ASSESSMENT OF CATECHOL INDUCTION AND GLUCURONIDATION IN RAT LIVER MICROSOMES

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ABSTRACT:
Catechols are substances with a 1,2-dihydroxybenzene group from natural or synthetic origin. The aim of this study was to determine whether catechols (4-methylcatechol, 4-nitrocatechol, 2,3-dihydroxynaphthalene) and the antiapoptotic drugs, entacapone and tolcapone, at doses 150 to 300 mg/kg/day, for 3 days, are able to enhance their own glucuronidation. The induction potency of catechols on rat liver UDP-glucuronosyltransferases (UGTs) was compared with that of a standard polychlorinated biphenyl (PCB) inducer, Aroclor 1254. The glucuronidation rate of these catechols was enhanced up to 15-fold in the liver microsomes of PCB-treated rats, whereas treatment with catechols had little effect. Entacapone, tolcapone, 4-methylcatechol, catechol, 2,3-dihydroxynaphthalene, and 4-nitrocatechol were glucuronidated in control microsomes at rates ranging from 0.12 for entacapone to 22.0 nmol/min/mg for 4-nitrocatechol. Using 1-naphthol, entacapone, and 1-hydroxypyrene as substrates, a 5-, 8-, and 16-fold induction was detected in the PCB rats, respectively, whereas the catechol-induced activities were 1.1- to 1.5-fold only. Entacapone was glucuronidated more efficiently by PCB microsomes than by control microsomes (Vmax/Km, 0.0125 and 0.0016 ml/min/mg protein, respectively). Similar kinetic results were obtained for 1-hydroxypyrene. The Eadie-Hofstee plots suggested the contribution of multiple UGTs for the glucuronidation of 1-hydroxypyrene (Km1, K1; Km2, K2; Km3 = 0.8, 9.7, and 63 μM, and Vmax1, Vmax2, Vmax3 = 11, 24, and 55 nmol/min/mg, respectively), whereas only one UGT could be implicated in the glucuronidation of entacapone (Km = 130 μM, Vmax = 1.6 nmol/min/mg). In conclusion, catechols are poor inducers of their own glucuronidation supported by several UGT isoforms. Their administration is unlikely to affect the glucuronidation of other drugs administered concomitantly.

Catechols comprise a large class of substances from natural or synthetic origin, including diverse endobiotic and xenobiotic compounds. They are also known to exhibit a variety of physiological, pharmacological, and toxicological properties. Structurally, they have a common catechol ring (1,2-dihydroxybenzene, pyrocatechol; Fig. 1). Occupational exposure to catechol, 4-methylcatechol, 4-nitrocatechol, and 2,3-dihydroxynaphthalene (DHN) and others is usually associated with industrial production of chemicals, or uses as metal ion complexation agents, or as antioxidants (IARC Working Group, 1999). Catechols are also present in dietary products (Carmella et al., 1982). Natural catechols from plant extracts, such as catechins and polyphenols from green tea, show anticancer activity (Colic and Pavelic, 2000).

Dopamine and other neurotransmitters are functionally important endogenous catechols. Entacapone and tolcapone are selective and reversible catechol-O-methyltransferase (COMT) inhibitors (Fig. 1), which prevent the peripheral O-methylation of levodopa and therefore enhance the bioavailability of this dopamine precursor in the brain. These nitrocatechols, developed as adjuncts to antiparkinsonian medication, prolong the duration and improve the efficacy of dopatherapy in patients with fluctuating Parkinson’s disease (Kurth and Adler, 1998; Männistö and Kaakkola, 1999; Poewe, 2004). Tolcapone has been withdrawn or its use restricted following findings of acute hepatic dysfunction in several patients and, in three cases, fatal fulminant hepatitis (Olanow, 2000; Spahr et al., 2000). In the light of the experience increasing worldwide (over 300,000 patient years), entacapone is potentially not a hepatotoxin (Brooks, 2004). In the rat model, high doses of tolcapone (500 mg/kg/day), but not of entaca-

ABBREVIATIONS: DHN, 2,3-dihydroxynaphthalene; COMT, catechol-O-methyltransferase; HPLC, high performance liquid chromatography; 1-OHP, 1-hydroxypyrene; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; h, human; r, rat; ALT, alanine aminotransferase; AST, aspartate aminotransferase; RT, retention time.
Catechol Induction and Glucuronidation in Rat Liver

