GLUCURONIDATION OF THE OXIDATIVE CYTOCHROME P450-MEDIATED PHENOLIC METABOLITES OF THE ENDOCRINE DISRUPTOR PESTICIDE: METHOXYCHLOR BY HUMAN HEPATIC UDP-GLUCURONOSYL TRANSFERASES

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ABSTRACT:

Methoxychlor, a currently used pesticide, is a proestrogen exhibiting estrogenic activity in mammals in vivo. Methoxychlor undergoes oxidative metabolism by cytochromes P450, yielding 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane (mono-OH-M) and 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane (bis-OH-M) as main metabolites. Since humans may be exposed to these estrogenic metabolites, which are potential substrates of UDP-glucuronosyltransferases (UGTs), their glucuronide conjugation was investigated with human liver preparations and individual UGTs. Incubation of both mono-OH-M and bis-OH-M with human liver microsomes formed monogluconorides. The structures of the glucuronides were identified by liquid chromatography/tandem mass spectrometry. Examination of cDNA-expressed recombinant human hepatic UGTs revealed that several catalyze glucuronidation of both compounds. Among the cDNA-expressed UGT1A enzymes, UGT1A9 seemed to be the main catalyst of formation of mono-OH-M-glucuronide, whereas UGT1A3 seemed to be the most active in bis-OH-M-glucuronide formation. Furthermore, the chiral selectivity of mono-OH-M glucuronidation was examined. The results of the incubation of single enantiomers generally agreed with the chiral analyses of mono-OH-M derived from the glucuronidase digestion of the glucuronides of the racemic mono-OH-M. There was a relatively slight but consistent enantioselective preference of individual UGT1A1, UGT1A3, UGT1A9, and UGT2B15 enzymes for glucuronidation of the S over the R-mono-OH-M, whereas in human liver microsomes differences were observed among donors in generating the respective R/S-mono-OH-M ratio. Since it was previously shown that human liver microsomes demethylate methoxychlor mainly into S-mono-OH-M, the observation that UGT1A1 isoforms preferentially glucuronidate the S-mono-OH-M suggests a suitable mechanism for eliminating this major enantiomer. This enantiomeric preference, however, is not extended to all samples of human liver microsomes that we tested.

Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] is a chlorinated hydrocarbon pesticide sharing structural similarity with DDT. Compared with DDT, methoxychlor has a short half-life and possesses low acute toxicity in mammals, apparently due to its rapid metabolism (Metcalfe, 1976); however, methoxychlor exhibits estrogenic activity in mammals in vivo (Osterhout et al., 1981; Bulger et al., 1985; Cummings and Gray, 1989). Based on this and other findings, methoxychlor has been classified as a prototype endocrine disruptor, and its toxicity is being extensively investigated (Bigsby et al., 1999; You et al., 2002).

In vitro and in vivo studies of methoxychlor metabolism have characterized several metabolites, some of which are estrogenic (Bulger et al., 1978; Kupfer and Bulger, 1979; Osterhout et al., 1981; Bulger et al., 1985; Hu and Kupfer, 2002a). The oxidative metabolism of methoxychlor catalyzed by the cytochrome P450 enzymes primarily yields a mono-[1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane (mono-OH-M)] and bis-demethylated product [1,1,1-trichloro-2,2-bis(4-hydroxy phenyl)ethane (bis-OH-M)]. These metabolites are the most potent estrogenic methoxychlor derivatives (Bulger et al., 1985; Gaido et al., 2000; Hu and Kupfer, 2002a,b).

Methoxychlor is prochiral, and, consequently, some of its metabolites are chiral. Therefore, the above-mentioned estrogenic activities may reflect the effect of the individual enantiomers present in the racemates of the synthetic preparations of these compounds. Additionally, it seems reasonable that the biological/pharmacological activities of methoxychlor metabolites will depend on the relative abundance of each enantiomer that has been generated through the enantiotopic selectivity of the cytochrome P450 isoforms catalyzing these reactions. Of interest is the observation that methoxychlor metabolism through cytochromes P450 in human liver microsomes yielded an S/R ratio of ca. 80/20% of the mono-OH-M (Hu and

ABBREVIATIONS: mono-OH-M, 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane; bis-OH-M, 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane; UGT, UDP-glucuronosyltransferase; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; ER, estrogen receptor.
Kupfer, 2002b), suggesting that the possible chiral preference of glucuronidation would influence the biological half-life of this major estrogenic metabolite.

