

UDP-glucuronosyltransferase Isoforms Catalyzing Glucuronidation of Hydroxy-Polychlorinated Biphenyls in Rat

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ABSTRACT

Polychlorinated biphenyls (PCBs) are highly toxic environmental contaminants that can cause irreversible damage in humans and wildlife. The mechanism of toxicity is not clear, but biotransformation products such as hydroxy PCBs (OH-PCBs) are a major concern. Efforts to elucidate the metabolism of PCBs and their metabolites, however, have paid little attention to the structure of the compound to be eliminated. The objectives of this study were to clarify organ tissue distribution of the glucuronidation activities toward OH-PCBs and to determine the UDP-glucuronosyltransferase (UGT) isoforms responsible for glucuronidation in relation to the OH-PCB structure. 2, 4, 6-Trichlorobiphenyl (PCB30) and 2, 3, 4, 5-tetrachlorobiphenyl (PCB61) were incubated in primary culture of rat hepatocytes, and the metabolites were identified by HPLC. Organ tissue glucuronidation activities toward ten OH-PCBs were investigated by reactions of microsomes prepared from brain, liver, small and large intestine, lung, kidney and testis tissues. To determine substrate specificity of the isoforms toward the OH-PCBs, rat UGT isoforms UGT1A1, UGT1A3, UGT1A5, UGT1A6, UGT1A7, UGT2B1, UGT2B3, and UGT2B12 were expressed in yeast strain AH22. Glucuronidation of the PCBs was found to be contingent on their hydroxylation. The organ tissues had strong glucuronidation activities toward the OH-PCBs tested; and most OH-PCBs were glucuronidated by UGT1A1, UGT1A6 and UGT2B1, all of which were substrate-specific. In conclusion, glucuronidation activities of UGT1A1, UGT1A6 and UGT2B1 toward OH-PCBs is relative to expression of the isoforms in each tissue, and glucuronidation intensity of the isoforms is relative to the structure of the OH-PCB to be glucuronidated.

Introduction

Polychlorinated biphenyls (PCBs) are highly toxic environmental contaminants that accumulate in lipid-rich body tissues of humans and wildlife through the food chain, exerting a detrimental impact on health. PCBs have adverse effects on neurodevelopment, such as delay in functions of the central nervous system (Jacobson et al., 1990) and lowering of IQs (Jacobson and Jacobson, 1997). *In vitro*, hydroxy-PCBs (OH-PCBs) interact with estrogen receptors, and this interaction can lead to either estrogenic or antiestrogenic activity (Connor et al., 1997; Andersson et al., 1999). In turtles, estrogenicity of OH-PCBs has been reported to induce reverse alteration of sex differentiation (Bergeron et al., 1994).

Although PCBs are ubiquitous in the environment, the mechanisms behind their adverse effects in the body are not fully understood. The toxicity may be linked partially to their biotransformation products, such as hydroxy-PCBs, as a result of hydroxylation by a diverse system of drug-metabolizing enzymes, i.e., the cytochrome P-450 monooxygenases (Matsusue et al., 1996; Letcher et al., 1999). Lans et al. (1994) have linked the neurodevelopment effects of the toxin to the binding of OH-PCBs to the thyroid hormone transport protein transthyretin. If the hydroxyl compound is *para* to the phenyl-phenyl bond and has adjacent chlorine atoms, the structure resembles that of the prohormone thyroxine (T₄) (Rickenbacher et al., 1986). This structural resemblance allows OH-PCB to bind with high affinity to the transthyretins (Lans et al., 1993). In rats, OH-PCB decreases circulating levels of thyroid hormones through competitive binding to the transthyretins, thus disrupting the transport of thyroid hormone and retinol (vitamin A) (Brouwer and

van den Berg, 1986). This is the presumed mechanism by which OH-PCBs are selectively retained in plasma and could conceivably be the mechanism behind PCB toxicity (Bergman et al., 1994) in the body. Not only can OH-PCB bind the protein to transport thyroxine but also to genes having a thyroxine binding site. Miyazaki et al. (2004), using reporter assay, reported that OH-PCB binds directly to the thyroid hormone responsive element, and this would disrupt expression of the downstream genes regulated by T3.

OH-PCB toxicity eludes easy evaluation in the animal body because the rate of OH-PCB metabolism differs according to the organ tissues, thus causing nonuniformity in overall OH-PCB concentrations in the body. Moreover, OH-PCBs themselves are composed of diversely structured isomers, which may affect metabolism of the compounds. In decades of research, however, such structural considerations have been largely neglected. To elucidate the mechanism governing adverse effects on the target tissues, it is expedient to clarify the metabolic system of the OH-PCBs before their arrival at target tissues.

The hydroxy metabolites of PCBs could be converted to either glucuronide conjugate or sulfate conjugate, mainly in the liver, and then excreted in the bile or urine. Glucuronide formation for lower chlorinated PCB congeners has been reported *in vivo* and *in vitro* (Norback et al., 1981; Schnellmann et al., 1984). In rat liver microsomes, glucuronidation efficiency (V_{\max}/K_m) seems to be relative to the substitution of chlorine atoms on the hydroxy-PCBs, as suggested in a study by Tampal et al. (2002). We hypothesized that the glucuronidation efficiency of each organ tissue toward the OH-PCBs depends on whether the structure of the given OH-PCB has affinity for UDP-glucuronosyltransferase (UGT) isoforms expressed in the tissue.

UGT is an intracellular membrane glycoprotein that catalyzes glucuronidation of bilirubin and steroids as well as xenobiotic compounds, including carcinogens (Dutton, 1980). UGTs have been classified into two major subfamilies, i.e., UGT1 and UGT2, on the basis of similarity in amino acid sequence (Burchell et al., 1991). In the UGT1 family, all mRNAs are produced from a single gene locus by individual promoters, followed by alternative splicing (Emi et al., 1995). The UGT1A transcripts contain individual amino-terminal domains followed by common carboxyl-terminal domains. Isoforms belonging to the UGT1 family primarily conjugate bilirubin or exogenous phenolic compounds (Bock et al., 1979; King et al., 2000). In contrast, UGT2 family members are encoded by individual genes characterized by six exons each (Mackenzie et al., 1997). The UGT2A subfamily consists of a single olfactory-specific isoform, and the UGT2B subfamily in the rat consists of six members: UGT2B1, UGT2B2, UGT2B3, UGT2B6, UGT2B8, and UGT2B12. Isoforms belonging to the UGT2 family conjugate steroids or bulky phenols (Bock et al., 1979; King et al., 2000; Turgeon et al., 2001).

The present study was conducted to determine the explicit UGT isoforms governing the elimination pathways of hydroxylated PCBs and to clarify the possible relationship between the OH-PCB structure and glucuronidation. To elucidate the metabolism, tissue distribution, accumulation and excretion of PCBs, we focused on organ-tissue distribution of the glucuronidation activities toward ten OH-PCBs and on the possible organ-tissue specificity of UGT isoforms responsible for the glucuronidation reactions.

Materials and Methods

Chemicals. Cholic acid was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), then purified further and converted to its sodium salt. UDP-glucuronic acid was obtained from Nakarai Pharmaceutical Co., Ltd. (Kyoto, Japan); polychlorinated biphenyls (PCBs) and hydroxyl polychlorinated biphenyls (OH-PCBs) from AccuStandard Inc. (New Haven, CT) (Table I), and high-performance liquid chromatography (HPLC)-grade acetonitrile from Labscan Co., Ltd. (Dublin, Ireland). HPLC-grade methanol, β -glucuronidase, and sulfatase (Type VIII) were obtained from Sigma-Aldrich (St. Louis, MO); and β -naphthoflavone was from Aldrich Chemical Co. Ltd. (Milwaukee, WI). All other reagents were of the highest grade available.

Animals. Male Sprague-Dawley rats, 9–13 weeks old (300–350 g), were used in all experiments. The rats were housed under standard conditions, given food and water *ad libitum*, and handled according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which are based on the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the United States.