Model catechols on the glucuronidation of catechols themselves and noncatecholic UGT probe substrates (1-naphthol, 1-hydroxypropene) in rats. The well known inducer, PCB, was used as a positive control. Hepatotoxicity of treatment doses was estimated by blood plasma transaminase tests [alanine and aspartate aminotransferases (ALT and AST)]. Since UGT1A9 and 1A6 isoforms have been previously shown by us to be greatly involved in catechol glucuronidation (Lautala et al., 2000; Antonio et al., 2002, 2003), the catechol drug entacapone and the PAH phenols, 1-naphthol and 1-hydroxypropene (1-OHP), were chosen as substrates.

Materials and Methods

Chemicals. All products were of the highest quality commercially available. 4-Methylcatechol was purchased from Merck (Darmstadt, Germany). Catechol, 4-nitrocatechol, 1-naphthol β-glucuronide, and bovine serum albumin were obtained from Sigma-Aldrich (St. Louis, MO); 1-naphthol and 2,3-dihydroxyxanthophenol were obtained from Aldrich Chemical Co. (Steinheim, Germany). Entacapone [(E)-2-cyano-3,4-dihydro-3-(3,4-dihydroxy-5-nitrophenyl) propanamide; OR-611] and tolcapone (3,4-dihydroxy-4-methyl-5-nitrobenzophenone; Ro-40–7592) were kindly provided by Orion Pharma (Espoo, Finland). Aroclor 1254 (RCS-088; Analabs, Creb Orchard, WV) was a PCB mixture from the Foxboro Company (North Haven, CT). 1-Pyrenyl β-d-glucuronide and entacapone 3-O-β-d-glucuronide reference compounds were synthesized and purified at the Helsinki University (Luukkaneen et al., 1999). Uridine 5′-diphosphosphate-glucuronic acid (UDPGA, sodium salt) and UDP-[U-14C]glucuronic acid (350 mCi/mmol) were obtained from Roche Diagnostics (Mannheim, Germany) and Amersham Biosciences Inc. (Saclay, France), respectively. 1-Hydroxypropene was obtained from Acros Organics (Geel, Belgium).

Animals and Study Design. Sixty male Wistar rats (271 ± 19 g, from the Helsinki University Breeding Centre) were housed in plastic cages bedded with aspen wood shavings in an air-conditioned room (23–25°C) with artificial lighting from 7:00 AM to 7:00 PM. They had free access to the pellet chow (Rat Chow, Altromin 1320; Altromin International, Lage, Denmark) and tap water. Five rats per group were treated with different catechols for 3 days by oral gavage (see Table 1). 4-Methylcatechol (100 or 150 mg/kg body weight/day) and 4-nitrocatechol (100 or 200 mg/kg/day) were administered in 5% sucrose syrup (2.5 ml/kg). Entacapone (200 mg/kg/day), tolcapone (200 mg/kg/day), and DHN (300 mg/kg/day) were administered in olive oil (3 ml/kg). Catechol- and vehicle-treated animals were killed 24 h after the third dose. PCB-treated rats and their controls were killed 5 days after a single 0.55 g/kg dose administered intraperitoneally in olive oil (2.5 ml/kg). Food was withdrawn 3 h before each treatment and before sacrifice. Blood (6–8 ml) was drawn under CO2 anesthesia by cardiac puncture with heparin-rinsed plastic needles. Blood plasma ALT and AST (alanine and aspartate aminotransferases, respectively) were determined with an automated photometer (Hitachi 747; Hitachi, Tokyo). Other biochemicals were determined with a commercial kit (Merck, Darmstadt, Germany). Liver per body weight was determined after weighing the liver and body weight.