Both mono-OH-M and bis-OH-M possess phenolic hydroxyl groups and are thus potential substrates of the UDP-glucuronosyltransferase (UGT) system (E.C. 2.4.1.17). Glucuronidation is a major pathway in the elimination of exogenous compounds (Evans and Relling, 1999; King et al., 2000; Tukey and Strassburg, 2000). This phase II metabolic reaction is catalyzed by UGT enzymes that transfer the glucuronosyl group from UDP-glucuronic acid to lipophilic compounds such as bile acids, steroid hormones, or environmental pollutants, thus facilitating their disposition and/or altering their therapeutic efficacy or toxic manifestation (Liston et al., 2001). Glucuronidation is primarily a detoxification pathway; however, metabolic activation is also possible [e.g., morphine 6-glucuronide is therapeutically more potent than morphine per se (Glare and Walsh, 1991)].

The UGT enzymes are found in a wide range of tissues, including the liver, intestine, lung, kidney, and colon (Sutherland et al., 1993; Tukey and Strassburg, 2001). These enzymes are mainly located in the endoplasmic reticulum and exhibit different but overlapping substrate specificities. Of interest is the finding that UGTs exhibit stereoselectivity of glucuronidation (Court et al., 2002).

UGT genes have been classified into families/subfamilies based on evolutionary divergence, with all known UGTs belonging to the UGT1A, 2A, and 2B subfamilies (Mackenzie et al., 1997). The UGT1A locus encodes nine functional enzymes: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10. Family 2 human enzymes include UGT2A1, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28 (Miners et al., 2002; Mackenzie et al., 2003). Although the expression of mRNA of different isoforms in human liver has been quantified (Congiu et al., 2002), information on the protein levels of each UGT enzyme in the tissues is inadequate because of the lack of monospecific antibodies suitable for Western blot analyses; consequently, the quantification of only UGT1A enzymes seems feasible because of the availability of common cross-reacting antibodies. To date, the following UGTs have been characterized in human liver: UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, and UGT2B17 and UGT2B28 (Levesque et al., 2001; Congiu et al., 2002; Lin and Wong, 2002).

In vivo studies on methoxychlor metabolism conducted in goats showed that most urinary methoxychlor metabolites were demethylated phenolic derivatives, and a major portion of these metabolites was conjugated with glucuronic acid (Davison et al., 1982). Since there is a potential exposure of humans to methoxychlor and studies on humans in vivo cannot be ethically performed, it was of interest to determine in vitro whether human UGTs catalyze glucuronidation of these environmental pollutants. Thus, our investigation was designed to examine whether human liver microsomes catalyze the glucuronidation of mono-OH-M and bis-OH-M. Additionally, the individual human cDNA-expressed UGTs responsible for the glucuronidation of these phenolic compounds have been characterized, and the potential chiral selectivity of the UGT enzymes toward the mono-OH-M enantiomers was investigated.

**Materials and Methods**

UDP-glucuronic acid, alamethicin, saccharic acid lactone (saccharolactone), dimethylsulfoxide, sodium hydroxide, and β-glucuronidase type B-1 from bovine liver (EC 3.2.1.31) were purchased from Sigma-Aldrich (St. Louis, MO). [3H]uridine diphosphate glucuronic acid (12.1 GBq/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Mono-OH-M was synthesized in our laboratory (Hu and Kupfer, 2002a). Bis-OH-M was kindly provided by Dr. J. Stambou (Illinois State Natural History Survey, Urbana, IL). Bilirubin was obtained from MP Biomedicals (Irvine, CA). HPLC grade methanol was purchased from J. T. Baker (Phillipsburg, NJ). A Prodigy ODS3 250 × 4.6-mm HPLC column was obtained from Phenomenex (Torrance, CA). A chiral-AGP 4 × 100-mm column was obtained from Chrom Tech, Inc. (Apple Valley, MN). Recombinant human UGT enzymes from baculovirus-infected insect cells (supersomes), provided as microsomal preparations and human liver microsomes, were purchased from BD Gentest (Woburn, MA).

**Western Blot Analysis: Antibody Development and Western Immunoblot.** Polyclonal antibodies, specific for the common region of all UGT enzymes, were developed by immunizing rabbits with a peptide sequence linked to keyhole limpet hemocyanin. The peptide sequence was from the C terminus region. Membrane proteins from insect cells were separated on a 10% SDS acrylamide gel using standard procedures. Proteins were transferred to nitrocellulose and detected with the chemiluminescent substrate kit from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Peroxidase-conjugated anti-rabbit secondary antibody was obtained from Sigma-Aldrich and diluted 1:2000 prior to use.