Isolation of Hepatocytes. Each rat was anesthetized with 60% urethane (0.3 ml/100 g), then the portal vein was cannulated and the liver was perfused for 10 min with EGTA solution (136.89 mM NaCl, 5.37 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 5.56 mM glucose, 0.017 mM phenol red, 10 mM HEPES, 0.5 mM EGTA per liter). The liver was subsequently perfused an additional 10 min with collagenase solution (136.89 mM NaCl, 5.37 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.81 mM MgSO₄, 5 mM CaCl₂, 4.17 mM NaHCO₃, 5.56 mM glucose, 0.017 mM phenol red, 10 mM

HEPES, 0.5 g Type I collagenase per liter). All perfusions were done at a constant rate of 10 ml/min. Immediately after perfusion, the liver was removed and individually minced in Hank's buffer solution; and the resulting solution was filtered through stainless mesh to dissociate the hepatocytes. The hepatocytes were resuspended in Hank's buffer solution and isolated by slow-speed centrifugation (50 g for 3 min, 3 times). Cell viability was determined by trypan blue staining.

Incubation of Hepatocytes with PCBs and OH-PCBs. Six-well tissue culture plates (Asahi Techno Glass Co., Ltd., Chiba, Japan) were coated with Matrigel (BD Biosciences, Bedford, MA) and the wells were prepared with Williams' medium E (Gibco Co., Ltd, Carlsbad, CA) supplemented with final concentrations of 5% fetal bovine serum (Asahi Techno Glass Co., Ltd.), 1 μ M insulin, 1 μ M dexamethazone, and 2 μ M glutamine. Approximately 10^6 cells were seeded into each well and cultured for 4 h. The monolayers were rinsed once, the culture medium was replaced with insulin-free and dexamethazone-free Williams' medium E, and the cells were cultured in the medium for 1 day.

The cultured cells were then separated into four batches for incubation with PCB30, PCB61, OH-PCB30 or OH-PCB61 (0.05 mM each) in the medium at 37 °C (2 ml) aerated with 5% CO₂ and 95% air. Both the PCB30 batch and PCB61 batch were incubated for 12 h and 24 h, and both the OH-PCB30 batch and the OH-PCB61 batch for 24 h. When PCB30 and OH-PCB30 were added independently to the media, about half (60%) the total added PCB30 (100 nmol) but none of the OH-PCB30 was taken up by the culture plate. When PCB61 and OH-PCB61 were added independently to the media, about half (49%) the

total added PCB61 (100 nmol) and a small amount (27%) of the total added OH-PCB61 (100 nmol) were taken up by the culture plate. The amount of each PCB derivative absorbed by the hepatocytes was calculated by subtracting the amount of each PCB derivative taken up by the culture plate from the total amount of PCB added to the culture medium.

High Performance Liquid Chromatography. In preparation for HPLC assay for the PCB metabolites, the medium was removed from the culture dish, the monolayer was rinsed with saline, and the hepatocytes were harvested into phosphate buffer (8.1 mM, pH 7.4). PCBs and their metabolites were extracted from the hepatocytes with 50% acetonitrile in 0.05 M acetate buffer solution (pH 4.5). The culture medium (150 μ l) was mixed with acetonitrile (50 μ l) and 0.5 M acetate buffer (20 μ l, pH 4.5), and the mixture was boiled for 5 min. This mixture and the extracts from hepatocytes were centrifuged independently for 10 min at 9000 g in room temperature. The supernatants were filtered through a disposable disc filter (HLC-DISK3; Kanto Co., Ltd., Tokyo, Japan) and the filtered samples were injected into the HPLC system (UV8020, DP8020, SD8022, CO8020; Tosoh, Japan) equipped with Tosoh TSK-gel 80TS C18 reverse-phase columns (4.6 mm x 30 cm). The column temperature was maintained at 40 °C and the samples were eluted on a constant flow rate at 1 ml/min.

PCB30 metabolites were eluted in two steps: first, with linear gradient of 24–85% methanol and 0.06–0.012% acetate ammonium for 30 min, and finally with 85% methanol and 0.012% acetate ammonium for 20 min. PCB61 metabolites were eluted with linear gradient of 24–100% methanol and 0.06–0% acetate ammonium for 50 min. Detection of the eluted metabolites was made by measuring absorption at 222 nm. Hydroxy-PCBs were quantified by

standard compound. PCB-glucuronide and PCB-sulfate were quantified according to the amount of the deconjugated hydroxy-PCB remaining after treatment of the reaction products as explained below.

Metabolite Verification. After the reactions of microsomes, the resultant medium (100 μ l) was mixed with β -glucuronidase (2.5 mg/ml) in 0.5 M acetate buffer solution (20 μ l, pH 4.5) and incubated for 30 min at 37 °C (Shibata et al., 2002). A separate aliquot of the medium (100 μ l) was mixed with sulfatase (Type VIII, 4 mg/ml) in 0.5 M acetate buffer solution (50 μ l, pH 4.5) and incubated for 9 h at 37 °C. Acetonitrile was added to the respective reaction solutions (40 μ l for β -glucuronidase reaction, and 50 μ l for sulfatase reaction), and each mixture was boiled for 5 min. These mixtures were centrifuged for 10 min at 9000 g in room temperature. The supernatant was filtered through a disposable disk filter (HLC-DISK3; Kanto Co., Ltd. Tokyo, Japan) and analyzed by HPLC for reaction products. For OH-PCB30 reaction products, the elution buffer used in HPLC contained 24–85% linear gradient of methanol and 0.06–0.012% linear gradient of acetate ammonium. For OH-PCB61 reaction products, the elution buffer contained 24–100% linear gradient of methanol and 0.06–0% linear gradient of acetate ammonium.

Tissue Preparation for Isolation of Microsomes. The rats were anesthetized by intraperitoneal injection of 60% urethane and euthanized by exsanguination. The brain, liver, small and large intestines, and one lung, kidney and testis each were excised and individually minced and homogenized with 4 volumes of 1.15% KCl solution (mass/vol.) containing 10 mM EDTA (pH 7.4). The homogenate was centrifuged at 9000 g for 15 min at 4 °C, and the supernatant fraction was centrifuged at 105,000 g for 60 min at 4 °C to isolate

the microsomes. Protein concentration was determined by the method of Lowry et al. (1951) with BSA as the standard.

PCB30 and PCB61 Metabolism in Microsomes. Liver microsomes were obtained from rats pretreated with β -naphthoflavone (100 mg/kg, i.p., per day for 4 days). PCB30 (0.5 mM) and PCB61 (0.5 mM) were independently incubated at 37 °C for 1 h with the liver microsomes in 50 mM potassium phosphate buffer (200 μ l) harboring 0.5 mM NADPH or both 0.5 mM NADPH and 1.5 mM UDP-glucuronic acid. Acetonitrile (200 μ l) was added to the reaction solution, and the mixture was centrifuged for 10 min at 9000 g in room temperature. The supernatant was filtered with a disposable disc filter (HLC-DISK3; Kanto Co., Ltd.) and analyzed by HPLC as described above, using the elution buffer (50–80% linear gradient of acetonitrile in 0.1% acetic acid solution). After gradient elution (20 min), the PCB30 products were eluted by 80% acetonitrile in 0.1% acetic acid solution for 15 min, and the PCB61 products for 20 min. Hydroxy-PCBs produced by the reaction were verified by adding standard compound to the resultant products (data not shown), and the PCB-glucuronide was confirmed by adding PCB-glucuronide obtained from glucuronidation in the liver microsomal assay using hydroxy-PCB as substrate.

UGT Enzyme Analysis. The microsomes of each organ tissue were activated with a final concentration of 0.05% sodium cholate. UDP-glucuronosyltransferase (UGT) activity was determined by incubation (0.5–2 h) of the microsomes of each organ tissue at 37 °C in 0.1 M Tris/HCl buffer (200 μ l, pH 7.4) containing lysophosphatidylcholine (50 μ g), $MgCl_2$ (5 mM), hydroxy-PCB (0.5 mM each), and UDP-glucuronic acid (3 mM). Linearity of the enzyme reactions was confirmed during incubation (data not shown).

Reaction products were eluted by HPLC with acetonitrile solutions.

