Investigation of the substrate specificity of a cloned expressed human bilirubin UDP-glucuronosyltransferase: UDP-sugar specificity and involvement in steroid and xenobiotic glucuronidation

Sahidan B. SENAFI, Douglas J. CLARKE and Brian BURCHELL*

Department of Biochemical Medicine, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, U.K.

A cloned human bilirubin UDP-glucuronosyltransferase (UGT) stably expressed in Chinese hamster V79 cells was used to assess the substrate specificity of the enzyme. The catalytic potential ($V_{max.}/K_{m(bilirubin)}$) of the enzyme with UDP-glucuronic acid (UDPGA) was 2-fold and 10-fold greater than that for UDP-xylose and UDP-glucose respectively. The formation of bilirubin mono- and di-conjugates was found to be dependent on time, UDP-sugar concentration and bilirubin concentration. *Ex vivo* studies demonstrated that the genetically engineered cell line was capable of the uptake and glucuronidation of bilirubin and the release of bilirubin glucuronide, indicating its usefulness in studying transport processes. Over 100 compounds, including drugs, xenobiotics and endogenous steroids, were tested as substrates for the enzyme to determine the chemical structures accepted as substrates. A wide diversity of xenobiotic compounds such as phenols, anthraquinones and flavones (many of which are in foodstuffs) were glucuronidated by the enzyme. The enzyme also had the capacity to glucuronidate oestriols and oestradiols sterioselectively. H.p.l.c. analysis of the regioselective glucuronidation of β-oestradiol (E_2) demonstrated that it was conjugated solely at its A-ring hydroxy group by the bilirubin UGT to form E_2–3-glucuronide, this was in contrast with human liver microsomes which formed 3- and 17-glucuronides of this oestrogen. Studies utilizing microsomes from a Crigler–Najjar patient and inhibition of E_2 glucuronidation with bilirubin indicated that the cloned expressed bilirubin UGT was the major human UGT isoform responsible for the formation of E_2–3-glucuronide, which is the predominant E_2 conjugate in human urine.

INTRODUCTION

Glucuronidation is a major pathway for the inactivation and excretion of both endobiotic compounds such as bilirubin and steroids as well as a multitude of xenobiotic compounds including drugs, carcinogens and others environmental pollutants [1]. In the past decade it has been established by substrate specificity, ontological, induction, purification and cloning studies that these glucuronidation reactions are catalysed by a multigene family of membrane-bound enzymes the UDP-glucuronosyltransferases (UGTs; EC 2.4.1.17) located in the endoplasmic reticulum [2]. Recently several human UGT cDNAs have been cloned and, on the basis of evolutionary divergence, have been split into two families termed UGT1 and UGT2 [3]. A single *UGT1* gene on chromosome 2 encodes human phenol and bilirubin UGTs [4,5]. The gene comprises at least six different exon 1s which encode the substrate-binding domains of the different isoforms, and exons 2–5 which encode the identical C-terminals of the UGT1 isoforms [4]. It is predicted that each exon 1 is differentially spliced to the exon 2 to 5 cluster in the primary RNA transcript to result in the formation of two bilirubin (UGT1*1 and UGT1*4) and two phenol (UGT1*02 and UGT1*6) UGT isoforms [3]. The *UGT2* gene family, which contains odorant- and steroid-metabolizing isoforms, in contrast with the UGT1 family is composed of independent genes [6,7] which are clustered on human chromosome 4 [8].

The most important physiological role of UGTs is the esterification of at least one of the two propionic acid side chains of the toxic haem breakdown product bilirubin, which is essential for the efficient biliary excretion of bilirubin. Failure to carry out this important task in humans results in potentially fatal consequences manifested in the inborn error Crigler–Najjar syndrome type 1 [9]. Although UDP-glucuronic acid (UDPGA) is the primary UDP-sugar co-substrate utilized for the esterification in mammals two other UDP-sugars, UDP-glucose and UDP-xylose, have also been demonstrated to be potential co-substrates [10]. Toxicologically it is important to determine whether exogenous compounds present in our diet, drugs or pollutants interact with bilirubin UGTs. The substrate specificity of bilirubin UGTs has received scant attention; only five compounds in addition to bilirubin are known as substrates for the enzymes [11–14]. Indirect evidence suggests that bilirubin UGTs may also interact with several other compounds which may be substrates, as administration or exposure to the xenobiotics ethinyloestradiol [15] and novobiocin [16] in humans causes elevated bilirubin levels and jaundice. Previously we have reported the cloning and stable expression of a cDNA encoding the major constitutive human bilirubin UGT, termed UGT1*1, in a fibroblast cell line [12]. In this report we utilize the cloned expressed human bilirubin UGT to study the kinetics and the formation of bilirubin conjugates using three different UDP-sugars and comprehensively assess the substrate specificity of the enzyme with over 100 different endogenous and exogenous compounds with UDPGA as a sugar donor.

MATERIALS AND METHODS

Maintenance of the genetically engineered cell line and preparation of cell extracts

Hamster lung fibroblast V79 cells stably expressing UGT1*1 (formerly termed HP3) cDNA [12] were grown in Dulbecco's modified Eagle medium (Gibco, Life Technologies, Paisley, U.K.) supplemented with 5% (v/v) fetal-calf serum, 50 units/ml penicillin, 50 mg/ml streptomycin (Gibco, Life Technologies, Paisley, U.K.) and 1 mg/ml geneticin (Sigma Chemical Co.,

Abbreviations used: UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; E_2, β-oestradiol.

