Arizona Center for Phytomedicine Research (Tucson, AZ, USA). It was >99% pure according to HPLC analysis. Hexahydro-curcumin and hexahydro-bisdemethoxy-curcumin were obtained by hydrogenation of the respective curcuminoids in methanol with a Pd on charcoal catalyst as described in detail previously [11] and were >95% pure according to HPLC analysis.

4-(Trifluoromethyl)umbelliferone (TFMU), E2, propofol (2,6-diisopropylphenol), UDPGA, β-glucuronidase (type B-1 from bovine liver), and all other chemicals and reagents were obtained from Sigma–Aldrich Chemical (Steinheim, Germany). HPLC-grade ACN was from Carl Roth (Karlsruhe, Germany).

2.2 Microsomal fractions

Human liver microsomes (HLM) were prepared from the liver of a 63-year-old white male (kindly provided by Dr. J. Weymann, former Knoll AG, Ludwigshafen, Germany) as described by Lake [24]. Pooled human intestinal microsomes (HIM) were purchased from Gentest (Woburn, MA, USA) and contained equal amounts of microsomes prepared from both the duodenum and jejunum section of each of the five donors (two females and three males of Caucasian and African American race, with ages ranging from 17 to 62 years). Supersomes, *i.e.*, microsomes from insect Sf-9 cells infected with a baculovirus strain containing the cDNA of human UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, or 2B7, were also from Gentest. Protein concentrations were measured according to Bradford [25] with BSA as standard.

2.3 Glucuronidation assays

For studies on their enzymatic activity, HLM, HIM, and individual human UGTs were incubated with the curcuminoids or with TFMU, E2, or propofol in a total volume of 0.2 mL of 50 mM tris buffer pH 7.5. A typical incubation contained 0.01–0.2 mg of the microsomal protein, which was first mixed with 5 μg of alamethicin in 50 μL buffer and placed on ice for 10 min. Subsequently, magnesium chloride (final concentration 10 mM), the β-glucuronidase inhibitor saccharolactone (10 mM), UDPGA (2 mM), and finally the substrate (20 μM) dissolved in DMSO (final concentration 2%) were added and the mixture incubated at 37°C for up to 2 h with linear product formation. Incubations were then placed on ice, and aliquots of 20–40 μL were directly injected into the HPLC without prior extrac-

tion. Only for E2, the following workup of the incubation mixture was required prior to HPLC analysis: 0.2 mL of 0.7 M glycine/HCl buffer pH 1.2 were added to yield pH 1.8, and the mixture was subsequently extracted three times with 0.5 mL each of ethyl acetate. The extract was evaporated to dryness under reduced pressure and dissolved in methanol.

Control incubations were conducted with HLM and HIM in the absence of UDPGA and with supersomes lacking a UGT isoform in the presence of UDPGA. All incubations were performed in triplicate.

When the inhibitory effect of propofol (at concentrations increasing from 0 to 200 μM) on the glucuronidation of curcumin (100 μM) by HLM (0.2 mg microsomal protein) was studied, the incubation time was shortened to 15 min and incubations were carried out in duplicate.

The amounts of glucuronides formed in the incubations were calculated from the area of the HPLC peaks of the glucuronides. As no standards were available for the glucuronides of the curcuminoids, TFMU, and propofol, it was assumed that the glucuronides had the same molar absorbance as the respective aglycones. E2-3-glucuronide was available as reference compound and exhibited half the molar extinction coefficient of E2.

For the identification of the glucuronide peaks in HPLC, enzymatic hydrolysis was carried out with some incubations. In these experiments, saccharolactone was omitted, and an aliquot of the incubation mixture was mixed with an equal volume of 0.15 M acetate buffer pH 5.0 containing 5000 Fishman units of β-glucuronidase and incubated for 2 h at 37°C prior to extraction with ethyl acetate and HPLC analysis. LC-MS analysis was also used for the identification of glucuronides.

2.4 Analysis of enzyme kinetics

For the kinetic studies, glucuronidation reactions were conducted with curcumin (0.1–50 μM) and HLM (0.01–0.1 mg microsomal protein) as described above. Glucuronidation activities for each substrate concentration were determined in triplicate. The kinetic data for curcumin glucuronidation were found to best fit the Michaelis–Menten equation: $V = V_{max} \times S/(K_m + S)$, where V_{max} is the maximum velocity, K_m the Michaelis–Menten constant, and S the substrate concentration. Kinetic parameters were estimated from the fitted curve by nonlinear regression analysis using the computer program OriginPro 6.0 (OriginLab Corporation, Northampton, MA, USA).