The 4-hydroxy-biphenyl products were eluted with the solution of 37% acetonitrile : 63% water : 0.1% acetic acid; HPCB3001N and HPCB3003N with the solution in a ratio of 50 : 50 : 0.1%; HPCB3006N, HPCB4001N, HPCB4004N, HPCB4008N and HPCB5007N with the solution in a ratio of 60 : 40 : 0.1%; and finally, HPCB4007N, HPCB5005N and HPCB6001N with the solution in a ratio of 68 : 32 : 0.1%.

Kinetic analysis. Kinetic parameters were determined according to the enzymatic activity against eight concentrations of hydroxy-PCB (0.006–0.5 mM) added to 200 μ l of 0.1 M Tris-HCl (pH7.4) containing 50 μ g lysophosphatidylcholine, and final concentrations of 5 mM MgCl₂, and 3 mM UDP-glucuronic acid. The reaction products were determined by HPLC analysis using acetonitrile elution buffer as described above. The apparent K_m values were determined by Lineweaver-Burk plot. Michaelis-Menten plots of the liver kinetics toward six representative hydroxy-PCBs are shown in supplemental data A, B.

UGT1A cDNA Preparation and Amplification. Total RNA was isolated from 0.2 g of each organ tissue preparation by applying TRIzol reagent (Gibco Co., Ltd., Carlsbad, CA), and single-strand cDNA was synthesized from the total RNA by Rever Tra Ace (Toyobo Co., Ltd., Osaka, Japan). The coding regions of the respective cDNA species were amplified by PCR oligonucleotide primers and Taq polymerase (Takara Co., Ltd., Japan). RT-PCR was used with exon-1 primers specific for each member of the rat UGT1A sequence and with one antisense primer specific for the common exon-2 sequence (Table II). UGT primers were designed to amplify different lengths of cDNA: UGT1A1, 945 base

pairs (bp); UGT1A2, 692 bp; UGT1A3, 681 bp; UGT1A5, 655 bp; UGT1A6, 794 bp; UGT1A7, 755 bp.

After preliminary incubation of the reaction mixture at 94 °C (2 min), amplification of the UGT1A first-exons was conducted in a 3-cycle protocol repeated 35 times: i.e., UGT1A1, UGT1A2, UGT1A3, UGT1A5, UGT1A6 and UGT1A7 were amplified, first, at 94 °C for 30 sec; second, at 55 °C for 30 sec; and third, at 72 °C for 1 min. Upon the 35th repetition, elongation followed at 72 °C for 1 min.

UGT2B cDNA Preparation and Amplification. UGT2B cDNA was prepared in a manner similar to that for the UGT1A cDNA. For recognizing a position within exon 1, RT-PCR was used with six exon-1-specific sense primers, each possessing a Spe I site (Table II). For recognizing a position within exon 5, RT-PCR was used with six antisense primers, each possessing a Spe I site (Table II). The primers allowed for annealing the beginning of exon 1 and the terminal of exon 5.

After the reaction mixture was incubated at 94 °C for 2 min, the UGT2B isoforms were amplified in their full lengths with KOD plus polymerase (Toyobo Co., Ltd. Osaka, Japan) in a three-cycle protocol repeated 35 times: i.e., UGT2B1 at 94 °C for 15 sec, at 50 °C for 30 sec, and at 68 °C for 90 sec; UGT2B2, UGT2B3, UGT2B4, UGT2B6, UGT2B8 at 94 °C for 15 sec, at 55 °C for 30 sec, and at 68 °C for 90 sec; UGT2B12 at 94 °C for 15 sec, at 52 °C for 30 sec, and at 68 °C for 90 sec. Upon the 35th repetition, elongation followed at 68 °C for 2 min.

Expression of UDP-Glucuronosyltransferase Isoforms. cDNAs of the rat UDP-glucuronosyltransferase isoforms UGT1A1, 1A3, 1A5, 1A6, 1A7,

2B1, 2B3, 2B12 obtained by RT-PCR were expressed in yeast AH22 cells according to slight modification of the method described previously (Yokota et al., 1999). Briefly, UGT2B3 was inserted into the yeast expression vector pGYR (Shinkyo et al., 2002) while the other seven isoforms were used with the vector pAAH5 (Yokota et al., 1999). Protein concentration was determined by the method of Lowry et al. (1951) with BSA as the standard. A UGT isoform expressed by yeast microsomes was used for glucuronidation of hydroxy-PCBs, and the resultant glucuronide conjugate was determined by HPLC using acetonitrile elution buffer as described above.

Immunoblot Analysis. Microsomes and other protein samples were subjected to SDS-PAGE through 10% acrylamide gel. The resulting polypeptide bands were transferred to nitrocellulose membranes, and polyclonal antibodies against the UGT isoforms (antibodies generously provided by Dr. S. Ikushiro, Toyama Prefectural University, Japan) were used for detecting the immunoreactive bands. Anti-UGT1A and anti-UGT2B1 antibodies can recognize the C-terminal region, i.e., G₅₁₆GKGRVKKSHKSKTH₅₂₉ and ₅₁₇CRKTANMGKKKKE₅₂₉ (Ikushiro et al., 1995; Ikushiro et al., 1997). For anti-UGT1A antibody, the staining intensity of each band was calculated by NIH imaging, and specific enzyme activities of the expressed UGT1A subfamily (UGT1A1, 1A3, 1A5, 1A6, 1A7) were corrected with UGT1A1 as standard.

Results

PCB30 and PCB61 Hydroxylation and Glucuronidation. For PCB30 and PCB61, HPLC chromatograms disclosed three main metabolites, i.e., 4-OH-PCB, glucuronide conjugate and sulfate conjugate (Fig. 1). PCB30 and PCB61 entered the hepatocytes within 12-h incubation, and large amounts of their metabolites were excreted into the respective culture media within 24-h incubation (Figs. 2 and 3). Of the PCB30 absorbed by the hepatocytes, 14.1% was excreted into the culture medium as glucuronide conjugate and 9.0% was excreted into the culture medium as sulfate conjugate (Fig. 2). When OH-PCB30 was added to the culture medium, the amount of excreted glucuronide conjugate increased dramatically to 38.1%, while the excreted sulfate conjugate showed a slight increase to 10.2% (Fig. 2). Of the PCB61 absorbed by the hepatocytes, 46.7% was excreted into the medium as glucuronide conjugate and 5.5% as sulfate conjugate (Fig. 3). When OH-PCB61 was added to the culture medium, the amounts of excreted glucuronide conjugate (46.6%) and sulfate conjugate (6.2%) were comparable to those found in the medium after addition of PCB61 (Fig. 3). For both PCB30 and PCB61, the glucuronide conjugate was the predominant metabolite, but OH-PCB was negligible in the hepatocytes as well as in the medium (Figs. 2 and 3). This suggests that glucuronidation of the PCBs was contingent on PCB oxidation.

Also in liver microsomes incubated in the presence of NADPH and UDP-glucuronic acid, glucuronidation was evident as a tandem reaction to oxidation. Upon incubation of either PCB30 or PCB61 with the liver microsomes in the presence of NADPH, 4-OH-PCB appeared in the incubation

buffer in 1 hour (Fig. 4). Upon addition of NADPH and UDP-glucuronic acid, the hydroxy-metabolite disappeared and the glucuronide conjugate appeared (Fig. 4), as in the primary cultured cells (Figs. 1, 2 and 3). This interchange between the dissipation of hydroxy-metabolite and the emergence of glucuronide conjugate indicate that the OH-PCB produced by cytochrome P-450 was conjugated immediately by UDP-glucuronosyltransferase.

UGT Activity toward Ten OH-PCBs. The liver, small and large intestines, lung, kidney, and testis differed markedly in their reactions toward the ten OH-PCBs tested and toward 4-hydroxy-biphenyl, as evidenced by a wide range of UGT activities including V_{\max} , K_m values, and intrinsic clearance (V_{\max}/K_m) for the enzyme reactions (Fig. 5). Liver microsomes had high levels of UGT activity toward the ten OH-PCBs and toward 4-hydroxy-biphenyl, and microsomes from the small and large intestines showed marked activity toward the 4-OH-PCBs having substitution of chlorine atoms on one or both sides of the hydroxy residue, i.e., HPCB5005N, HPCB5007N, and HPCB6001N (Fig. 5). The kidney and testis had low levels of UGT activity, but all ten OH-PCBs were nevertheless glucuronidated in spite of the low activity. 4-OH-PCB30 and 4-OH-PCB61, however, were glucuronidated solely by the liver microsomes (Fig. 5).