* To whom correspondence should be addressed.

Poole, Dorset, U.K.). The presence of geneticin was found to be necessary to maintain the expression of human bilirubin UGT by the transfected cells. The confluent cells were harvested after rinsing with ice-cold PBS (135 mM Na_2HPO_4/10 mM NaCl) at pH 7.4. Freshly harvested cells were disrupted by four 5s sonic bursts using a Soniprobe sonicator type 1130A (Dave Instrument Ltd., England), this was found to increase the bilirubin UGT activity approx. 2-fold. The resulting homogenate was divided into 3 mg protein amounts and frozen immediately at −70 °C and used for enzyme assays within 2 months.

Liver samples

Human liver samples were obtained with ethical permission from a normal subject and a Crigler–Najjar patient (B1) as described previously [17].

Protein estimation

Protein estimations were carried out essentially as described by the procedure of Lowry et al. [18] with BSA fraction V (Boehringer–Mannheim, U.K.) as standard.

Enzyme assays

Substrates, as well as other chemicals, were purchased from Sigma (Poole, Dorset, U.K.), Aldrich (Dorset, U.K.), BDH (Poole, Dorset, U.K.) or Fluka (Derbyshire, U.K.) and were the highest grade available.

For the quantification of total bilirubin conjugates and percentages of bilirubin mono- and di-conjugate formation two sets of assays were run in parallel. One set was used to estimate specific activity of bilirubin UGT as described by Heirwegh et al. [19] and the other set was analysed by h.p.l.c. to separate mono- and di-conjugates as described by Odell et al. [20] in order to calculate the percentage of mono- and di-conjugates.

Time-course studies of bilirubin conjugation were carried out using saturation concentrations of bilirubin (120 μM) and different co-substrates (10 mM) with 300 μg of protein. The reaction was stopped at 0, 5, 10, 15, 20 and 30 min. The effect of variable concentrations of UDP-sugar (0–20 mM) and bilirubin (10–200 μM) on the formation of mono- and di-conjugates were carried out as above with a 30 min incubation period. Untransfected cells were used in control experiments.

A universal t.l.c.-based assay system was used for substrate specificity studies as described elsewhere, using a 30 min incubation period [21,22]. For screening compounds as substrates for bilirubin UGT, final acceptor substrate and UDPGA concentrations of 500 μM and 2 mM were used respectively.

The K_m and $V_{max.}$ values with bilirubin, octylgallate and β-oestradiol were determined using the aforementioned assay methods using 10–1000 μM acceptor substrate and 0–40 mM UDPGA and 30 min incubation periods.

H.p.l.c. analysis of β-oestradiol glucuronide isomers

The regioselective glucuronidation of β-oestradiol at 3-OH and 17-OH positions was determined by h.p.l.c. analysis. Incubations containing 10 mg of protein, 500 μM β-oestradiol, 2 mM UDPGA, 30 mM $MgCl_2$, and 50 mM Tris/maleate (pH 7.4) in a total volume of 1.0 ml were conducted for 30 min at 37 °C. The reaction was stopped by the addition of 0.6 ml of acetonitrile and proteins were pelleted by centrifugation at 12000 *g* for 5 min. The acetonitrile of the supernatant phase was removed by vacuum centrifugation at 40 °C (Speedivac, Savant Ltd, U.K.) and the resultant aqueous phase loaded on a 100 mg C18 Bond-Elut solid-phase extraction column (Varian Harbor City, CA, U.S.A.) that had been previously washed with 1.0 ml of acetonitrile and 1.0 ml of 0.1 M ammonium acetate (pH 4.5). The column was then washed with 1.0 ml of 0.1 M ammonium acetate (pH 4.5) and the β-oestradiol and conjugates were eluted with 1.0 ml of acetonitrile. This acetonitrile fraction was evaporated to dryness by vacuum centrifugation at 40 °C and was reconstituted with 100 μl of h.p.l.c. mobile phase [15% (v/v) acetonitrile/85% (v/v) 0.1 M ammonium acetate, pH 4.5] This was subjected to h.p.l.c. analysis on a 5 μM pore diam. Spherisorb C8 column (250 mm × 4.6 mm; Phase Separations Ltd, Clwyd, U.K.) fitted to a Beckman System Gold system using the following conditions. The initial solvent conditions were acetonitrile/0.1 M ammonium acetate (pH 4.5) (3:17, v/v) followed by linear gradients to (9:11, v/v) over 30 min, then (13:7, v/v) over 3 min at which it was held for a further 5 min. Data were collected and analysed using Beckman System Gold software. Authentic standards of β-oestradiol, β-oestradiol-3-glucuronide and oestradiol-17β-glucuronide (purchased from Sigma) eluted at 37.5 min, 20 min and 21 min respectively and were detected at 280 nm. Incubations with β-glucuronidase were as described previously [13].

Ex vivo conjugation of bilirubin

For the *ex-vivo* study with the cell line expressing the human bilirubin transferase incubations were carried out adding stock 366 μM bilirubin/300 μM BSA to 30–40% confluent cells growing in tissue culture medium to a final bilirubin concentration of 5 μM. Samples (0.5 ml) of tissue-culture medium were taken after 0, 3, 6, 9 and 15 h and analysed by a diazotization procedure and h.p.l.c. analysis as described previously.