2.5 HPLC analysis

An HP 1100 system equipped with a binary pump, a photodiode array detector (DAD), and HP Chemstation software for data collection and analysis (Agilent Technologies, Waldbronn, Germany) was used, and separation was carried out on a 250 × 4.6 mm id, 5 μm, RP Luna C18(2) column (Phenomenex, Torrance, CA, USA). Eluent A was deionized water adjusted to pH 3.0 with formic acid and eluent B was ACN, with a flow rate of 1 mL/min. Different linear gradients were applied for the separation: gradient I for curcuminoids: 0–35 min 30–70% B; gradient II for hexahydro-curcuminoids: 0–6 min 15% B, 6–7 min 15–30% B, 7–17 min 30–35% B, 17–32 min 35–45% B; gradient III for TFMU: 0–6 min 15% B, 6–7 min 15–30% B, 7–24 min 30–65% B; gradient IV for E2: 0–2 min 17% B, 2–9 min 17–45% B, 9–16 min 45–50% B, 16–22 min 50–100% B; gradient V for propofol: 0–15 min 30–55% B, 15–20 min 55–75% B, 201–32 min 75–100% B. Curcuminoids were monitored at 420 nm, hexahydro-curcuminoids and E2 at 280 nm, TFMU at 325 nm, and propofol at 220 nm. The LOQs, calculated as three times the LOD after the injection of a 40 μL aliquot of the incubation mixture, were 3 pmol for curcuminoids and 15 pmol for hexahydro-curcuminoids.

2.6 LC-MS/MS analysis

LC-MS and MS/MS experiments were performed using an Agilent HP 1100 HPLC system equipped with an MSD-Trap-SL IT mass spectrometer operated in the negative ESI mode as previously described [14].

3 Results and discussion

3.1 Glucuronidation of curcuminoids in human hepatic and intestinal microsomes

Human hepatic and intestinal microsomes were incubated with curcumin, demethoxy-curcumin, bisdemethoxy-curcumin, hexahydro-curcumin, and hexahydro-bisdemethoxy-curcumin, and the reaction mixture was analyzed by HPLC/DAD directly after incubation, because of the known instability of curcuminoids and their glucuronides [14, 26]. The UGT substrates TFMU and E2 were included for comparison. It is known that two different glucuronides of E2, *i. e.*, at positions 3 and 17, are generated in humans [27]. For the calculation of the enzyme activities, the sum of all glucuronides formed from each substrate was used. The results are depicted in Fig. 2.

When curcumin was incubated with HLM, two monoglucuronides were observed. As previously reported [14], the major product carries the glucuronic acid at the phenolic and the minor metabolite at the alcoholic hydroxyl group. These two monoglucuronides can easily be separated by HPLC, and their exact chemical structures could be elucidated by LC-MS/MS after hydrogenation [14]. As curcuminoids are symmetrical molecules, their phenolic monoglucuronides exhibit only one HPLC peak [14].

The formation of the phenolic glucuronide of curcumin followed Michaelis–Menten kinetics. The kinetic param-

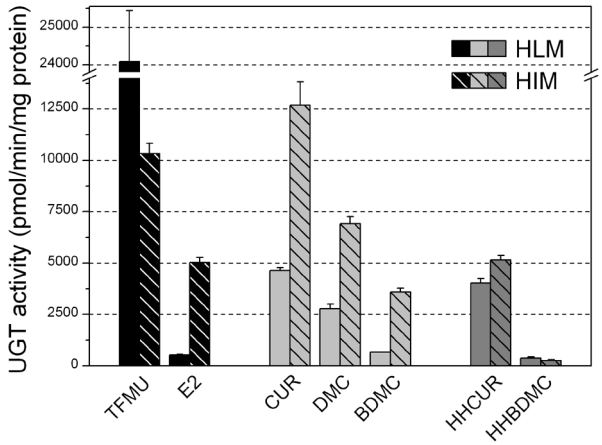


Figure 2. Activities of microsomes from human liver (HLM) and human intestine (HIM) for the glucuronidation of various curcuminoids and standard substrates. Rates of glucuronidation were determined at 20 μM concentrations of each substrate and are expressed as pmol/min/mg protein (mean ± SD of three independent experiments). The sum of all glucuronides was used for substrates generating more than one glucuronide. TFMU, 4-(trifluoromethyl)umbelliferone; E2, 17β-estradiol; CUR, curcumin; DMC, demethoxy-curcumin; BDMC, bisdemethoxy-curcumin; HHCUR, hexahydro-curcumin; HHBDMC, hexahydro-bisdemethoxy-curcumin.