Substrate Specificities of UGT Isoforms. All the UGT isoforms except UGT2B8 were expressed in the liver, though in various levels of intensity, as shown by the amplified cDNAs (Fig. 6). UGT1A1 was expressed in all but the brain and lung, UGT1A6 was expressed in all the organ tissues tested, and UGT1A7 in all but the brain and kidney (Fig. 6). Except for UGT2B8 and UGT2B12, the isoforms belonging to the UGT2B subfamily were expressed

solely in the liver. UGT2B8 was not expressed in hepatic or in extrahepatic tissue. UGT2B12 was expressed not only in the liver but also in the kidney (Fig. 6). The isoforms expressed in brain, liver, lung, small and large intestines, kidney, and testis, i.e., UGT1A1, UGT1A3, UGT1A5, UGT1A6, UGT1A7, UGT2B1, UGT2B3 and UGT2B12, were also expressed in yeast AH22 cells, as shown by immunoblotting analysis (Fig. 7).

The various UGT isoforms displayed vastly different characteristics toward the ten OH-PCBs and toward 4-hydroxy-biphenyl, as evidenced by the intrinsic clearance (V_{\max}/K_m) calculated from the V_{\max} and K_m values (Fig. 8). UGT2B3 and UGT2B12, which are responsible for the glucuronidation of testosterone and 4-methylumbelliferone, respectively, showed no activity toward any of the OH-PCBs tested. UGT1A1 glucuronidated the OH-PCBs having substitutions of chlorine atoms on one or both sides of the hydroxy position (HPCB5005N, HPCB5007N, and HPCB6001N). UGT1A6 showed a preference for the OH-PCBs with substitution of hydroxy residues in the *ortho*- or *meta*- positions and expressed a high level of activity toward 2-hydroxy-2', 3', 4', 5'-tetrachlorobiphenyl (HPCB4001N). On the other hand, UGT2B1 had a high level of glucuronidation activity toward 4-hydroxy-PCBs having either no substitution or a single substitution of chlorine atoms on the sides of the hydroxy position (HPCB3006N and HPCB4008N) (Fig. 8).

Discussion

This study had five main findings. First, PCB glucuronidation was contingent on hydroxylation of the parent PCB (OH-PCB). Second, the liver as well as extrahepatic organ tissues had strong glucuronidation activities toward the ten OH-PCBs tested. Third, UGT1A1, UGT1A6 and UGT2B1 were the isoforms responsible for glucuronidation of most of the OH-PCBs. Fourth, each of the isoforms UGT1A1, UGT1A6 and UGT2B1 had unique substrate specificity to certain organ tissues. Finally, glucuronidation intensity of these three UGT isoforms was relative to the structure of each OH-PCB compound to be eliminated

PCB Hydroxylation, a Prerequisite to OH-PCB Glucuronidation.

Our study bears out that, in rats, elimination of environmental PCBs from the body depends on oxidation of the parent PCB, as intense glucuronidation is contingent on oxidation. 2, 4, 6-Trichlorobiphenyl (PCB30) and 2, 3, 4, 5-tetrachlorobiphenyl (PCB61) are metabolized to 4-hydroxy-PCB30 (4-OH-PCB30) and 4-hydroxy-PCB61 (4-OH-PCB61), respectively. Because these two OH-PCBs are especially potent in estrogenic activity (Carlson and Williams, 2001), which is known to cause irreversible damage in humans and wildlife, these compounds were selected as the focus of the present investigation. Both 4-OH-PCB30 and 4-OH-PCB61 are conjugated mainly by glucuronic acid and are also manifested as sulfate conjugate, as demonstrated by assay of our primary cultured hepatocytes. Furthermore, the glucuronide conjugate is formed in the presence of NADPH and UDP-glucuronic acid, as shown by the microsomal assays. Because of impending harm from the hydroxylated PCBs, intense glucuronidation is necessary but, paradoxically, the

hydroxylation itself is a prerequisite in the mechanism of their glucuronidation.

In spite of their indispensable role in glucuronidation, however, OH-PCBs inhibit sulfation, which is one of the important elimination pathways. In rats, Schuur et al. (1998a; 1998b) showed that OH-PCBs inhibit a sulfotransferase isoform responsible for sulfation toward thyroxine. In renal excretion of the estrogenic steroid 17β -estradiol, human estrogen sulfotransferase (hEST), a metabolic enzyme expressed in cytosol, catalyzes sulfation of the steroid (Coughtrie et al., 1998). Curiously, however, certain OH-PCBs can inhibit the activity of hEST (Kester et al., 2000), even though the IC_{50} values of many OH-PCBs are approximately 40–to–50-fold lower than the K_m value of 17β -estradiol sulfotransferase (Kester et al., 1999). This phenomenon may be explained by the crystal structure of hEST containing OH-PCB, which is a structure allowing OH-PCBs to bind to the enzyme (Shevtsov et al., 2003).

OH-PCB can bind not only to the metabolic enzyme hEST but also to genes having a thyroxine binding site. Miyazaki et al. (2004), in a study using reporter assay, reported that OH-PCBs bind directly to the thyroid hormone responsive element. In our study, however, the OH-PCBs were glucuronidated in the organ tissues, thus suggesting it is strongly unlikely that the glucuronide conjugate would bind to the thyroid hormone responsive element, because the PCB structure would change. In support of this view, we found that, in primary cultured cells of rat liver, PCBs are metabolized mainly to the glucuronide conjugate. This result is in line with work documenting the fate of glucuronide conjugate *in vivo* (Anderson et al., 1977; Norback et al., 1981). In rat *in vivo*, the glucuronide conjugate of PCBs is excreted in the urine and feces and is the

main metabolite of PCBs (Anderson et al., 1977), and in rhesus monkey the glucuronide conjugate is eliminated in biliary excretion (Norback et al., 1981).

The lipophilic nature of PCBs enables their intracellular invasion into the endoplasmic reticulum. Accordingly, in the present study UGTs, which are expressed in the endoplasmic reticulum, immediately converted the OH-PCBs to glucuronic acid conjugate, as shown by OH-PCB reaction with liver microsomes in the presence of NADPH and UDP-glucuronic acid. In our primary cultured hepatocytes as well, conjugation of the OH-PCBs with glucuronic acid was contingent on PCB hydroxylation. These results support the findings by Oberg et al. (2002) that in rats given a single oral dose of PCBs, the PCBs accumulate in large amounts in the liver immediately after uptake through the gastrointestinal tract and then recede drastically. Together with a growing body of research, our study has clarified that elimination of PCBs from the body depends on their hydroxylation and, in turn, on glucuronidation of the OH-PCB.

Organ Tissues and UGT Isoforms Associated with OH-PCB

Glucuronidation. In conjunction with the literature (Anderson et al., 1977; Norback et al., 1981; Oberg et al., 2002), our results indicate that hepatic as well as extrahepatic organ tissues can glucuronidate OH-PCB and that, as we suspected, the UGT isoforms are the isoforms largely responsible for the OH-PCB glucuronidation. UGT1A1, UGT1A6 and UGT2B1 are the isoforms predominantly governing OH-PCB glucuronidation, as disclosed by our tests on ten OH-PCBs.

OH-PCB Glucuronidation Relative to Organ Tissue Specificity.

Each of the isoforms UGT1A1, UGT1A6 and UGT2B1 has specificity toward certain organ tissues in the body, as demonstrated by this study. Depending on

the organ tissue and the compound to be glucuronidated, generally UGT2B1 has the strongest glucuronidation activity, followed by UGT1A6; but UGT1A1 is able to compensate for its low activity because of the wide range of specificity for the various tissues.