RESULTS AND DISCUSSION

Study of the kinetics of formation of bilirubin mono- and di-conjugates

The excretion of the toxic haem breakdown product bilirubin from the body is of paramount importance [2,9]. In human bile bilirubin glucuronides predominate; however, biliary bilirubin xylosides and glucosides have also been identified [23,24]. Whereas the UDP-sugar specificity of bilirubin UGT has been studied in purified rat liver preparations [25] because of the lability of the human enzyme and scarcity of tissue this has not been characterized in humans apart from in crude liver homogenates [10,26]. Studies by Motoyama [26] with 50 human liver samples demonstrated that there was a significant correlation ($r^2 = 0.974$) between bilirubin UGT and bilirubin xylosyltransferase activity, suggesting that a single enzyme was responsible for these activities. The recent stable expression of a cDNA clone encoding the major constitutive human bilirubin UGT in a tissue culture expression system in this laboratory [12] has enabled us to determine the UDP-sugar preferences of this important enzyme. Kinetic analysis of the conjugation of bilirubin performed by bilirubin UGT provided information about the rate of reaction and affinity characteristics of the enzyme for several UDP-sugars (Table 1). The K_m for bilirubin was similar for each of the UDP-sugars (Table 1). The relatively high K_m and low $V_{max.}$ obtained for UDP-glucose compared with the other two UDP-sugars tested indicated that it was the poorest substrate among the three UDP-sugars. The lower K_m for UDP-xylose (0.32 mM) compared with UDPGA (0.41 mM) indicated that UDP-xylose was the most effective UDP-sugar at low concentrations; however, the

Table 1. Kinetic parameters of human bilirubin UDPGT using different UDP-sugars

Values are the means of three separate experiments. Range of data obtained was $< \pm 6.0\%$.

UDP-sugar	$V_{max.}$ (nmol · min^{-1} · mg^{-1})	$K_{m(UDP\text{-}sugar)}$ (mM)	$K_{m(Bilirubin)}$ (μM)	$V_{max.}/K_{m(UDP\text{-}sugar)}$ (ml · min^{-1} · mg^{-1})	$V_{max.}/K_{m(Bilirubin)}$ (ml · min^{-1} · mg^{-1})
UDPGA	0.43	0.41	24	1.05	0.018
UDP-xylose	0.35	0.32	22	1.09	0.010
UDP-glucose	0.06	3.80	25	0.02	0.002

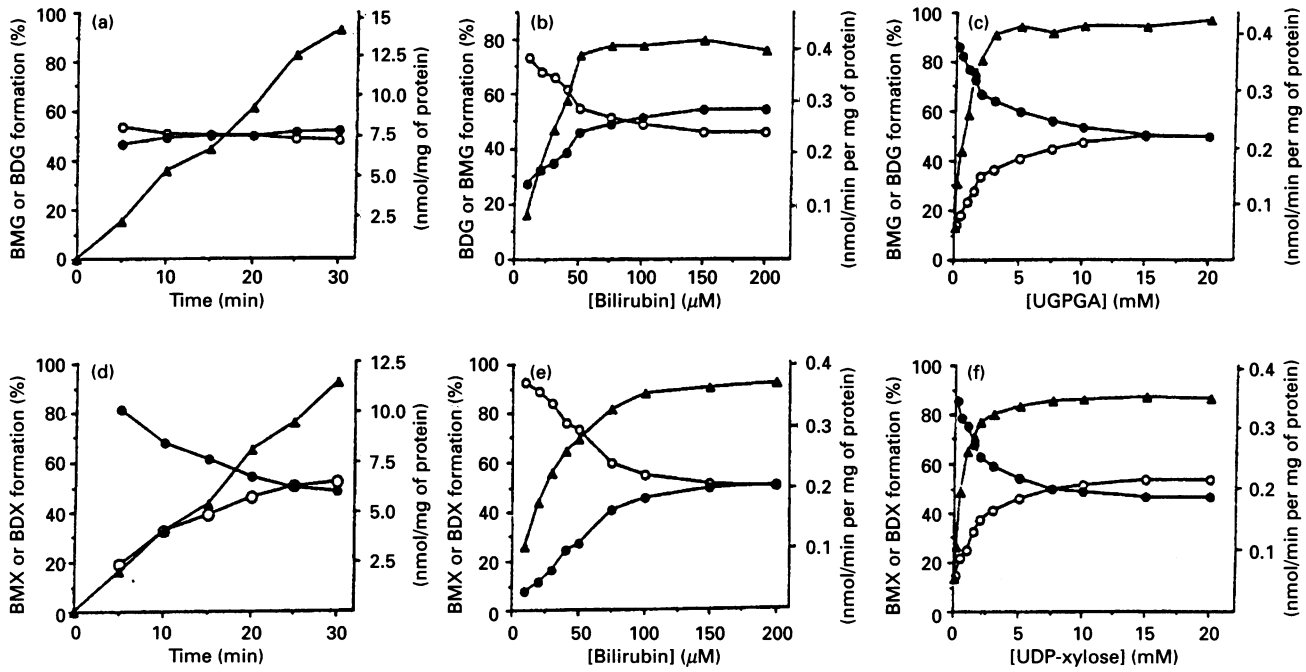


Figure 1 Formation of bilirubin mono- and di-conjugates by human bilirubin UDP-glucuronosyltransferase

The effect of incubation time (**a** and **d**), bilirubin concentration (**b** and **e**) and UDP-sugars concentration (**c** and **f**) on the formation of bilirubin mono- and di-conjugates. Closed and open circles represent mono- and di-conjugates respectively. Closed triangles represent total conjugate formation. Data obtained represent the average from three separate experiments. Range of data obtained was within $\pm 5.7\%$ of the average. Abbreviations: BMG, bilirubin monoglucuronides; BDG, bilirubin diglucuronides; BMX, bilirubin monoxylosides; BDX, bilirubin dixylosides.