TABLE 1

Acute effects in male Wistar rats dosed with different catechol compounds or a PCB agent (Aroclor 1254)

<table>
<thead>
<tr>
<th>Treatment and Daily Dosages</th>
<th>No. of Rats</th>
<th>Rat Weight</th>
<th>Blood Plasma</th>
<th>Liver Wet</th>
<th>Liver per Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g</td>
<td>IU</td>
<td>ALT AST</td>
<td>g %</td>
</tr>
<tr>
<td>Control (olive oil), 2.5 ml/kg (1 day)</td>
<td>5</td>
<td>252 ± 11</td>
<td>58 ± 11</td>
<td>109 ± 19</td>
<td>11.9 ± 2.2</td>
</tr>
<tr>
<td>Aroclor 1254, 0.5 g/kg (1 day)</td>
<td>5</td>
<td>255 ± 6</td>
<td>47 ± 15</td>
<td>149 ± 24*</td>
<td>18.5 ± 1.9**</td>
</tr>
<tr>
<td>Control (5% aqueous sucrose), 2.5 ml/kg (3 days)</td>
<td>5</td>
<td>260 ± 9</td>
<td>57 ± 11</td>
<td>107 ± 7</td>
<td>11.9 ± 1.0</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>100 mg (0.81 mmol/kg) (3 days)</td>
<td>5</td>
<td>258 ± 20</td>
<td>61 ± 5</td>
<td>110 ± 26</td>
</tr>
<tr>
<td>150 mg (1.21 mmol/kg) (3 days)</td>
<td>5</td>
<td>269 ± 11</td>
<td>70 ± 11</td>
<td>85 ± 6</td>
<td>11.8 ± 0.8</td>
</tr>
<tr>
<td>4-Nitrocatechol</td>
<td>100 mg (0.64 mmol/kg) (3 days)</td>
<td>5</td>
<td>260 ± 14</td>
<td>59 ± 9</td>
<td>90 ± 11</td>
</tr>
<tr>
<td>200 mg (1.29 mmol/kg) (3 days)</td>
<td>5</td>
<td>264 ± 25</td>
<td>60 ± 7</td>
<td>90 ± 19</td>
<td>12.9 ± 1.7</td>
</tr>
<tr>
<td>Control (olive oil), 3 ml/kg (3 days)</td>
<td>5</td>
<td>279 ± 20</td>
<td>63 ± 14</td>
<td>92 ± 12</td>
<td>12.7 ± 1.5</td>
</tr>
<tr>
<td>2,3-Dihydroxyxanthophenol, 300 mg (1.87 mmol/kg) (3 days)</td>
<td>5</td>
<td>284 ± 15</td>
<td>72 ± 4</td>
<td>122 ± 8*</td>
<td>13.3 ± 1.5</td>
</tr>
<tr>
<td>Control (olive oil), 3 ml/kg (3 days)</td>
<td>5</td>
<td>293 ± 4</td>
<td>73 ± 10</td>
<td>75 ± 21</td>
<td>12.3 ± 0.9</td>
</tr>
<tr>
<td>Entacapone, 200 mg (0.66 mmol/kg) (3 days)</td>
<td>5</td>
<td>283 ± 8</td>
<td>75 ± 5</td>
<td>88 ± 8</td>
<td>12.3 ± 1.5</td>
</tr>
<tr>
<td>Tolcapone, 200 mg (0.73 mmol/kg) (3 days)</td>
<td>5</td>
<td>276 ± 10</td>
<td>76 ± 8</td>
<td>87 ± 13</td>
<td>11.8 ± 1.4</td>
</tr>
</tbody>
</table>

All catechols were administered intragastrically. Aroclor was administered intraperitoneally.

Values are the mean ± S.D.; those that differ from the respective control group by Student’s t test (two-tail) are: *p < 0.05, **p < 0.01.

Fig. 1. Structures of the catechols and PCBs.
perfused in situ with phosphate-buffered saline, removed, weighed, cooled in ice, and stored frozen (−70°C). The experiments were approved by the local Ethical Committee for Animal Studies (the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes were followed).

**Preparation of Microsomes from Rat Liver.** The thawed livers were homogenized in 2 volumes (w/v) of cold 0.250 M sucrose/1 mM Tris-HCl (pH 7.4) buffer with a Potter-Elvehjem glass homogenizer (Thomas tubes with Teflon pestle). Enzyme fractions were prepared at 5°C by differential ultra-centrifugation. The supernatant of the first centrifugation (10,000g, 15 min) was run at 106,000g for 60 min to obtain as a pellet the microsomal fraction. The pellet was homogenized in 10 ml of buffer and centrifuged again at 106,000g for 60 min. The microsomes were resuspended in the buffer to give a protein content of 10 to 20 mg/ml, divided in small aliquots, and stored frozen in Eppendorf tubes (−70°C) until use. The protein concentrations were determined (Lowry et al., 1951) using commercial protein standards (Precise Eiweiss/Protein; Roche Diagnostics).