Organ-tissue specificity of UGT1A1

In this study, UGT1A1 could glucuronidate 4-hydroxy-2', 3, 3', 4', 5'-pentachlorobiphenyl (HPCB5005N), 4-hydroxy-2', 3, 4', 5, 6'-pentachlorobiphenyl (HPCB5007N), and 4-hydroxy-2', 3, 3', 4', 5, 5'-heptachlorobiphenyl (HPCB6001N). Coincidentally, these three compounds (HPCB5005N, HPCB5007N and HPCB6001N) are all characterized by substitution of chlorine atoms on one or both sides of the 4-hydroxy position (see Table I). UGT1A1 activities toward these compounds are comparable in the rat liver, small and large intestines, kidney and testis, but different in the lung, as borne out by the V_{\max} values in this study. V_{\max} values toward HPCB5005N, HPCB5007N and HPCB6001N in lung tissue, which does not express the isoform UGT1A1, were lower than the V_{\max} values in liver, intestines, kidney and testis, where UGT1A1 is expressed. Similarly, K_m values toward HPCB5007N and HPCB6001N were the same for liver, intestines, kidney and testis but different for the lung. The V_{\max} and K_m values toward HPCB5005N, HPCB5007N and HPCB6001N in liver, intestines, kidney and testis indicate that glucuronidation of these three compounds is mediated by the isoform UGT1A1 in the liver, intestine, kidney and testis but not in the lung.

Organ-tissue specificity of UGT1A6

With the sole exception of the brain tissue, all other organ tissues in our study (liver, small and large intestines, lung, kidney and testis) showed UGT activity toward the 4-hydroxy compounds HPCB3001N, HPCB3003N and HPCB4001N. The substrate specificities of liver, small and large intestines, kidney and testis toward HPCB3001N, HPCB3003N and HPCB4001N are consistent with the tissue specificity of expressions of UGT1A6, the sole UGT isoform found to be expressed in every one of the organ tissues tested. UGT1A6, however, shows a preference for OH-PCBs having substitutions of hydroxy residues in either the *ortho*- or *meta*- position, such as 2-hydroxy-2', 3', 4', 5'-tetrachlorobiphenyl (HPCB4001N). Notably, UGT activity toward HPCB4001N was detectable in microsomes from the brain (data not shown), whereas the K_m values for the reaction with HPCB4001N were the same in all organ tissues other than the brain (i.e., liver, small and large intestines, lung, kidney and testis). Plausibly, then, HPCB4001N glucuronidation can be mediated by UGT1A6 in all the rat organ tissues tested in this study.

Organ-tissue specificity of UGT2B1

Of the UGT2B family of isoforms, which are generally associated with glucuronidating bulky phenols such as 4-hydroxy-biphenyl (Bock et al., 1979), UGT2B1 was able to glucuronidate all the 4-OH-PCBs tested in our study except for HPCB6001N. Liver tissue demonstrated intense glucuronidation activity toward 4-OH-PCB30 (HPCB3006N), 4-hydroxy-2', 3, 4', 6'-tetrachlorobiphenyl (HPCB4008N) and 4-hydroxy-biphenyl; and curiously, all these compounds possess either a single substitution or no substitution of chlorine atoms on the sides of the 4-hydroxyl residue. Moreover, the hepatic activity toward these

three compounds (HPCB3006N, HPCB4008N and 4-hydroxy-biphenyl) is consistent with that demonstrated by UGT2B1. In the liver, the K_m values toward HPCB3006N and HPCB4008N were the same as the value for UGT2B1, indicating that glucuronidation toward HPCB3006N and HPCB4008N is mediated by UGT2B1 expressed in the liver. In extrahepatic tissues expressing UGT1A1, however, glucuronidation toward HPCB4008N would be mediated by UGT1A1, given that UGT1A1 is able to glucuronidate a compound if the compound possesses the substitution of chlorine atoms on the sides of the hydroxy position, as in the case of HPCB4008N. Conversely, it is plausible that the reason HPCB3006N eludes glucuronidation by extrahepatic tissues is that HPCB3006N lacks the substitution of chlorine atoms on the sides of the hydroxy position, thus precluding glucuronidation by UGT1A1.

On the other hand, the UGT2B1 glucuronidation activity toward HPCB5007N is weak. In spite of sharing a structure similar to that of HPCB3006N (4-OH-PCB30) and HPCB4008N, the compound HPCB5007N substitutes chlorine atoms on both sides of the 4-hydroxy residues. The weak UGT2B1 glucuronidation activity toward HPCB5007N, therefore, would indicate that the UGT2B1 activity site is obstructed by the chlorine atoms on dual sides of the 4-hydroxy residues.

While UGT2B1 has intense glucuronidation activity toward the nonplanar PCBs such as HPCB3006N (4-OH-PCB30) and HPCB4008N, the activity of the isoform is weak toward the planar OH-PCBs such as HPCB4007N and HPCB5005N, having a single chlorine atom at the 2' position. Thus, UGT2B1 is rendered incapable of accommodating a planar compound.

Role of the OH-PCB Structure.

This study has borne out that while glucuronidation activity toward OH-PCB in the respective organ tissues depends on the intensity of the tissue-specific expressions of UGT1A1, UGT1A6 and UGT2B1, the glucuronidation by these isoforms is either enhanced or precluded according to the structure of the compound to be eliminated.

Sandau et al. (2000), studying xenobiotic PCBs in organ tissues from humans, reported that 20% of total PCB in human blood consists of hydroxy metabolites, predominantly 4-OH-PCB109 (2, 3, 3', 4', 5 -*penta* CB), 4-OH-PCB146 (2, 2', 3, 4', 5, 5' -*hexa* CB) and 4-OH-PCB187 (2, 2', 3, 4', 5, 5', 6 -*hepta* CB). Of these compounds, Tampal et al. (2002) have shown that, in rat liver microsomes, 4-OH-PCB146 and 4-OH-PCB187 are glucuronidated to a less extent than other OH-PCBs. In the light of our present results, we surmise that the poor glucuronidation of 4-OH-PCB146 and 4-OH-PCB187 reported by Tampal et al. (2002) could be attributed to the structure shared by these two OH-PCBs. Given that glucuronidation intensity of the UGT isoforms in our study was relative to the structure of the compound to be glucuronidated, it is plausible that the OH-PCB structure impedes or precludes UGT2B1 glucuronidation activity toward those two OH-PCBs in spite of the intense glucuronidation activity generally exhibited by UGT2B1 in the liver.

We strongly suspect that the reason the three major hydroxy metabolites accumulating in human blood (4-OH-PCB109, 4-OH-PCB146 and 4-OH-PCB187) elude glucuronidation by UGT2B1 in the liver microsomes is that whereas these three OH-PCBs have substitutions of chlorine atoms on both sides of the 4-hydroxyl residue, the UGT2B1 preference is allied with

compounds having either no substitution or a single substitution of a chlorine atom on the sides of the hydroxy position, as indicated by our present results. Because UGT1A1 favors 4-hydroxy-PCBs having substitutions of chlorine atoms on one or both sides of the hydroxy position, 4-OH-PCB109, 4-OH-PCB146 and 4-OH-PCB187 would likely be glucuronidated by UGT1A1 expressed in liver, small and large intestines, lung, kidney and testis, in spite of the weak glucuronidation activity associated with UGT1A1.

Although most OH-PCBs in this study were highly vulnerable to the intense glucuronidation activities of UGT1A1, UGT1A6 and UGT2B1, the compound HPCB4007N remained persistent and was only marginally glucuronidated by either UGT1A1 or UGT2B1. Given that the liver microsomes alone had robust glucuronidation activity toward HPCB4007N, on conjecture we propose that UGT isoform(s) other than those used in our study may be at work toward glucuronidating the toxic planar OH-PCB HPCB4007N in the liver. Further studies are warranted.

A limitation of the present work was that a few of the other known UGT isoforms were not expressed in our yeast AH22 cells, leaving that fraction of the total gamut of UGT isoforms remaining to be tested for tissue specificity and glucuronidation activity in relation to the OH-PCB structures.

Conclusion.

Results of this study support our hypothesis that in rat organ tissues the glucuronidation efficiency toward OH-PCB is dependent on whether the UGT isoforms expressed in each given tissue show a preference for the structure of the target OH-PCB. Providing new insight into the mechanism behind PCB

toxicity in the body, this study has established (i) that hydroxylation of the parent PCB is necessary to facilitate glucuronidation, (ii) that in each organ tissue the glucuronidation activity toward the OH-PCB depends on the organ-specific expression patterns of UGT1A1, UGT1A6, and UGT2B1 and (iii) that the glucuronidation intensity of these three isoforms is relative to the structure of the compound to be glucuronidated.