$V_{max.}$ with UDPGA was $\sim 20\%$ higher compared with that with UDP-xylose at saturating UDP-sugar concentration (Table 1). The ratio of transfer rates of glucuronosyl:xylosyl:glucosyl with saturating amounts of substrates by the cloned expressed enzyme was 1:0.8:0.1 which is close to that found by Fevery et al. [10] for human liver homogenates (1:0.6:0.07), suggesting that this isoform is the major enzyme responsible for the biosynthesis of the different types of bilirubin conjugates found in bile. As there is a less than 2-fold difference in the catalytic potential of the enzyme to utilize UDPGA and UDP-xylose as sugar donors this suggests that as the levels of bilirubin glucuronides in bile are more than 20-fold greater than bilirubin xylosides [23,24] UDP-xylose must be present at very low levels in human liver in comparison with UDPGA levels.

The rate of formation of bilirubin glucuronides and xylosides enabled the accurate quantification of mono- and di-conjugates over a 30 min time course (Figures 1a and 1d). These results indicated that a single bilirubin UGT enzyme was able to catalyse the formation of both mono- and di-conjugates of bilirubin using UDPGA and UDP-xylose as co-substrates. The low catalytic rate measured for UDP-glucose prevented the quantification of mono- and di-glucosides. The conjugation of bilirubin in the presence of either UDPGA or UDP-xylose in the assay system was linear during the 30 min incubation time. The time-course experiments demonstrated that there were only slight differences in the proportion of bilirubin monogluronides and bilirubin diglucuronides (40–60% over the entire incubation period) formed during 30 min incubation, indicating that the hydrophilic monoglucuronidated bilirubin was rapidly conjugated by the enzyme (Figure 1a). In contrast, by following the time course of formation of bilirubin xylosides we found that monoxylosides were the predominant conjugate formed throughout the time course, indicating that they were only slowly converted into dixylosides by the enzyme (Figure 1d). This data suggests that the enzyme has different affinities for the two monoconjugated species.

Increasing the concentration of bilirubin or UDP-sugar utilized in the assay system changed the relative amounts of bilirubin mono- and di-conjugates formed with both UDPGA and UDP-xylose. At low concentrations of bilirubin (10–50 μM) the

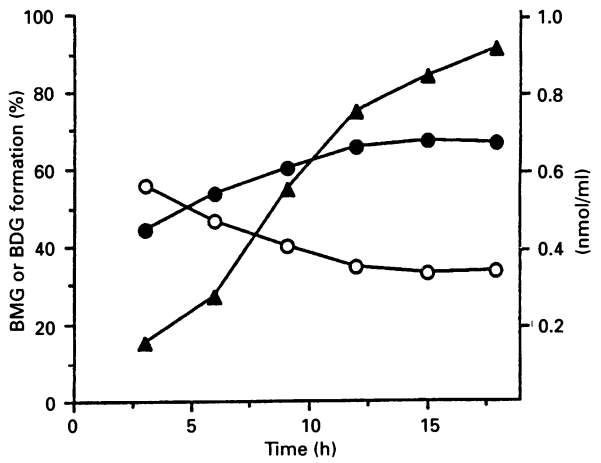


Figure 2 *Ex vivo* formation of bilirubin glucuronides by whole recombinant cell lines expressing bilirubin UDP-glucuronosyltransferase

Samples were analysed 3, 6, 9, 12, 15 and 18 h after addition of bilirubin as described in the Materials and methods section. Open and closed circles represent mono- and di-conjugates respectively. Closed triangles represent total conjugate formation. Data obtained represent the average from three separate experiments. Range of data obtained was within ± 9.3% of the average. Abbreviations: BMG, bilirubin monoglucuronides; BDG, bilirubin diglucuronides.

diconjugates were dominant. As the level of bilirubin increased from 10 μM to 75 μM, the proportion of diconjugate decreased with concomitant increase in monoconjugates formed and almost equal amounts of mono- and di-conjugate were formed when bilirubin concentration exceeded 75 μM (Figures 1b and 1e). Studies with different concentrations of UDP-sugars were also carried out to determine the effect on bilirubin conjugation. Low concentrations of UDP-sugar (UDPGA or UDP-xylose) resulted in the formation of higher relative amounts of monoconjugate in comparison with diconjugate. When the concentration of UDP-sugar exceeded 15 mM, however, approximately equal amounts of both conjugates were observed (Figures 1c and 1f).