**Blood Plasma Enzyme Analyses.** Blood specimens were centrifuged at 3000g for 20 min (at 20°C) to obtain the plasma samples stored at −70°C until use. The activity of ALT and AST in plasma was kinetically determined by recommended methods (ECCLS, 1988) using enzyme reagent kits from Medix Biochemica (Kauniainen, Finland).

**Glucuronidation Activity Analyses.** *Entacapone glucuronidation.* The activity was determined by an HPLC method. The incubation mixture (final volume 250 μl, in Eppendorf tubes) contained 50 mM potassium/sodium phosphate buffer (pH 7.4), 8 mM MgCl2, 250 μM entacapone (added in assay buffer), and 40 to 80 μg of microsomal protein. The reaction was started with 50 μl of 25 mM UDPGA (5 mM), incubated for 15 min at 37°C, and stopped with 25 μl of cold 4 M perchloric acid. The tubes were placed in an ice bath for 10 min (protein precipitation) before being mixed and centrifuged (13,000 rpm, 5 min). The resulting supernatants were injected into a Shimadzu HPLC/CLASS VP 5.021 chromatography system (Shimadzu, Kyoto, Japan) equipped with a Nova-Pak C18 column (4 μm, 150 × 3.9 mm; Waters, Milford, MA), an SPD-10AV UV-visible detector (at 305 nm), and an SIL-10A autoinjector. Acetanilide/0.5% acetic acid in Millipore H2O (25:75, v/v) was used as the mobile phase for isocratic runs at a flow rate of 1 ml/min. Entacapone 3-O-β-D-glucuronide was used for product identification (RT = 3.1 min; see Fig. 2) and quantitation by peak area calculation. Calibration was based on a seven-point (0–4 μM) concentration curve (r ≥ 0.99). The enzyme reaction was linear with incubation time (≤60 min) and protein concentration (25–150 μg/250 μl).

**1-OHP glucuronidation assay.** The reaction mixture contained 25 μl of rat liver microsomes (0.3–3 μg of protein) in 1% bovine serum albumin, 120 μl of 50 mM potassium/sodium phosphate buffer/0.15 M KCl (pH 7.4) with 10 mM MgCl2, 100 μl of 50 mM potassium/sodium phosphate buffer/0.15 M KCl (pH 7.4), with 11 mM UDPGA (samples) or without UDPGA (controls). The reaction was started (after a 5- to 7-min preincubation at 37°C) with 5 μl of 1-OHP in dimethyl sulfoxide, incubated for 10 min, stopped with 50 μl of cold 15% ZnSO4 solution, and cooled for 15 min in an ice bath (protein precipitation) before addition of 500 μl of acetonitrile. The tubes were mixed by vortex (10 s), sonicated (2 min), and centrifuged (13,000g, 10 min, at 20°C). The supernatants were analyzed as described previously (Luukkanen et al., 2001) in a Shimadzu HPLC/CLASS VP 5.021 chromatography system (Shimadzu) using an RF-10AXl fluorescence detector (λex 242 nm, λem 382 nm) and 60% acetonitrile in a 0.5% aqueous acetic acid for isocratic reverse-phase C18 runs. 1-Pyrene β-D-glucuronide was used for product identification (RT = 2.5 min) and quantitation by six-point (0–56 nM) linear calibration curves (r ≥ 0.99) and peak area calculation. The enzyme reaction was linear with incubation time (≤30 min) and protein concentration (0.3–4 μg/250 μl).

**1-Naphthol glucuronidation.** This activity was determined using 70 to 100 μg of rat liver microsomal protein per 250-μl reaction mixture and a 500 μM 1-naphthol substrate concentration; but, otherwise, the reaction conditions were identical to those of the 1-OHP UGT incubation assay described above. The supernatants were diluted (1:31) in 30% acetonitrile/0.5% aqueous acetic acid (eluent) for isocratic analysis (5-μl injection; flow rate 0.9 ml/min) with a reverse-phase C18 guard cartridge (Spherisorb S5 ODS2, 30 × 4 mm) and an analytical column (S3 ODS2, 150 × 4.6 mm, Spherisorb; Waters, Wexford, Ireland) using an RF-10Axl fluorescence detector (λem 290 nm, λex 332 nm) and an SIL-10A Dvp autoinjector in a Shimadzu HPLC/CLASS VP 6.12 SP2 chromatography system. 1-Naphthol β-D-glucuronide was used for product identification (RT = 4.1 min) and quantitation by six-point linear calibration curves (0, 0.1, 0.3, 0.6, 0.8, 1.0, and 1.5 μM; r = 1.00) and peak area calculation. The enzyme reaction was linear with incubation time (≤20 min; protein 150 μg/250 μl) and protein concentration (37–300 μg/250 μl; 10-min incubation).