The relative level of expressed UGT protein is shown in Table 1. To better assess the relative contribution of individual UGT enzymes, glucuronidation activity toward mono-OH-M and bis-OH-M was normalized according to the level of expressed UGT1A protein in supersomes as determined by Western blot analysis. Because of the lack of information about the actual enzyme content of UGT2B supersomes (specific antibodies for UGT2B isoforms are not available), the activities of these enzymes—although presented in the figures as per microsomal protein of the insect cells—were not quantitatively evaluated.

**Incubations.** Incubations were conducted in 4-ml vials under an atmosphere of air in a Dubnoff metabolic shaking incubator. The incubation contained the following constituents in 200 μl of final volume: 100 mM TRIS buffer (pH 7.4), 62.5 μM of mono-OH-M or 31.3 μM of bis-OH-M, individually expressed UGT enzymes at a concentration of 0.5 mg protein/ml (supersomes) or human liver microsomes (0.06 mg of protein/ml) and 2.5 mM UDP-glucuronic acid added in 12 mM MgCl2. When [3H]UDP-glucuronic acid was used, the radioactivity was 300,000 dpm per incubation tube. Alamethicin and microsomes were preincubated for 15 min on ice; however, when individually expressed UGT enzymes were used, incubations were conducted for 60 min to obtain a sufficient amount of the metabolite for identification. Reactions were termi-

### Table 1 Western blot analysis of UGT1A isoform expression in supersome preparations

<table>
<thead>
<tr>
<th>UGT1A</th>
<th>Intensity/μg</th>
<th>Relative Level of Expressed Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td>81,615</td>
<td>1.00</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>43,258</td>
<td>0.53</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>55,038</td>
<td>0.67</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>36,969</td>
<td>0.45</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>59,924</td>
<td>0.73</td>
</tr>
</tbody>
</table>

* Intensity of bands per microgram of protein of individual UGT1A isoforms reflecting their expression in the supersomes used in the current study.

* Assigning a level of 1 to the most expressed UGT (i.e., UGT1A1). The relative levels of the other UGT1A isoforms were calculated from the respective intensities.
nated by the addition of 400 µl of ice-cold methanol/0.5% acetic acid. The incubation mixture was centrifuged in an Eppendorf 5415C centrifuge at 14,000 rpm. The aliquots were evaporated to dryness at room temperature under a stream of nitrogen gas. The residues were dissolved in 0.3 ml of water, passed through a 0.20-µm polytetrafluoroethylene filter, and the filtrate was subjected to HPLC analysis to identify the metabolites and to determine the rates of product formation. In incubations using human liver microsomes, two compounds, bilirubin and 1-naphtol, which are potential inhibitors of UGT1A1- and UGT1A9-mediated mono-OH-M glucuronidation activity, respectively, were tested for their inhibitory effect of mono-OH-M glucuronidation. The compounds were dissolved in dimethylsulfoxide/0.05 N NaOH (50:50), with the pure solvent serving as control. The final concentration of dimethylsulfoxide in the medium was 0.5%.

Metabolite Analysis and Purification. Analyses of glucuronides were conducted in the following HPLC apparatus: Waters 6000 pump, Spectroflow 757 UV detector, and a Packard 500 TR radioactivity detector. A novel chromatographic method has been developed in our laboratory for the detection of both mono-OH-M and bis-OH-M glucuronides. Analyses were performed using a 5-µm Phenomenex Prodigy ODS3 column (250 x 4.6 mm). The compounds were detected by their UV absorption at 230 nm and radioactivity. For mono-OH-M incubations, the eluant consisted of methanol/water/ acetic acid (72:27.5:0.5); the flow rate was 1 ml/min for 0 to 14.5 min and then increased to 1.5 ml/min. Retention times were 15 min for mono-OH-M glucuronide and 19 min for mono-OH-M. In the case of bis-OH-M incubations, the eluant consisted of methanol/water/acetic acid (60:39.5:0.5); the flow rate was 1 ml/min for 0 to 15.5 min and then increased to 2 ml/min. Retention times were 17 min for the bis-OH-M glucuronide and 22 min for the bis-OH-M. The mono-OH-M and bis-OH-M glucuronides were collected off the HPLC, and the solvent was evaporated under a stream of nitrogen gas at room temperature. The isolated metabolites were dissolved in water and stored at −20°C for analysis and subsequent use in the glucuronidase digestion procedure or LC/MS/MS determination.