The results obtained by varying either bilirubin or UDP-sugar may reveal the situation *in vivo*. The plasma level of bilirubin is always maintained at a very low concentration (< 17 μM) to avoid any hazardous effect of free bilirubin, such as kernicterus [9]. In this study we have shown that at physiological concentrations of bilirubin and UDPGA (0.5 mM) [1,9], the formation of bilirubin diglucuronides predominated, which reflects the *in vivo* situation where more than 80% of the bilirubin conjugates in bile are bilirubin diglucuronides [24].

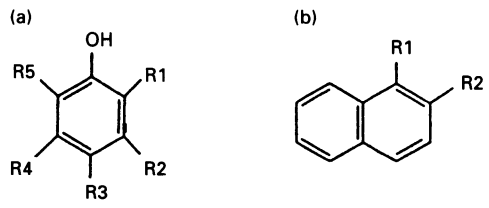
Ex vivo conjugation of bilirubin in fibroblast cells expressing bilirubin UGT

When bilirubin was added to the medium of fibroblast cells heterologously expressing the human bilirubin transferase, bilirubin glucuronides could be detected in the culture medium after 3 h and accumulated during the 18 h course of the experiment (Figure 2). H.p.l.c. analysis revealed the formation of both mono- and di-glucuronides (Figure 2). No xylosides or glucosides were detected indicating that the levels of UDP-xylose and UDP-glucose must be absent or very low in comparison with UDPGA levels, or that transport processes are not present in this cell line for their excretion.

This demonstrated that as well as the *in vitro* conjugation of bilirubin the genetically engineered cell line expressing the bilirubin UGT was capable of *ex vivo* uptake and metabolism of bilirubin and the transport of bilirubin glucuronides out of the cell (Figure 2), suggesting it may be a good model for studying the transport of bilirubin and its conjugates.

Table 2 Glucuronidation of different phenols by human bilirubin UGT

Values are the means of three separate experiments. Range of data obtained was < ± 6.0%. Acceptor substrate and UDPGA concentrations were 500 μM and 2 mM respectively.



Name	General structure	Substitutions R_1	R_2	R_3	R_4	R_5	Activity (nmol · min^{-1} · mg of protein)
Octylgallate	A	OH	H	$COOC_8H_{17}$	H	OH	1.90
Propylgallate	A	OH	H	$COOC_3H_7$	H	OH	0.72
4-Nitrophenol	A	H	H	NO_2	H	H	0.20
Eugenol	A	OCH_3	H	CH—CH=CH_2	H	H	0.17
Methyl-4-hydroxybenzoate	A	H	H	$COOCH_3$	H	H	0.13
Ethyl-4-hydroxybenzoate	A	H	H	$COOC_2H_5$	H	H	0.09
Propyl-4-hydroxybenzoate	A	H	H	$COOC_3H_7$	H	H	0.05
4-n-Ethylphenol	A	H	H	C_2H_5	H	H	0.04
4-n-Propylphenol	A	H	H	C_3H_7	H	H	0.02
4-tert-Butylphenol	A	H	H	$C(CH_3)_3$	H	H	0.01
α-Naphthol	B	OH	H				0.42
β-Naphthol	B	H	OH				0.18

Table 3 Compounds which were not glucuronidated by human bilirubin UGT

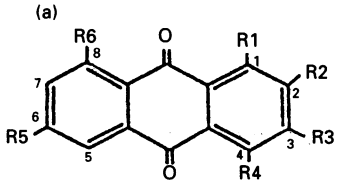
Drugs and other compounds of medicinal use	Drugs and other compounds of medicinal use	Endogenous compounds	Xenobiotic compounds
(−)-Morphine	Imipramine	3,5-Di-iodothyronine	(+)-Isomenthol
3-Azido-3-deoxythymidine	Ketoprofen	4-Hydroxyestrone	3-tert-Butyl-anisole
4-Hydroxytamoxifen	Labetalol	5-Hydroxytryptamine	4-Hydroxyacetylphenol
Acetylsalicylic acid	Larprofen	Aldosterone	4-Hydroxybiphenyl
Amitriptyline	Lorazepam	Androsterone	4-n-Amylphenol
Ampicillin	Mitoxantrone	Biliverdin	4-Phenylazophenol
Carbamazepine	Naloxone	Corticosterone	Borneol
Carvacrol	Naproxen	Dopamine	Carveol
Chloramphenicol	Novobiocin	Estrone	Catechin
Clofibrate	Oxazepam	Etiocholanolone	Citronellol
Clopamide	Phenobarbital	Hyodeoxycholic acid	Eosin
Cyclizine	Phenolphthalein	Retinoic acid	Ethcrynic acid
Cyproheptadine	Probenicid	Testosterone	Gallic acid
Dapsone	Propanolol		Menthol
Daunomycin	Propofol		Monoethyl-hexylphthalate
Diethylstilbestrol	Rifampicin		*N*-Acetyl-*p*-aminophenol
Digitoxigenin mono-digitoxide	Suprofen		*β*-Napthoflavone
Doxorubicin	Temazepam		Nopol
Fenoprofen	Tetracycline		Phenol Red
Fenoterol	Thymol		Toulic acid
Fluoroscein	Tripelennamine		
Frusemide	Tyrimide		
Ibuprofen			

Substrate and UDPGA concentrations used were 500 μM and 2 mM respectively.