**Glucuronidation of catechol, 4-methylcatechol, 4-nitrocatechol, DHN, entacapone, and tolcapone.** A thin-layer chromatographic method was used for this purpose (Antonio et al., 2002). Briefly, microsomes (100 μg of protein) were incubated in 100 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl2 in the presence of radiolabeled UDP-[14C]glucuronic acid (2.5 mM, 0.1 μCi) and 0.5 mM catechol substrate dissolved in dimethyl sulfoxide for 1 h at 37°C. The reaction was stopped by addition of 40 μl of ethanol in ice. The precipitated proteins were removed by centrifugation at 10 min at 4000g and the supernatant was loaded onto LK60F silica gel plates (250-μm thickness; Whatman, Clifton, NJ). The plates were developed with a mobile phase composed of n-butanol, acetone, acetic acid, 25% aqueous ammonia, and water (70:50:18:1.5:60 v/v), dried, and sprayed with 1% (v/v) 2-(4-butylphenyl)-5-(4-biphenyl-yl)-1,3-oxadiazole in toluene. The radioactivity associated with the glucuronide was visualized by autoradiography with BioMax MR-2 Kodak films (Sigma-Aldrich) after 3 days of exposure at −20°C. The silica gel was scapped and the radioactivity was measured by liquid scintillation counting with a Tri-Carb 2100 TR spectrometer, with Fluorosafe Safe Ultima Gold (PerkinElmer Life and Analytical Sciences, Rungis, France) as scintillation cocktail.

**Enzyme Kinetics.** Apparent kinetic constants, Km and Vmax, toward entacapone and 1-OHP were determined by fitting the initial glucuronidation velocities (V) and substrate (S) concentrations to the Michaelis-Menten equation by a nonlinear least-squares method (Leonora v1.0; Cambridge University Press, Oxford, UK). The constants were determined also by the Eadie-Hofstee method, by plotting the observations (V versus VS).

**Results**

Effects of Catechol and PCB Pretreatment in the Rats. The effects of administration of catechols were investigated after a 3-day treatment of rats with relatively high oral doses of 4-methylcatechol, 4-nitrocatechol, DHN, entacapone, or tolcapone (Table 1). A high dose of 4-nitrocatechol caused diarrhea in rats. DHN had a clear narcotic and paraplegic effect in one of five rats, but only after the first administration. Plasma ALT/AST levels were analyzed for possible treatment-related hepatotoxicity. A slight elevation of AST (33%) was observed in DHN-exposed rats. Liver weight was not increased in the catechol-treated groups, whereas the PCB-exposed rats exhibited a
hepatomegaly (increases in liver weights, 55%) and increases in plasma AST levels (37%) (Table 1).

**Glucuronidation of Catechols, 1-Naphthol, and 1-OHP in Liver Microsomes of Rats Pretreated with Model Catechols or PCB.**

Induction of catechol glucuronidation upon exposure to five different model catechols (Fig. 1) was assessed by using the inducing agents themselves as probe substrates in vitro. The specific glucuronidation activity toward these catechols and the catechol itself was determined at a 0.5 mM aglycone and a 2.5 mM glucuronic acid substrate level. The results are shown in Fig. 3. The catechol glucuronidation rates, when calculated from the means of the four vehicle-treated subgroups, were in control rat liver as follows (mean ± S.D., n = 4): 0.12 ± 0.04, 0.54 ± 0.16, 5.7 ± 1.1, 6.8 ± 1.2, 7.0 ± 0.5, and 22.0 ± 1.1 nmol/min/mg microsomal protein for entacapone, tolcapone, 4-methylcatechol, catechol, DHN, and 4-nitrocatechol, respectively. A multiphasic difference was consistently observed between the conjugation rates of the COMT inhibitors and the small catechols. The difference displayed between entacapone and 4-nitrocatechol was greater in vehicle-treated (183-fold) and catechol-treated rat groups (77- to 245-fold) than in PCB rats (14-fold), because the PCB treatment stimulated the metabolism of entacapone more than that of 4-nitrocatechol (15-fold versus 1.3-fold). The inducing effect of catechols was weak. No autoinduction was observed.