Although it remains to be clarified why the human body cannot stave off 4-OH-PCB109, 4-OH PCB146 and 4-OH PCB187 from their tendency to accumulate in the blood, a conceivable reason suggested by this study, we believe, is that the glucuronidation activity of UGT1A1 is of low intensity, not adequate enough to eliminate these persistent compounds in the organ tissues. However, all three of the compounds (4-OH-PCB109, 4-OH PCB146 and 4-OH PCB187) possess a structure characterized by substitution of chlorine atoms on both sides of the 4-hydroxy position, which is the structure favored by UGT1A1 in our experiments. In other OH-PCBs sharing this structure (e.g., HPCB5007N and HPCB6001N), the structure confers increased glucuronidation activity on the organ-tissues expressing UGT1A1. Given that OH-PCBs with this structure are vulnerable to glucuronidation by UGT1A1, as borne out by this study, we propose that if the expression level of UGT1A1 could somehow be elevated by pharmaceuticals, nutraceuticals or other technique, then the increased expression level would facilitate glucuronidation and the harmful OH-PCBs such as 4-OH-PCB109, 4-OH-PCB146 and 4-OH-PCB187 would be measurably alleviated from the body.

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Footnotes

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Legends for Figures

Fig. 1. HPLC chromatograms of PCB 30 (panel A, B, C) and PCB61 (panel D, E, F) metabolites in primary cultures of rat hepatocytes. The primary cultures were independently incubated with 0.05 mM 2, 4, 6-trichlorobiphenyl (PCB30) and 0.05 mM 2, 3, 4, 5-tetrachlorobiphenyl (PCB61) for 24 h. PCB metabolites excreted into the culture medium (A, D); deconjugated metabolites treated with sulfatase (B, E); deconjugated metabolites treated with β -glucuronidase (C, F). PCB-glucuronide (PCB30 peak a, 28.6 min; PCB61 peak a, 40.0 min). PCB-sulfate (PCB30 peak b, 30.2 min; PCB61 peak b, 40.7 min). 4-hydroxy-PCB (PCB30 peak c, 37.0 min; PCB61 peak b, 49.9 min). Parent PCB (PCB30 peak d, 47.1 min; PCB61 peak d, 54.2 min).

Fig. 2. PCB30 and metabolites in the culture medium (left) and hepatocytes (right) after exposure to 50 μ M PCB30 (A, B, C, D) or 4-hydroxy-PCB30 (E, F). Exposure was 12 h (A, B) or 24 h (C, D, E, F). Data are shown as relative amounts (%) of the PCB absorbed by hepatocytes. OH-PCB30: 4-Hydroxy-PCB30; PCB30-GA: PCB30-glucuronide; PCB30-S: PCB30-sulfate.

Fig. 3. PCB61 and metabolites in the culture medium (left) and hepatocytes (right) after exposure to 50 μ M PCB61 (A, B, C, D) or 4-hydroxy-PCB61 (E, F). Exposure was 12 h (A, B) or 24 h (C, D, E, F). Data are shown as relative amounts (%) of the PCB absorbed by hepatocytes. OH-PCB61: 4-Hydroxy-PCB61; PCB61-GA: PCB61-glucuronide; PCB61-S: PCB61-sulfate.

Fig. 4. HPLC chromatograms of PCB30 metabolites (panel A) and PCB 61

metabolites (panel B) in rat liver microsomes. Reaction was done with NADPH and UDP-glucuronic acid (chromatogram A), with NADPH (chromatogram B) or without either NADPH or UDP-glucuronic acid (chromatogram C). Arrows indicate glucuronidated PCB (peak a, 6.3 min on panel A; 9.1 min on panel B), 4-hydroxy-PCB (peak b, 19.5 min on panel A; 23.6 min on panel B) and parent PCB (peak c, 28.6 min on panel A; 35.7 min on panel B).

Fig. 5. Glucuronidation toward ten hydroxy-PCBs and 4-hydroxy-biphenyl in rat microsomes from the liver, small and large intestines, lung, kidney, and testis. Data are presented as mean \pm SE. K_m values are indicated in upper parentheses, and V_{max}/K_m values in italicized brackets below. 4-HB: 4-Hydroxy-biphenyl.

Fig. 6. Expression of UDP-glucuronosyltransferase isoforms in rat brain (A), liver (B), lung (C), large intestine (D), kidney (E) and testis (F). RT-PCR was conducted with specific primers as described in Materials and Methods. The expected PCR products are illustrated in Fig. 1. M: Marker; bp: base pairs.

Fig. 7. Western blot analysis of UDP-glucuronosyltransferase isoforms expressed on AH22 yeast cells. Immunoreaction was performed using antibodies against the common sequence of UGT1A family (panel A) and against UGT2B1 (panel B). Rat liver microsome was used for positive control (lane Ms).

Fig. 8. Glucuronidation toward ten hydroxy-PCBs and 4-hydroxy-biphenyl in

the microsomes of AH22 yeast cells expressing UDP-glucuronosyltransferase.

Data are presented as mean \pm SE. K_m values are indicated in upper parentheses, and V_{max}/K_m values in italicized brackets below. 4-HB:
4-Hydroxy-biphenyl.

Supplemental data. Michaelis-Menten plots of liver kinetics toward six representative hydroxy-PCBs. The kinetics of liver UGT toward HPCB3006N (A), HPCB4008N (B), HPCB4007N (C), HPCB5007N (D), HPCB4001N (E), and HPCB6001N (F) are shown.

TABLE I

The polychlorinated biphenyls used in this study

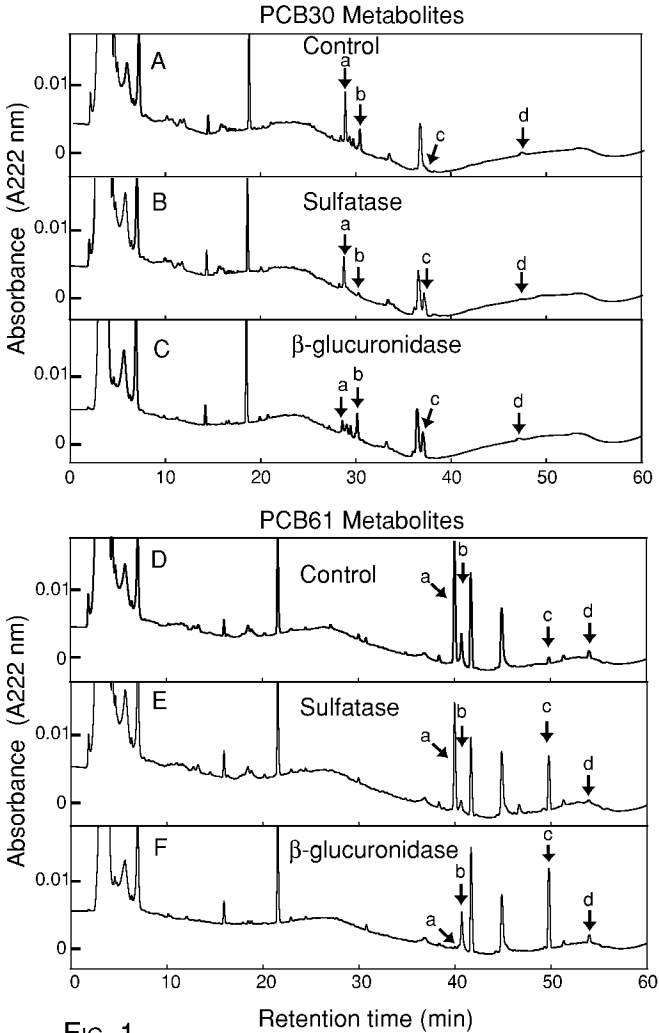
IUPAC name	Common name	Structure
2, 4, 6-trichlorobiphenyl	PCB30	
2, 3, 4, 5-tetrachlorobiphenyl	PCB61	
4-Hydroxy-2', 4', 6'-trichlorobiphenyl	HPCB3006N (OH-PCB30)	
2-Hydroxy-2', 4', 6'-trichlorobiphenyl	HPCB3001N	
3-Hydroxy-2', 4', 6'-trichlorobiphenyl	HPCB3003N	
4-Hydroxy-2', 3, 4', 6'-tetrachlorobiphenyl	HPCB4008N	
4-Hydroxy-2', 3, 4', 5, 6'-pentachlorobiphenyl	HPCB5007N	
4-Hydroxy-2', 3', 4', 5'-tetrachlorobiphenyl	HPCB4007N (OH-PCB61)	
2-Hydroxy-2', 3', 4', 5'-tetrachlorobiphenyl	HPCB4001N	
3-Hydroxy-2', 3', 4', 5'-tetrachlorobiphenyl	HPCB4004N	
4-Hydroxy-2', 3, 3', 4', 5'-pentachlorobiphenyl	HPCB5005N	
4-Hydroxy-2', 3, 3', 4', 5, 5'-heptachlorobiphenyl	HPCB6001N	
4-Hydroxy-biphenyl	4-HB	

TABLE II

Sequence of PCR primers for amplification of rat UDP-glucuronosyltransferase isoforms

Individual sense (F) primers and antisense (R) primers were used for amplification of UGT1A family (upper Table) and UGT2B family (lower Table).