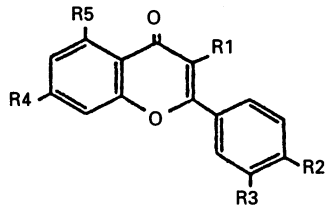
Table 4 Glucuronidation of substrates of mainly natural origin with anthraquinone and flavone structure by human bilirubin UGT

Values are the means of three separate experiments. Range of data obtained was < ± 6%.
Acceptor substrate and UDPGA concentrations used were 500 μM and 2 mM respectively.

(a)



(b)



Name	General structure	Substitutions R_1	R_2	R_3	R_4	R_5	R_6	Activity (nmol · min^{-1} · mg of protein^{-1})
Anthraquinone	A	H	H	H	H	H	H	0
Anthraflavic acid	A	H	OH	H	H	OH	H	1.72
Emodin	A	OH	H	OH	H	CH_3	OH	0.35
Alizarin	A	OH	OH	H	H	H	H	0.16
Alizarin-S	A	OH	OH	SO_3H	H	H	H	0
Chrysophanic acid	A	OH	H	H	H	CH_3	OH	0
1,8-Dihydroxyanthraquinone	A	OH	H	H	H	H	OH	0
Quinizarin	A	OH	H	H	OH	H	H	0
Anthrarufin	A	H	H	H	OH	H	OH	0
Quercetin	B	OH	OH	OH	OH	OH		0.57
Fisetin	B	OH	OH	OH	OH	H		0.57
Naringenin	B	H	OH	H	OH	OH		0.51
Galangin	B	OH	H	H	OH	OH		0.08
3-Hydroxyflavone	B	OH	H	H	H	H		0.37
7-Hydroxyflavone	B	H	H	H	OH	H		0.05

Investigation of the substrate specificity of human bilirubin UGT

In addition to the studies of UDP-sugar specificity we have used a series of 118 structural analogues (Tables 2–6) to gain the first insights into types of chemical structures that are potential substrates accepted by bilirubin UGT. A universal t.l.c. assay was employed to test the ability of this isoform to glucuronidate these structurally diverse compounds utilizing a final acceptor

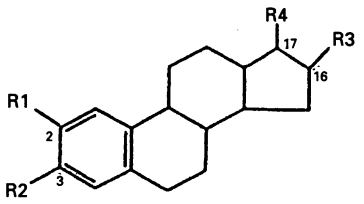
Table 5 Kinetic parameters of bilirubin UGT and phenol UGT1*02 isoforms towards octylgallate

Abbreviation: OGAL, octylgallate.

Isoenzyme	$V_{max.}$ (nmol · min^{-1} · mg^{-1})	$K_{m(UDPGA)}$ (mM)	$K_{m(OGAL)}$ (μM)	$V_{max.}/K_{m(UDPGA)}$ (ml · min^{-1} · mg^{-1})	$V_{max.}/K_{m(OGAL)}$ (ml · min^{-1} · mg^{-1})
Bilirubin UGT	2.15	1.81	162	1.19	0.013
Phenol UGT1*02	6.44	0.41	158	15.71	0.041

Table 6 Glucuronidation of oestrogens by human bilirubin UGT

Values are the means of three separate experiments. Range of data obtained was < ± 6%. Acceptor substrate and UDPGA concentrations used were 500 μM and 2 mM respectively.



	Substitutions						
	R_1	R_2	R_3		R_4		
			Orientation		Orientation		
Name			α	β	α	β	Activity (nmol · min^{-1} · mg of protein^{-1})
Oestriol	H	OH	OH	H	H	OH	0.11
16-Epioestriol	H	OH	H	OH	H	OH	0
17-Epioestriol	H	OH	OH	H	OH	H	0
β-Oestradiol	H	OH	H	H	H	OH	0.10
α-Oestradiol	H	OH	H	H	OH	OH	0
2-Hydroxyoestriol	OH	OH	OH	H	H	OH	0.21
Ethinyloestradiol	H	OH	H	H	—C≡CH	OH	0.33

substrate concentration of 500 μM for screening. A total of 27 compounds, including bilirubin, were found as substrates for the cloned human bilirubin transferase (Tables 2–6). Six compounds were glucuronidated at higher rates than bilirubin using the conditions employed.

(a) Xenobiotics as substrates of human bilirubin UGT

Thirteen xenobiotic phenols were glucuronidated by the enzyme (Table 2). Polyphenolic gallates, which are used as food additives due to their antioxidant properties [27], were the most rapidly conjugated of substrates by the enzyme being 2–5-fold higher than the rate with bilirubin when measured at substrate concentrations of 500 μM (Tables 1 and 2). Interestingly, increasing the carbon chain length in the ester side chain from three in propylgallate to eight in octylgallate resulted in enhanced glucuronidation. In contrast, increasing the carbon chain length or complexity at the *para* position to the phenolic hydroxy group in esters of hydroxybenzoic acid (methyl, ethyl or propyl) or alkyl-substituted phenols (ethyl, propyl, or tert-butyl) resulted in a reduction in the capacity of the enzyme to conjugate the phenolic hydroxy group (Table 2). The enzyme was also demonstrated to have a preference for the conjugation of the hydroxy group at carbon 1 of naphthols rather than carbon 2 which was conjugated at a 2-fold lower rate. Unlike other enzymes of the UGT1 family [22] the human bilirubin UGT was unable to catalyse the glucuronidation of phenols with alkyl substituents *ortho* to the phenolic hydroxy group, such as carvacrol or thymol, nor had it the ability to conjugate aliphatic alcohols (citronellol or nopol) (Table 3). Carboxylic acid drugs (fenoprofen, suprofen) or phthaleides (phenolphthalein, eosin), which are good substrates for several UGT2 enzymes [27,28], were not glucuronidated by bilirubin UGT either (Table 3). The 45 drugs and medicinal compounds listed in Table 3, many of which are extensively glucuronidated in humans [2], were not substrates for the enzyme using the conditions employed.