The ability of catechols to induce glucuronidation metabolism in rat liver was determined by the substrates entacapone, 1-naphthol, and 1-OHP as shown in Table 2. Aroclor 1254 treatment enhanced the glucuronidation of 1-naphthol, entacapone, and 1-OHP by 5-, 8-, and 16-fold, respectively, whereas the catechols exhibited only a weak or no effect at all. Some slight increases were observed in rats treated with 4-methylcatechol (low dose), DHN, entacapone, or tolcapone in the activities toward 1-naphthol (24–30%), entacapone (25–50%), and 1-OHP (11–39%). For the UGT enzyme induction studies shown in Table 2, the substrate concentrations selected for screening of UGT and 1-OHP (11–39%). For the UGT enzyme induction studies shown in Table 2, the substrate concentrations selected for screening of UGT by 1-OHP and entacapone are based on the enzyme kinetics shown in Figs. 4 and 5. The 1-OHP UGT assay is extremely sensitive, allowing activity measurement at low substrate concentrations. When the glucuronidation activity was determined at a concentration as low as 1 μM, the turnover was 16 times faster in PCB microsomes than in control microsomes. At the higher probe concentrations, 15, 100, and 150 μM, the glucuronidation rates were increased 10-fold, 4-fold, and 5-fold, respectively (Table 2).

**Kinetics of Entacapone and 1-OHP Glucuronidation in Rat Liver Microsomes.** The initial velocities of the glucuronidation of 1-OHP (Fig. 4) or entacapone (Fig. 5) were measured in liver microsomes (pooled from five rats per treatment) under conditions with a substrate turnover of ≤10% at tested aglycone concentrations. The apparent Km and Vmax values were determined by the Michaelis-Menten equation for hyperbolic enzyme kinetics. These plots and the corresponding Eadie-Hofstee plots are presented in Figs. 4 and 5. Characterization of 1-OHP glucuronidation in microsomes from control (olive oil) or DHN-, entacapone-, or tolcapone-treated rats gave results consistent with monophasic Michaelis-Menten kinetics. The apparent Km and Vmax values were similar after these treatments, ranging from 6.0 to 6.4 μM and from 8.3 to 10.1 nmol/min/mg, respectively. Variance in intrinsic clearance was small (Vmax/Km, 1.3–1.8 ml/min/mg). For PCB microsomes, the Km was 7.6 μM and the Vmax 22.1 nmol/min/mg (Vmax/Km = 2.9 ml/min/mg). The monophasic or multiphasic nature of the Eadie-Hofstee plots (yielding one or three straight lines) suggested that 1-OHP glucuronidation was catalyzed in catechol compound-treated rats predominantly by one isoenzyme but in PCB microsomes by several forms. Up to three components could be resolved for 1-OHP glucuronidation data: Km1,
Entacapone glucuronidation kinetics were analyzed in control and PCB microsomes, giving the results: $K_m = 237 \mu M, V_{max} = 0.37 \text{ nmol/min/mg} (V_{max}/K_m = 1.6 \times 10^{-3} \text{ ml/min/mg})$ and $K_m = 130 \mu M, V_{max} = 1.62 \text{ nmol/min/mg} (V_{max}/K_m = 12.5 \times 10^{-3} \text{ ml/min/mg})$, respectively. The changes observed in kinetic constants suggested UGT enzyme induction. Despite the present findings implicating an increase in catalytic affinity toward entacapone, only one component was resolved by the Eadie-Hofstee method (Fig. 5).

**Discussion**

UGTs are a superfamily of membrane-bound enzymes, which catalyze the transfer of glucuronic acid from the donor substrate, UDP-GA, on hydroxyl groups of a variety of substances (Clarke and Burchell, 1994; Burchell et al., 1995, 1998; Mackenzie et al., 1997; Tukey and Strassburg, 2000). The reaction leads to the formation of hydrophilic glucuronides, which can be excreted into bile and urine (Wikberg et al., 1993; Jorga et al., 1999; Radominska-Pandya et al., 1999; Antonio et al., 2002, 2003; Keski-Hynninen, 2002). In this work, we determined, using dedicated thin-layer chromatography or HPLC methods, the glucuronidation activity of different catecholic substrates, and of the antiparkinsonian drugs tolcapone and entacapone. The average rates at which these catechols were glucuronidated in liver microsomes of control rats differed up to 183-fold, entacapone being glucuronidated at the lowest rate, whereas 4-nitrocatechol was actively glucuronidated. These data revealed the importance of the structure of the chemical group on the catechol ring on the glucuronidation reaction.