UGT1A Primers	Sequence
UGT1A1-F	5'-TGG TGT GCC GGA GCT CAT GTT CG-3'
UGT1A2-F	5'-GGA AGA ATA TCA GCG GGA AAT ACT GGG C-3'
UGT1A3-F	5'-GGC ACC ACT TGC TGG GCC ACC TTC-3'
UGT1A5-F	5'-GTG GTC TTT GAA ACA GGC AAC TAT GTG-3'
UGT1A6-F	5'-CCT CAG TGA ACG CGG ACA CGA C-3'
UGT1A7-F	5'-CAG TTG GCA GCT GGG AAA ACC A-3'
Common-R	5'- CTG GAA TCT CTG AGA CCA TGG ATC-3'
UGT2B Primers	Sequence
UGT2B1-F	5'-CGC ACT AGT ATG TCT ATG AAA CAG ACT TCA-3'
UGT2B1-R	5'-CGC ACT AGT CTA CTC TTT CTT CTT CTT-3'
UGT2B2-F	5'-CGG ACT AGT ATG CCC AGA AAG TGG ATT TC-3'
UGT2B2-R	5'-CGG ACT AGT CTA CTC ATT CTT CAT TTT CT-3'
UGT2B3-F	5'-CGG ACT AGT ATG CCT GGG AAG TGG ATT TC-3'
UGT2B3-R	5'-CGG ACT AGT CTA CTC ATT CTT CAT TTT C-3'
UGT2B6-F	5'-CGG ACT AGT ATG CCA GGA AAA TGG ATT TTT GC-3'
UGT2B6-R	5'-CGG ACT AGT CTA CTC ATT CTT CAT TTT C-3'
UGT2B8-F	5'-TGA TCA CTA GTA TGC CTC AGA AGT GGA TTT-3'
UGT2B8-R	5'-CTT AAA CTA GTC TAG TCC CTT TTA TTC TTT-3'
UGT2B12-F	5'-TGC ACT AGT ATG TCT GGG AAG TG-3'
UGT2B12-R	5'-CAT ACT AGT CTA CTC ATT CTT TGT-3'



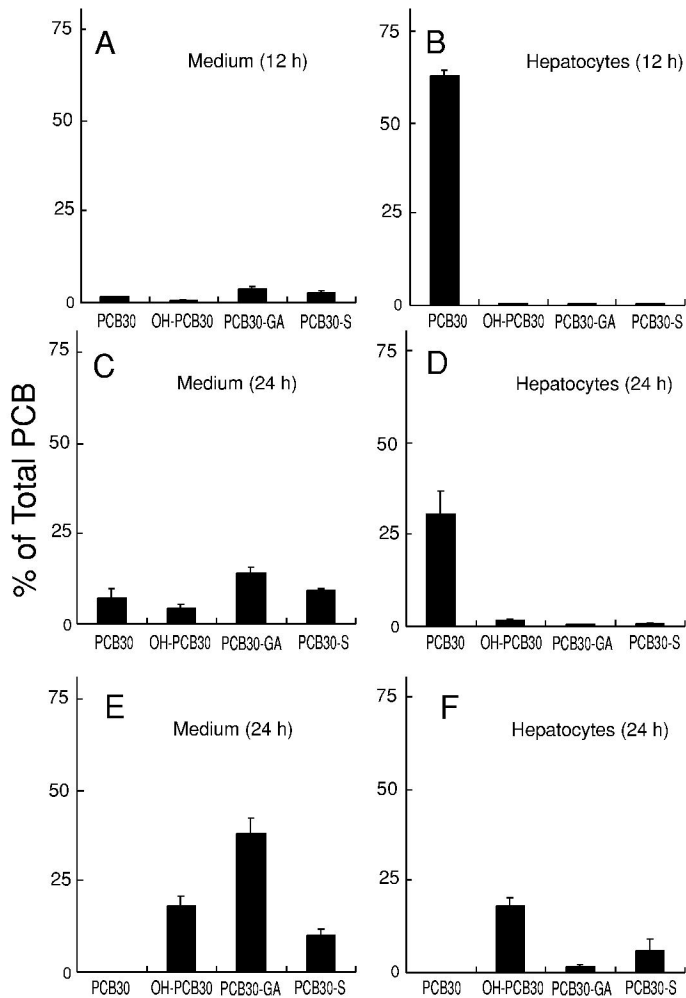


FIG. 2.

Metabolites

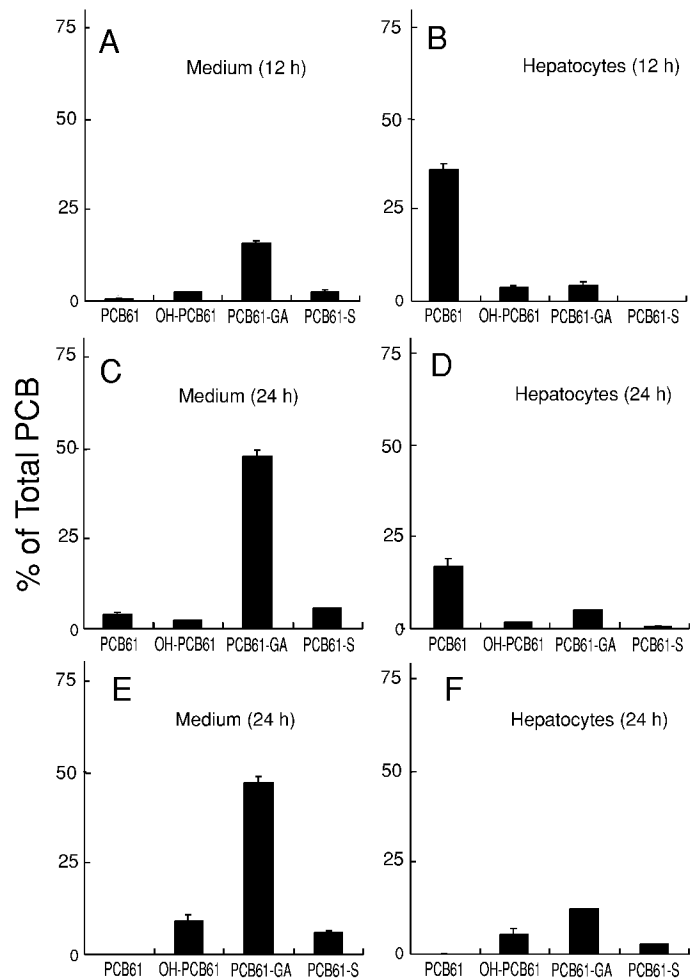


FIG. 3. Metabolites

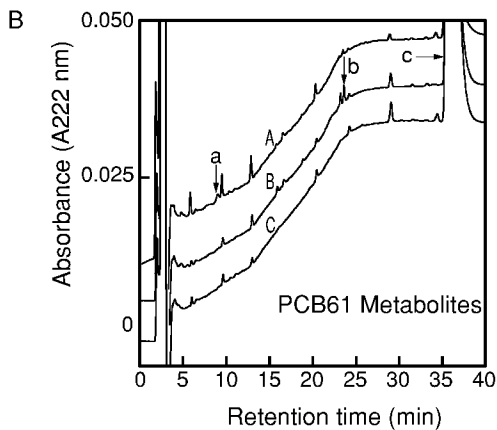
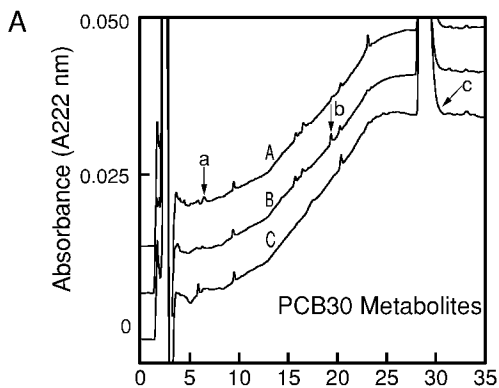


FIG. 4.