Comparison of the structures of the three anthraquinones anthraflavic acid, emodin and alizarin, which were substrates for the enzyme, with anthrarufin, 1,8-dihydroxyanthraquinone, chrysophanic acid and quinizarin, which were not substrates, gave an insight into the structure of anthraquinones accepted as substrates for the enzyme (Table 4). Hydroxy groups at positions 2, 3 and 6 of the anthraquinone structure appeared to be accessible to enzymic conjugation with glucuronic acid whereas

hydroxy groups closer to the quinoic carbonyl oxygens at positions 1, 4 or 8 appeared not to be accessible. Such a result might be explained by steric hinderance or an interaction of the hydroxy groups with the carbonyl groups. The reason for anthraflavic acid being more rapidly glucuronidated than emodin and alizarin might in part be due to the fact that it has two hydroxy groups in preferred positions (positions 2 and 6) for conjugation, thus it may be diconjugated.

Examination of the different flavones glucuronidated indicated that quercetin, fisetin and naringenin, which have hydroxy groups at the R2 position (Table 4), were conjugated at higher rates. The reason why 3-hydroxyflavone was glucuronidated at a 4-fold higher rate than galangin (3,5,7-dihydroxyflavone) is difficult to interpret, indicating the complexity of the structural and/or electronic configuration of flavones that govern their rates of glucuronidation. Obviously the use of more structural analogues may help solve such apparent anomalies.

As the highest glucuronidation rate by the transferase among the substrates tested was with octylgallate, which was also a good substrate for phenol UGT1*02 (formerly termed HP4) [22], this compound was used in a series of experiments to determine the kinetics of octylgallate glucuronidation and to indicate which enzyme might assume major responsibility for catalysis of glucuronidation of this substrate *in vivo*. The kinetic parameters of bilirubin and a bulky phenol UGT towards octylgallate were compared (Table 5). The catalytic potential ($V_{max.}/K_{m(octylgallate)} \times V_{max.}/K_{m(UDPGA)}$) of the bulky phenol-metabolizing isoform was approx. 40-fold higher compared with the bilirubin isoform. This suggests that the bilirubin isoform is unlikely to play an important role in the glucuronidation of octylgallate *in vivo*.

(b) Steroids and other endogenous compounds as substrates of human bilirubin UGT

Previously we have demonstrated that this human bilirubin UGT is the major enzyme responsible for the glucuronidation of the oral contraceptive drug ethinyloestradiol at the 3-OH position in human liver [13]. In this study we also assessed the ability of the enzyme to glucuronidate endogenous steroids (Table 6). The corticoids, corticosterone and aldosterone, and the androgen testosterone were not substrates for the enzyme (Table 3). Of the 17 steroids tested only four that had both 17β- and 3β-hydroxy substituents were glucuronidated (Tables 3 and 6). This suggests that hydroxy moities at these positions are necessary structural requirements for steroids to fit the active site of the enzyme. Besides hydroxy groups at C-3 and C-17, the stereochemistry of hydroxy groups at C-16 and C-17 in oestriols also affected glucuronidation (Table 6). The hydroxy group had to be in the β-position at C-17 and the α-position at C-16 for the oestrogen to be glucuronidated. To date although five human liver UGT isoforms have been demonstrated to glucuronidate oestrogens such as oestriols, oestrone, ethinyloestradiol and catechol oestrogens [13,28–33] none has the ability to conjugate β-oestradiol like the bilirubin UGT. The K_m and $V_{max.}$ of the enzyme for β-oestradiol were 75 μM and 0.11 nmol/min per mg respectively, indicating that it was a poorer substrate for the enzyme in comparison with bilirubin and octylgallate (Tables 1 and 5). Interestingly β-oestradiol may be glucuronidated at two positions by virtue of the fact that it possesses an hydroxy group at carbons 3 and 17 respectively [34]. In human urine β-oestradiol glucuronides account for 12–36 % of the total oestrogens excreted and the 3-glucuronide was the predominant conjugate [35]. Until now the regioselective glucuronidation of β-oestradiol by human liver enzyme preparations *in vitro* has not been studied, this