β-Glucuronidase Digestion of Mono-OH-M and Bis-OH-M Glucuronides. Glucuronidase treatment was used to identify the metabolites as being glucuronides and to determine the ratio of S- and R- enantiomers of the mono-OH-M moiety (formed after the digestion of the mono-OH-M glucuronide from incubation of the racemic mixture). The reaction mixture in a final volume of 500 µl contained the glucuronide metabolite dissolved in water, 1000 U β-glucuronidase from bovine liver in 0.1 M phosphate buffer, pH 5.0, and was incubated for 18 h at 37°C. The reaction was terminated by adding 1 ml of ice-cold methanol/0.5% acetic acid. The reaction products were analyzed by HPLC as described above. The mono-OH-M peak was collected off the HPLC, and the solvent was evaporated at 40 to 45°C under a stream of nitrogen gas. Then the mono-OH-M was dissolved in 20% acetonitrile/water and kept at −20°C for further chiral analysis.

To examine the effect of incubation conditions on mono-OH-M glucuronide (e.g., possibly eliciting a non-β-glucuronidase-related degradation), incubations were carried out under the same conditions as described above except that β-glucuronidase was added after the termination of the incubation. Additionally, the possibility of alteration of configuration of the mono-OH-M enantiomers during β-glucuronidase treatment was examined in the following experiment. The racemic mixture of mono-OH-M was incubated with β-glucuronidase under the conditions described above. Then the resulting mixture was resolved on a chiral column, and the ratio of the enantiomers was determined. Since there was no change in the ratio of R- and S-mono-OH-M after glucuronidase treatment, the possibility of alteration of configuration of mono-OH-M during β-glucuronidase treatment was ruled out. Additionally, experiments separating the R- and S-mono-glucuronides showed that only one enantiomer of glucuronide was formed with the incubation of a single R-enantiomer of mono-OH-M. Indeed, glucuronidase digestion of this single enantiomer of mono-OH-M glucuronide yielded only R-mono-OH-M, indicating that no alteration of configuration of the mono-OH-M moiety occurs during glucuronidase digestion.

Most recently, we were able to directly separate the enantiomers/diastereomers of mono-OH-M glucuronides with a chiral column (Fig. 1). To examine whether a chiral conversion may be occurring during glucuronidase digestion, an incubation of racemic mono-OH-M with UDP-glucuronic acid was carried out. The formed glucuronides were divided into two parts. One part underwent chiral analysis so that the racemization ratio of the glucuronides formed could be determined. With the other part of the sample, digestion with glucuronidase enzyme was carried out, and the racemization ratio of the released mono-OH-M was determined using chiral separation. Since the ratio of enantiomers of the glucuronides by direct chiral separation was essentially the same as the enantiomeric ratio of the mono-OH-M after glucuronidase digestion, the possibility of a chiral conversion during glucuronidase-catalyzed reaction could be ruled out.

Enantiotopic Analysis of Metabolites. Aliquots of the mono-OH-M in 20% acetonitrile/water were injected onto a chiral HPLC column for enantio-meric analysis. The R- and S-mono-OH-M enantiomers were separated on a chiral-AGP column (100 x 4.0 mm, 5 µm, at λ = 230 nm) with acetonitrile/water (18:82) at a flow rate = 0.9 ml/min. Retention times were 14 min for R-mono-OH-M and 17 min for S-mono-OH-M (Hu and Kupfer, 2002b). LC/MS Determination of Glucuronide Formation. The mono-OH-M and Bis-OH-M glucuronides, generated in incubations of mono-OH-M or bis-OH-M with UDP-glucuronic acid and human liver microsomes, were collected off the HPLC (incubation and analysis method described above), and the solvent was evaporated under a stream of nitrogen gas at room temperature. The isolated metabolites were dissolved in water and subjected to LC/MS/MS analysis. Studies were conducted in a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Electrospray ionization was used with a
capillary temperature of 175°C, source voltage of 4.50 kV, capillary voltage of −45 V, and tube lens onset of −55 V. Analyses were conducted in the negative ion mode performing full scan MS and MS/MS/ion plots to detect mono-OH-M (m/z 505) and bis-OH-M glucuronide (m/z 491) (Fig. 3, A and B).

Chromatographic separation was obtained using an Aquasil C18 100 × 1-mm column (Keystone Scientific Inc., Bellefonte, PA) with a 5-μm particle size. The eluant contained 69% methanol and 1% acetic acid. The flow rate was 50 μl/min. The retention time was 4 min for bis-OH-M monoglucuronide and 7 min for mono-OH-M glucuronide.