eters obtained from three independent experiments over a concentration range of 0.1–50 μM curcumin were $K_m = 0.9 \pm 0.2$ μM and $V_{max} = 5123 \pm 190$ pmol/min/mg protein. The low K_m and high V_{max} values indicate a high affinity and high capacity of hepatic UGTs for curcumin. In addition, the low K_m suggests that curcumin may have the potential to inhibit the glucuronidation of other UGT substrates. Basu *et al.* [28] observed a transient and concentration-dependent inhibition of UGTs in human colon cancer cells by curcumin and reported that curcumin inhibited the glucuronidation of eugenol, capsaicin, bilirubin, and mycophenolic acid *in vitro*. Whereas glucuronidation of bilirubin represents an important detoxification step and its inhibition may be harmful, decreased glucuronidation of the immunosupressive agent mycophenolic acid is expected to increase the therapeutic index *in vivo*.

In contrast to HLM, only the phenolic curcuminoid glucuronides were generated by HIM. For both types of microsomes, curcumin was the best and bisdemethoxy-curcumin the poorest substrate, and hexahydro-bisdemethoxy-curcumin was barely glucuronidated at all (Fig. 2). For curcumin and its demethoxy-congeners, HIM had about three-fold higher activity than HLM, whereas hexahydro-curcumin and hexahydro-bisdemethoxy-curcumin were glucuronidated with similar activity by HIM and HLM. Thus, the gastrointestinal tract may play an important role in the first pass effect of dietary curcuminoids, and systemic bioavailability is likely to be reduced due to presystemic elimination through UGTs. The low systemic bioavailability of orally ingested curcumin has been reported in clinical trials with colorectal cancer patients, resulting in nanomolar hepatic concentrations of curcumin and its conjugates as compared to colorectal concentrations in the micromolar range [6, 7, 29]. Thus, the concentrations used in our *in vitro* study reflect the *in vivo* conditions in the intestine and colon rather than in the liver.

For the comparison of HIM and HLM, it should be pointed out that HIM were pooled microsomes obtained from several donors of diverse race, gender, and age, whereas HLM were from one individual. This raises the question of how representative the activity of our HLM was. For that reason, the glucuronidation activities of our HLM for various commonly used substrates were compared with data published in the literature. For the glucuronidation of E2 at position 3, the reported average activity of HLM obtained from donors of diverse age and gender was 797 ± 573 pmol/min/mg protein, and proved to be about ten-fold lower in HLM than in HIM [27]. In our HLM, the activity for the formation of E2-3-glucuronide was 426 ± 41 pmol/min/mg protein and 12-fold lower than in our pooled HIM, which is in good agreement with the literature data. Likewise, the activity for the glucuronidation of propofol was very similar between our HLM, *i.e.*, 182 pmol/min/mg protein, and that reported for pooled HLM, *i.e.*, 200 pmol/min/mg protein [30]. Therefore, our HLM exhibit activities for the glucuronidation of E2 and propofol well within the range reported in the literature and appear to represent typical HLM.

3.2 Activities of recombinant UGTs for the glucuronidation of curcuminoids

In order to clarify which UGT isoforms contribute to the glucuronidation of curcuminoids and their hexahydro-metabolites, the activities of nine human UGTs were studied. The nonspecific UGT substrate TFMU and the specific substrates propofol and E2 were included as controls. Propofol is selectively glucuronidated by UGT1A9 and E2 at position 17 by UGT2B7. Position 3 of E2 is glucuronidated mostly by UGT1A1 and with lower activity by other UGTs, *e.g.*, 1A3 and 1A8 [31].