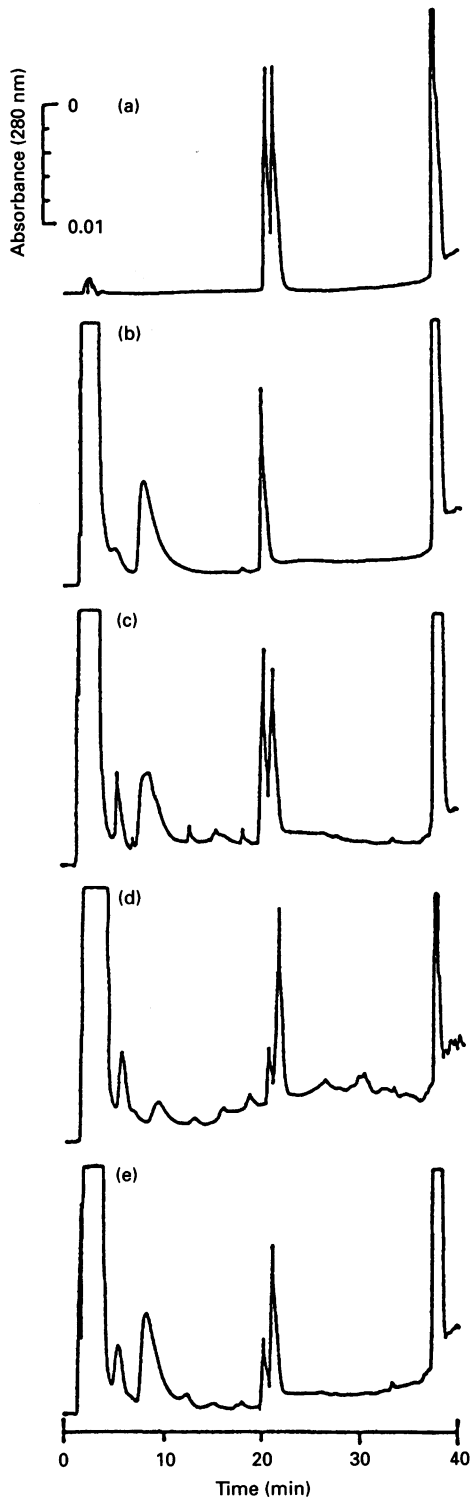


Figure 3 H.p.l.c. analysis of the regioselective glucuronidation of β-oestradiol by cloned human bilirubin UGT and human liver microsomes

Chromatogram (**a**) was generated from h.p.l.c. analysis of authentic standards of unconjugated β-oestradiol, β-oestradiol-3-glucuronide and oestradiol-17β-glucuronide. Chromatograms (**b**)–(**e**) were generated from incubating the following samples with β-oestradiol as described in the Materials and methods section. (**b**) V79 cells stably expressing human bilirubin UDP-glucuronosyltransferase; (**c**) normal human liver microsomes; (**d**) Crigler–Najjar liver microsomes; and (**e**) human liver microsomes including 200 μM bilirubin. Unconjugated β-oestradiol, β-oestradiol-3-glucuronide and oestradiol-17β-glucuronide were eluted at 37.5, 20 and 21 min respectively. The peaks eluting between 2 and 12 min were found in the corresponding blank incubations without β-oestradiol (data not shown).

therefore prompted us to study the process in more detail utilizing the cloned enzyme and human liver microsomes. H.p.l.c. analysis using a system that separated E_2 glucuronide isomers indicated that the glucuronide synthesized by incubating E_2 with the bilirubin UGT had a retention time identical with an authentic E_2-3-glucuronide standard (Figures 3a and 3b), representing conjugation at the phenolic hydroxy group of the A-ring as opposed to the aliphatic 17-hydroxy position on the D-ring. As expected from the *in vivo* studies human liver microsomes had the capacity to form both the 3- and 17-glucuronides, the E_2-3-glucuronide:E_2-17β-glucuronide ratio being 1.2 (Figure 3c). The authenticity of the glucuronides formed was further confirmed by β-glucuronidase treatment, which abolished the peaks (results not shown). The use of Crigler–Najjar liver microsomes, which are genetically devoid of bilirubin UGT activity [9], or the addition of bilirubin to an incubation with β-oestradiol and normal human liver microsomes, resulted in a decreased formation of the E_2-3-glucuronide such that the E_2-3-glucuronide:E_2-17β-glucuronide ratio was 0.30 and 0.45 respectively (Figures 3d and 3e). This suggested that bilirubin UGT was the enzyme responsible for the majority of the E_2-3-glucuronide formation in human liver. It is therefore interesting to speculate that bile ductular cholestasis which has been described in several Crigler–Najjar patients [9] might be due to the genetic deficiency of bilirubin UGT which results in the formation of mainly cholestatic E_2-17β-glucuronide (Figure 3d) rather than the non-cholestatic E_2-3-glucuronide as in normal humans [36].

In addition to steroids several other endogenous compounds such as neurotransmitters and bile acids, which are substrates for other UGT isoenzymes [2], were not substrates of the bilirubin transferase (Table 3).

Conclusions

We conclude that human bilirubin UGT is the major UGT isoform responsible for the glucuronidation, xylosidation and glucosidation of bilirubin in human liver. Previously we have shown that human bilirubin UGT is involved in thyroid hormone and ethinyloestradiol glucuronidation. In this report we also show that it is capable of the glucuronidation of endogenous oestrogens (β-oestradiol and oestriols) and a diverse variety of xenobiotic compounds (phenols, anthraquinones and flavones) of which many are found in our diet, e.g. eugenol (cloves), gallates (food preservatives), emodin (rhubarb) and naringenin (grapefruit), indicating the functional diversity of this enzyme. This suggests that these compounds have the potential to cause jaundice (hyperbilirubinaemia) by acting as competitive substrates with the major endogenous substrate bilirubin for the active site of the enzyme. Further study of structural analogues by kinetic analysis should lead to a better description of the substrate-binding site of this important enzyme.

This work was supported by grants from the Medical Research Council, the Wellcome Trust and the National University of Malaysia.

Received 9 February 1994/7 April 1994; accepted 13 April 1994

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