Results

In preliminary investigations, incubations using male rat liver microsomes demonstrated glucuronidation of both mono-OH-M and bis-OH-M, the major phenolic estrogenic metabolites of methoxychlor (not shown). Consequently, the current study was initiated to determine whether human liver preparations metabolize the mono-OH-M and bis-OH-M into the corresponding glucuronides. Also, it was of interest to determine whether glucuronidation involving UDP-glucuronic acid was enantioselective, using one of the mono-OH-M enantiomers preferentially. The lack of authentic standards of mono-OH-M and bis-OH-M glucuronides necessitated an indirect approach (described below). Therefore, a novel HPLC method with UV detection at λ = 230 nm was developed to detect mono-OH-M and bis-OH-M glucuronide formation. Incubations with [14C]UDP-glucuronic acid (radiolabeled for metabolite detection and quantification) resulted in a radioactive peak that disappeared after β-glucuronidase digestion. Additionally, we confirmed the formation of the monoglucuronide metabolites after the incubation of mono-OH-M and bis-OH-M with human liver microsomes and UDP-glucuronic acid using LC/MS and LC/MS/MS analyses (Figs. 2 and 3, A and B). The
LC/MS measurement showed a full scan mass spectrum with an intense deprotonated molecular ion [M-H]⁻ (m/z 505 for mono-OH-M glucuronide and 491 for bis-OH-M glucuronide), consistent with the molecular ion of mono-OH-M and bis-OH-M monoglucuronide, respectively. Additionally, the isotopic pattern of the molecular ions was characteristic of a compound containing three chlorines. Moreover, MS/MS fragmentation resulted in the formation of a characteristic fragment with m/z 175 corresponding to glucuronic acid moiety (data not shown).

**Effect of Saccharolactone on the Rate of Formation of Glucuronide Metabolites**

When 10 mM saccharolactone was included in the incubation mixture of human liver microsomes, both mono-OH-M and bis-OH-M glucuronidation activity decreased (Fig. 4). Additionally, in HG-3 human donor sample and pooled human liver microsomes, the inhibition rate of mono-OH-M and bis-OH-M-glucuronidation was significantly different. Therefore, it can be concluded that the rate of inhibition by saccharolactone was substrate and in some cases microsomal source-dependent.

**Glucuronidation of Mono-OH-M and Bis-OH-M**

**Glucuronidation by Individual cDNA-Expressed UGTs.** Metabolites generated by incubation of mono-OH-M and bis-OH-M with human liver microsomes were analyzed by HPLC and detected by their UV absorbance at λ = 230 nm. The following recombinant UGT isoforms (supersomes) were used to determine their ability to catalyze glucuronidation of mono-OH-M and bis-OH-M: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B15, and UGT2B17.

**Mono-OH-M Glucuronidation.** At a 62.5 μM concentration of mono-OH-M, several UGT1A isoforms (UGT1A1, UGT1A3, UGT1A9, UGT2B7, and UGT2B15, normalized for their differences in expression in supersomes), exhibited activity toward mono-OH-M glucuronidation, with UGT1A9 seemingly the predominant catalytic isoform with approximately 2 times greater activity than any other UGT1A isoform (Fig. 5A).

The potency of mono-OH-M glucuronidation activity was UGT1A9 ≫ UGT1A3 ≫ UGT1A1. In addition, UGT2B15 and UGT2B7 were also capable of glucuronidating mono-OH-M; however, only few or no glucuronide conjugates were detected in...
incubations with UGT1A4, UGT1A6, UGT2B4, and UGT2B17 enzymes.

**Bis-OH-M Glucuronidation.** The bis-OH-M glucuronidation activity at a 31.3 μM concentration of bis-OH-M was ranked as being UGT1A3 > UGT1A9 > UGT1A1 > UGT1A4. Additionally, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 enzymes demonstrated considerable activity (Fig. 5B); however, no conjugates could be detected in incubations with the UGT1A6 isofrom.

**Glucuronidation by Human Liver Microsomes.** Mono-OH-M and Bis-OH-M Glucuronidation. Metabolites generated by incubation of mono-OH-M and bis-OH-M with human liver microsomes and UDP-glucuronic acid (Fig. 6) were analyzed by HPLC and detected by their radioactivity and LC/MS/MS. The formation of glucuronides of mono-OH-M and bis-OH-M was time-dependent and linear up to 30 min. The LC/MS/MS analysis revealed that incubation of bis-OH-M yielded a monoglucuronide as the main metabolite.