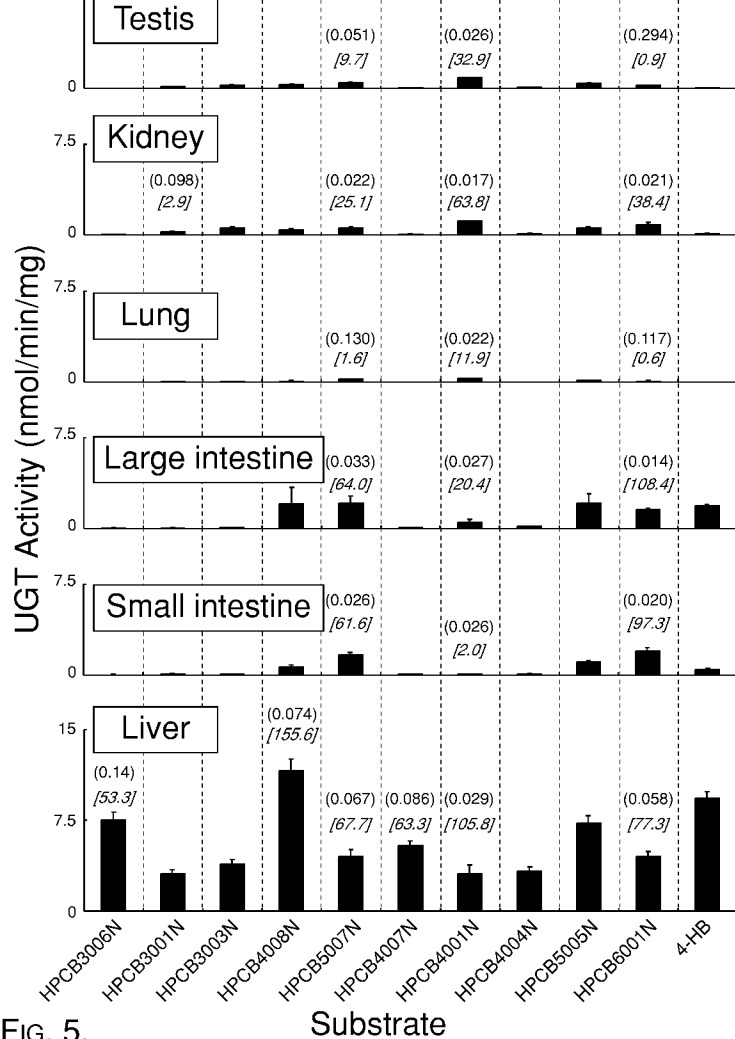


FIG. 5.

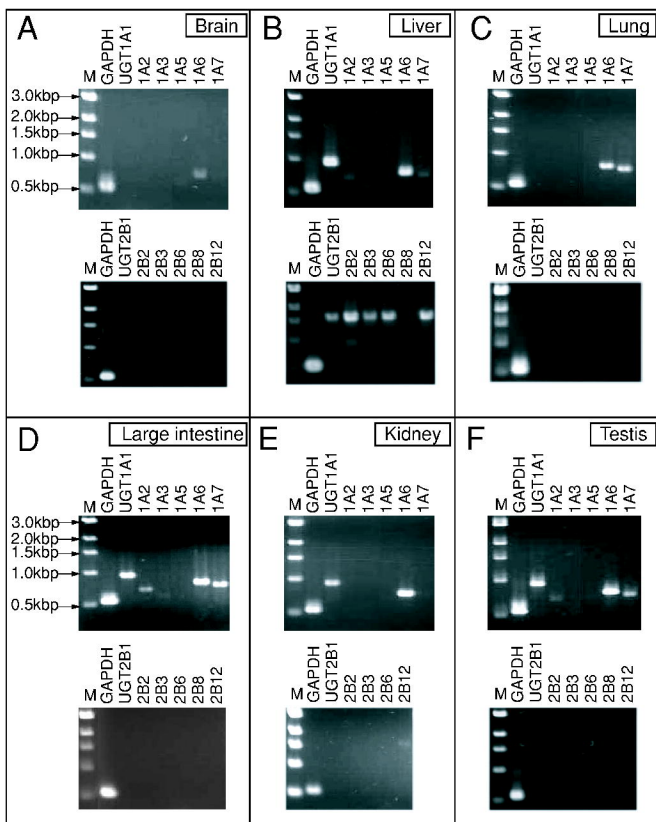


FIG. 6.

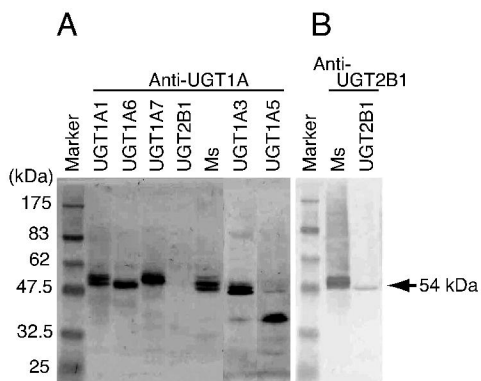


FIG. 7.

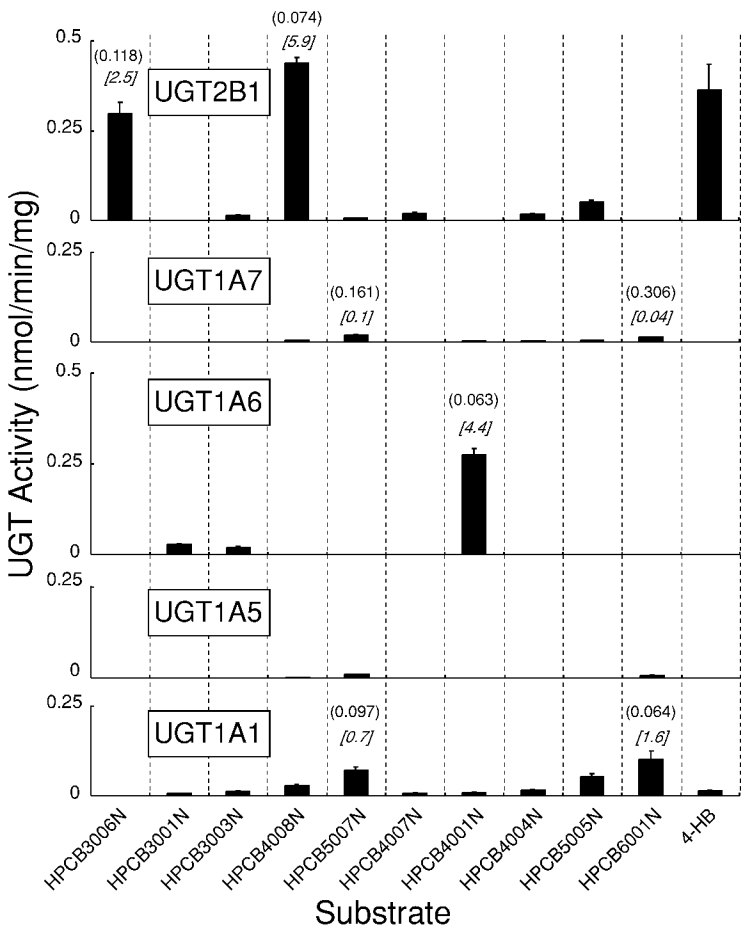


FIG. 8.