The majority of the UGT isoforms, *i.e.*, 1A1, 1A3, 1A7, 1A8, 1A9, 1A10, and 2B7, were able to catalyze the glucuronidation of the three curcuminoids and of hexahydro-curcumin, although with markedly different activities (Fig. 3). Glucuronidation of curcumin and demethoxy-curcumin was mainly catalyzed by UGT1A1, 1A8, and 1A10. Bisdemethoxy-curcumin appeared to be a less favorable substrate than the other curcuminoids for all UGTs, in particular for UGT1A8. For hexahydro-curcumin, UGT1A8, 1A9, and 2B7 were the isoforms with the highest activities. In contrast, hexahydro-bisdemethoxy-curcumin proved to be a poor UGT substrate and its conjugation was only marginally catalyzed by UGT1A1, 1A9, and 2B7. UGT1A6

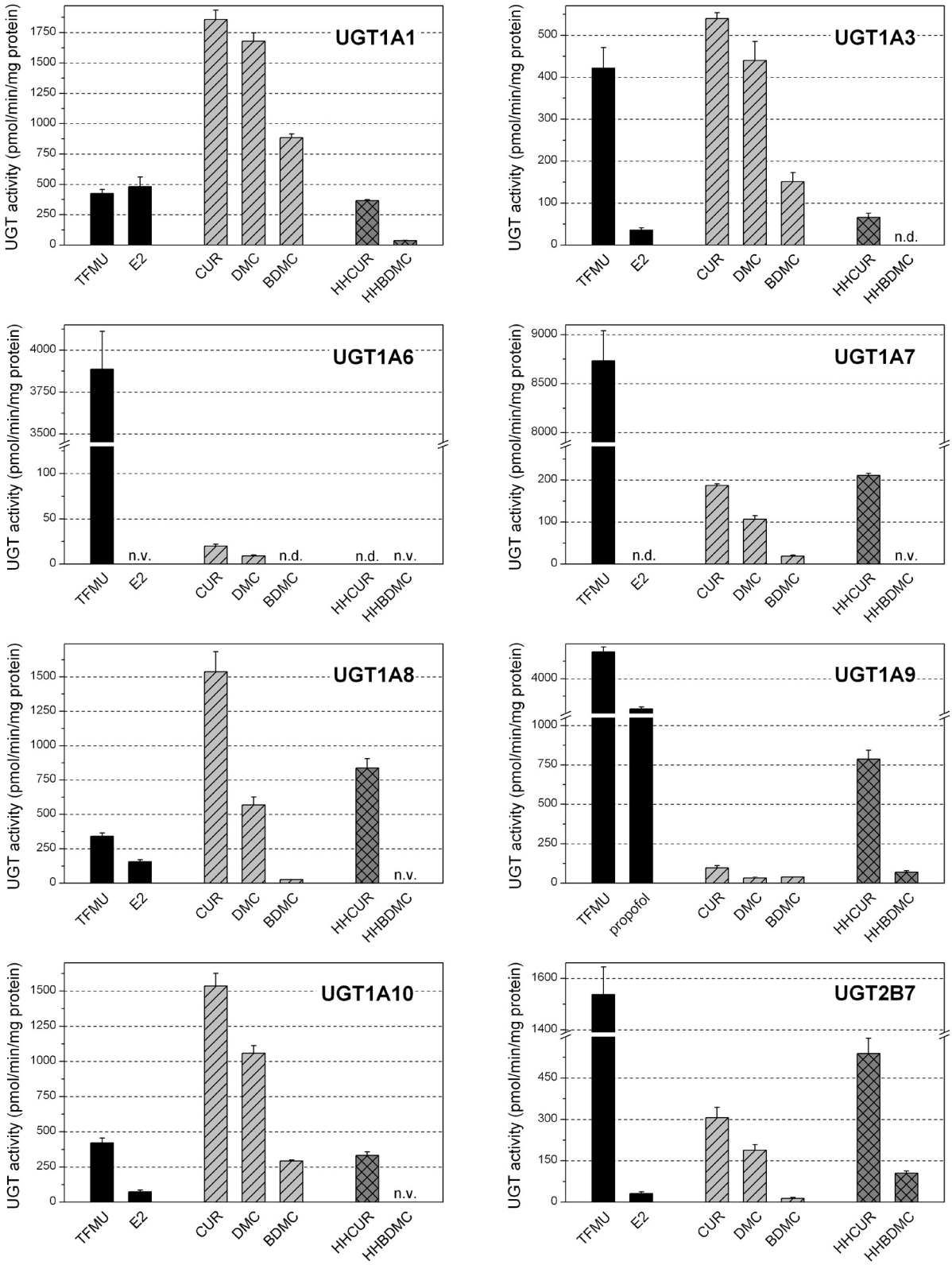


Figure 3. Activities of supersomes expressing human recombinant UGT isoforms for the glucuronidation of various curcuminoids and standard substrates. Rates of glucuronidation were determined at 20 μM concentrations of each substrate and are expressed as pmol/min/mg protein (mean ± SD of three independent experiments). The sum of all glucuronides was used for substrates generating more than one glucuronide. For a definition of labels, see the caption to Fig. 2. n.d., not determined; n.v., not verifiable.