Human liver microsomes from three different donors and a pool of human microsomes from 22 human donors were used in this investigation. These microsomal preparations were selected to provide differences in their catalytic activity of the UGT1A1, UGT1A4, and UGT1A9 enzymes (Table 2).

The contribution of UGT1A1 and UGT1A9 enzymes to mono-OH-M glucuronidation in human liver microsomes was investigated in the following experiments. Bilirubin, a specific substrate of UGT1A1 (Lin and Wong, 2002), was used as a possible competitive inhibitor of UGT1A1 to examine the potential involvement of UGT1A1 in mono-OH-M glucuronidation, whereas 1-naphthol, an inhibitor of UGT1A6 and UGT1A9 activity but not UGT1A1 and UGT1A3 (Hanioka et al., 2001), was used to examine the contribution of UGT1A9 enzyme to mono-OH-M glucuronidation, since UGT1A6 did not significantly catalyze mono-OH-M glucuronidation. Incubation of mono-OH-M in the presence of 2 μM bilirubin diminished mono-OH-M glucuronidation activity in pooled human liver microsomes by 4.5 ± 2.7% (i.e., mean ± S.D.) and in human liver microsomes of HG-95 donor by 3.5 ± 2.0%. In the presence of 1-naphthol (20 μM), the mono-OH-M glucuronidation was inhibited by 10.3 ± 2.0% in pooled human liver microsomes and 15.9 ± 8.9% in donor HG-95 liver microsomes. A comparison of human liver microsomes among individual donors revealed a statistically significant difference (p = 0.03) in mono-OH-M glucuronidation activity. The difference among individual donors in bis-OH-M glucuronidation activity was statistically highly significant (p < 0.0001), with an approximately 3-fold difference between the highest and lowest values.

**Chiral Selectivity in Mono-OH-M Glucuronidation**

For the determination of the chiral selectivity of UGT isoforms toward mono-OH-M glucuronidation, two methods were used. The first was a direct method involving incubation of the single enantiomers R- and S-mono-OH-M with human liver microsomes and with those UGT isoforms exhibiting substantial activity toward mono-OH-M glucuronidation; the enantiotopic selectivity by UGTs was estimated by comparing the ratio of the glucuronidated metabolites formed. The second method used an indirect method in which the mono-OH-M glucuronides generated by incubation of racemic mono-OH-M were digested with β-glucuronidase yielding mono-OH-M. In the latter case, the enantiomeric ratio of the mono-OH-M was determined using chiral separation of the S- and R-mono-OH-M.

**Incubation with R- and S-Mono-OH-M. Individually Expressed UGTs.** UGT1A1, UGT1A3, UGT1A9, and UGT2B15 were tested for their affinity toward the S- and R-mono-OH-M enantiomers. All of these UGTs showed preference for the S-mono-OH-M (Fig. 7A). The extent of chiral selectivity was in the following order: UGT1A1 >
UGT1A3 > UGT1A9 > UGT2B15, with the chiral selectivity of UGT2B15 being statistically not significant. UGT1A9 and UGT2B15, the most efficient in catalyzing mono-OH-M glucuronidation, proved to be the least enantioselective toward individual mono-OH-M enantiomers (i.e., 60.1 and 55.7% in favor of the S-enantiomer, respectively).

**Human Liver Microsomes.** Human liver microsomes of three individual donors and a pool from 22 donors were used to test the chiral selectivity of the enzymes toward the S- and R-mono-OH-M. As shown in Fig. 7B, only a slight difference was observed in two individual donors (HG-3 and HH-31) in favor of the S-mono-OH-M enantiomer. This difference was greater in the case of HG-95 and pooled human liver microsomes; however, unexpectedly, there was a greater selectivity toward the R-enantiomer.

**β-Glucuronidase Digestion of the Mono-OH-M Glucuronide Formed by Incubation of Racemic Mono-OH-M with UDP-Glucuronic Acid, Followed by Chiral HPLC Analyses of the Released Mono-OH-M.** The mono-OH-M glucuronide generated by incubation of racemic mono-OH-M was digested with β-glucuronidase. After 18 h of incubation, ca. 95% of mono-OH-M glucuronide was converted to free mono-OH-M. The ratio of the different enantiomers was determined using chiral HPLC separation. A chromatogram of the chiral separation is shown in Fig. 8.

**Individually Expressed UGTs.** Only a slight difference was observed between the S- and R-mono-OH-M glucuronidation, as the chiral separation revealed (Fig. 9A). Digestion of glucuronides resulted in a slightly elevated formation (statistically not significant) of S- compared with R-mono-OH-M formation. The same—although more pronounced—enantionic preference was found in experiments of single S- or R-mono-OH-M incubations.

**Human Liver Microsomes.** Differences among individuals were observed in their preference toward the individual enantiomers of mono-OH-M glucuronidation (Fig. 9B). In accordance with the results of the R- and S-mono-OH-M incubation, glucuronide digestion of sample HH-31 yielded more S- than R-mono-OH-M, whereas digestion of glucuronides generated by the liver microsomes of HG-95 donor resulted in more R- than S-mono-OH-M. Also, more R-mono-OH-M was formed after digestion of the monoglucuronide derived from the racemic mono-OH-M when monoglucuronides were generated by HG-3 liver microsomes. S-mono-OH-M was present in a slightly higher amount after digestion of monoglucuronides generated by the pooled human liver microsomes. The latter two results are not consistent with the enantioselective results of the incubations obtained with the single enantiomers (S- or R-mono-OH-M).

**Discussion**

Methoxychlor, a currently used pesticide, has been extensively studied regarding its endocrine and reproductive toxicity in animals. It was therefore important to establish the nature of the major pathways of methoxychlor metabolism. We have investigated the formation of O-glucuronides of the mono- and bis-demethylated methoxychlor metabolites, i.e., of mono-OH-M and bis-OH-M, using human liver microsomes and cDNA-expressed human UGT isoforms. The capability of preparation of the chiral methoxychlor metabolite (mono-OH-M) in our laboratory and our ability to separate the enantiomers of mono-OH-M on a chiral column has permitted the investigation of the potential stereoselectivity of glucuronidation of UGT isoforms toward the chiral metabolites of methoxychlor.

In several reported studies, saccharolactone, a β-glucuronidase inhibitor, was included in the incubation mixture in an attempt to inhibit the enzymatic hydrolysis of the conjugate formed during the incubation; however, the significance of β-glucuronidase activity, which is abundant in mammalian liver, seems to be substrate and microsomal source-dependent. For instance, acylglucuronides were found to be more susceptible to the effects of the β-glucuronidase than phenolic glucuronides (Brunelle and Verbeeck, 1996). Additionally, it was suggested that the use of β-glucuronidase inhibitor may not be necessary in human liver microsomes, since humans exhibit only a low level of β-glucuronidase activity (Brunelle et al., 1996). Recently, Alkhafy and Frye (2001) reported that the addition of saccharolactone to incubations of acetaminophen with human liver microsomes diminished the velocity of acetaminophen glucuronidation by approximately 45%. In a study comparing cell lines in the presence and absence of saccharolactone, UGT1A1 and UGT1A9 showed less activity in the presence of saccharolactone, whereas a slight increase in activity was observed in the case of UGT2B15 (Ethell et al., 2001). It should be noted, however, that all differences in activity were below 5% in that study; however, in our study, it was found that the addition of saccharolactone diminished mono-OH-M and bis-OH-M glucuronidation activity by 20 to 40%. The inhibitory effect of saccharolactone was substrate and, in two liver samples (HG-3 and pooled), microsomal source-dependent, indicating a certain degree of variability in the inhibitory effect of saccharolactone. The mechanism by which saccharolactone diminishes glucuronidation is not known; however, a shift in the pH of the incubation mixture by saccharolactone is a possibility, but we made no further attempt to resolve this capricious saccharolactone effect and consequently did not include saccharolactone in subsequent incubations.

In human liver microsomes, the highest observed difference among individual donors was approximately 3-fold in both mono-OH-M and bis-OH-M glucuronidation. Additionally, the enzymatic activity of the pooled human liver microsomes (from 20 livers) was in the range of the three individual donors, indicating that the individual liver samples reflected the activity of a cross-section of a sizable population pool.