formed only trace amounts of curcumin and demethoxy-curcumin glucuronide (Fig. 3). With UGT1A4, no glucuronide formation of curcumin and hexahydro-curcumin was detectable even when the protein concentration was increased five-fold, although UGT1A4 converted E2 to the 17-glucuronide with an activity of 10 pmol/min/mg protein. The lack of activity of this UGT isoform for curcuminoids may be expected because UGT1A4 primarily catalyzes the glucuronidation of amines [15].

For most UGT isoforms, similar glucuronidation patterns as observed with HLM and HIM (Fig. 2) were noted, and none of the curcuminoids represented a specific UGT substrate. Whereas curcumin was glucuronidated with the highest activity of all congeners by most UGTs, it is notable that UGT1A9 and 2B7 preferred hexahydro-curcumin (Fig. 3). For UGT1A9, this is in agreement with its reported preference of nonplanar phenolic substrates [32], because hexahydro-curcumin contains a saturated aliphatic chain and hence is less planar than curcumin.

UGT1A1 represents an important enzyme of the human liver and intestine. UGT1A3, 1A9, and 2B7 are also active in hepatic and extrahepatic tissues. In contrast, UGT1A7, 1A8, and 1A10 are exclusively expressed in tissues of the gastrointestinal tract [21, 22]. The high activity of UGT1A8 and 1A10 is supported by the markedly higher activity of intestinal microsomes for the glucuronidation of curcumin than hepatic microsomes (Fig. 2) and suggests that these two intestinal UGTs may be important for the glucuronidation of curcuminoids *in vivo*. UGT1A8 and 1A10 are also known to exhibit high activities for other bioactive plant products such as coumarins like scopoletin, flavonoids like genistein, and anthraquinones like anthraflavic acid [33].

All UGT isoforms except UGT1A9 exclusively formed the phenolic glucuronide of the curcuminoids. UGT1A9 generated both the phenolic and the alcoholic monoglucuronides, and the relative proportion of the alcoholic glucuronide increased from curcumin to bisdemethoxy-curcumin (Table 1). In HLM, the activity for the formation of the alcoholic curcumin glucuronide was 45 ± 9 pmol/min/mg protein. Because propofol, which is a specific substrate for UGT1A9 with a reported K_m of 170 μM [32], was glucuronidated by HLM with an activity of 128 ± 17 pmol/min/mg protein, it was assumed that glucuronidation of curcumin may be reduced in the presence of propofol. In fact, when competition experiments were performed, the HPLC chromatograms indicated a decrease in the formation of the alcoholic curcumin glucuronide as propofol concentration was increased. For example, the formation of the alcoholic curcumin glucuronide was inhibited by 40 and 58% at 50 and 100 μM propofol, respectively, whereas the formation of the phenolic curcumin glucuronide was only slightly affected by propofol, confirming that UGT1A9 contributes mainly to the formation of the alcoholic curcumin glucuronide. This also explains why rat liver microsomes were unable to generate the alcoholic glucuronide of curcumin in a previous study [14], because the gene for the homologous UGT isoform Ugt1a9-ps in the rat represents a pseudogene which leads to an inactive transcript [17]. Although the produced amounts were low, the formation of the alcoholic glucuronide may play an important role in human kidney. Sutherland *et al.* [34] have shown that UGT1A9 mRNA levels in human kidney are up to three-fold higher than in human liver. Moreover, the kidney has been shown to be capable of glucuronidation of a wide range of drugs including propofol [35].

Table 1. Glucuronidation of curcuminoids by UGT1A9

Curcuminoid	Enzymatic activity (pmol/min/mg protein)	
	Phenolic glucuronide	Alcoholic glucuronide
Curcumin	77 ± 13	20 ± 4
Demethoxy-curcumin	15 ± 1	15 ± 1
Bisdemethoxy-curcumin	9 ± 1	29 ± 1

4 Concluding remarks

In summary, our study has identified the human isoforms of UGTs involved in the glucuronidation of curcuminoids and provided evidence for a major contribution of intestinal UGT enzymes along with isoforms localized in the liver. The data indicate that while the liver is still an important organ for both systemic and first-pass metabolism, the gastrointestinal tract may significantly participate in the first-pass glucuronidation of curcuminoids.

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