We also observed that, among the UGT1A enzymes examined, UGT1A9 demonstrated the highest catalytic activity toward mono-OH-M glucuronidation, with UGT1A3 and UGT1A1 enzymes also exhibiting, albeit decreased, mono-OH-M glucuronidation activity. In a study of mRNA levels of UGT isoforms of 18 human donors, UGT1A9 mRNA was the least expressed mRNA of a UGT isoform (Congiu et al., 2002). Therefore, UGT1A9—although apparently the most effective in mono-OH-M glucuronidation compared with other recombinant UGT isoforms—may not be the main catalyst of mono-
OH-M glucuronidation in human liver microsomes. Indeed, the inhibition by 20 
\(\mu\)M 1-naphthol in human liver microsomes was only ca. 15%. In that experiment, 1-naphthol was used as an inhibitor of UGT1A9-mediated mono-OH-M glucuronidation activity because it inhibited 7-ethyl 10-hydroxycamptothecin glucuronidation by 86% in recombinant human UGT1A9 and exhibited 30% inhibition of UGT1A6 but showed no inhibitory effect toward UGT1A1 and UGT1A3 enzymes (Hanioka et al., 2001). However, since UGT1A6 does not participate significantly in mono-OH-M glucuronidation, the inhibition of mono-OH-M glucuronidation in human liver microsomes by 1-naphthol seems to solely reflect the inhibition of UGT1A9. The relatively low extent of inhibition of mono-OH-M glucuronidation by 1-naphthol indicates a significant but relatively minor involvement of UGT1A9 in mono-OH-M glucuronidation in human liver microsomes.

Bilirubin (2 
\(\mu\)M), which inhibited 7-ethyl 10-hydroxycamptothecin glucuronidation by 60% in incubations with human recombinant UGT1A1 but did not inhibit recombinant UGT1A3, UGT1A6, or UGT1A9 enzymes (Hanioka et al., 2001), only slightly inhibited mono-OH-M glucuronidation; this suggested only a minor role of UGT1A1 in mono-OH-M glucuronidation by human liver microsomes.\(^1\)

Greater enantiomeric selectivity was observed in the case of incubation with S- and R-mono-OH-M compared with the results of enantiomeric analyses from the incubation of racemic mono-OH-M (after \(\beta\)-glucuronidase digestion). Additionally, in the case of the HG-3 sample and pooled human liver microsomes, these results were not consistent with those obtained from the incubation of the single enantiomers. Possible reasons for these results could be due to the following: in the incubation of the racemic mixture, in addition to the potential different conversion rates of the enantiomers, there could be competition between the R- and S-mono-OH-M for the binding to the active site as well, whereas in the incubations of individual enantiomers, only the single enantiomer conversion rates are reflected. Our experiments, in which we separated the two glucuronides derived from the mixture of glucuronides formed during incubation of the R,S-enantiomers of mono-OH-M (using the same chiral column as in the separation of the R,S-enantiomers of mono-OH-M), confirmed this assumption.

All individual UGTs showed higher activity toward the S-mono-OH-M than the R-mono-OH-M, whereas human liver microsomes either lacked preference or were more active toward the R-mono-OH-M. The cause of this discrepancy might suggest the involvement of an unknown, hitherto undiscovered UGT in mono-OH-M and bis-OH-M glucuronidation. Alternatively, this phenomenon might be due to the differences in membrane environment of the cDNA expression system of the recombinant UGTs and that of human liver microsomes. Indeed, it has been shown that the type of phospholipid environment influences glucuronidation (Magdalou et al., 1982). Additionally, experimental data suggest that UGTs may function as oligomers (Lin and Wong, 2002), which in turn may alter the enantiomeric selectivity.

1 It should be noted that, since bilirubin is a substrate of UGT1A1, the absence of inhibition by bilirubin might merely reflect that mono-OH-M is a better substrate than bilirubin; however, the mRNA levels of UGT isoforms in human liver indicated that UGT1A1 is one of the least abundant UGT isoforms. Therefore, this finding and our observation that UGT1A1 is not highly active in mono-OH-M glucuronidation suggest that UGT1A1 plays a minor role in mono-OH-M glucuronidation in human liver microsomes.
that the ER isoforms do not exhibit enantiomeric preferences for the mono-OH-M enantiomers.

Of additional interest are our earlier findings that human liver microsomes preferentially demethylate methoxychlor into the S-enantiomer of mono-OH-M, forming approximately 80% of the S and 20% of the R (Hu and Kupfer, 2002b). Our current observation that UGT1A isoforms preferentially glucuronidate the S-mono-OH-M suggests a suitable mechanism for elimination of the major enantiomer, i.e., the S-mono-OH-M. This enantiomeric preference, however, is not extended to all samples of the human liver microsomes that we tested.

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References
UDP-glucuronosyltransferases (UGTs). UGT1A9 is more resistant to detergent inhibition than other UGTs and was purified as an active dimeric enzyme. *J Biol Chem* **278**:3536–3544.


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