Compared with the effects by the highly potent inducer, PCB, the glucuronidation rate of catechols showed only marginal alterations in response to the present catechol treatments. Under the experimental conditions used here, catechols appeared to present no inducing properties. The data on the inductive effect of PCB on catechol glucuronidation could be favorably compared with those of Antonio et al. (2002), who determined the induction potency of 3-methylcholanthrene, phenobarbital, or the peroxisome proliferator, clofibrate, on the glucuronidation of 41 catechols. The results indicated that only 3-methylcholanthrene could enhance catechol glucuronidation, thus indicating that UGT isoforms (UGT1 family) could be involved in catechol glucuronidation. The PAH type of induction, e.g., by 3-methylcholanthrene, β-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-p-dioxin, PAH, or PCB, is regulated by aryl hydrocarbon receptor, which functions as a ligand-activated transcription factor in over-expression of the forms rUGT1A6, rUGT1A7, hUGT1A6, and hUGT1A9 (Bock et al., 1998, 1999). Other typical coefficients achieved by using Aroclor 1254 as the inducing PCB agent (Safe, 1994) are a phenobarbital type of induction and hepatomegaly, which accounted for a 55% increase in the liver weights (Table 1).
Chrysene had an intermediary effect (a 2-fold increase). Finally, the PAHs, naphthalene, phenanthrene, and pyrene, caused no effect (E. Elovaara and L. Luukkanen, unpublished results).

Entacapone was glucuronidated in rat liver microsomes by an as yet uncharacterized UGT. When we compare the apparent $K_m$ and $V_{max}$ values shown in Fig. 5 with the kinetic parameters reported in human liver microsomes ($K_m = 47 \mu M, V_{max} = 6.8 \text{ nmol/min/mg}$) by Lautala et al. (2000), it appears that entacapone is conjugated by rUGTs less efficiently than by forms expressed in human liver (UGT1A9). Moreover, a manifold induction of entacapone glucuronidation was consistently detected in PCB-treated animals (Table 2; Fig. 3). Enzyme kinetic data (Fig. 5) showed that the apparent $K_m$ value for entacapone-metabolizing activity was lower in PCB rats (130 $\mu M$) than that in control rats (237 $\mu M$).

1-Naphthol was used as a phenol UGT marker substrate for rat UGT1A6. As shown by Jackson et al. (1988), 1-naphthol is one of the most rapidly glucuronidated substrates by this enzyme, which exhibits a restricted specificity toward planar phenols. Also, with this substrate, a significant UGT induction was detected in PCB-treated rats (Table 2). This finding is in agreement with that reported for Aroclor 1254 by Oesch et al. (1992). We have also found that 1-naphthol glucuronidation is enhanced in rat liver 3- to 4-fold upon exposure to PAH-type inducers such as $\beta$-naphthoflavone, benzo(a)pyrene, or 3-methylcholanthrene, whereas exposure to PAHs like naphthalene,
phenanthrene, or pyrene has no effect (E. Elovaara and J. Mikkola, unpublished results).

1-OHP is a sensitive probe for detecting UGT induction by chemical inducers of the PAH type. We have shown that 1-OHP (at a 3 μM concentration) is glucuronidated 5 and 8 times faster in liver microsomes of rats treated by 3-methylcholanthrene and PCB, respectively, than by control microsomes (Luukkanen et al., 1997). 1-OHP is also a UGT-selective substrate as inferred from enzyme kinetic data obtained in rats treated with different model inducers: control < pyrazole < acetone < phenobarbital < 4,4′-methylenebis-(2-chloroaniline) < 3-methylcholanthrene < cresolose < PCB (Vmax/Km, 0.11, 0.19, 0.22, 0.43, 0.46, 2.05, 2.41, and 3.01 ml/min/mg, respectively). Application of 1-OHP as a selective marker of the PAH-type inducible phenol UGT forms (low Km, high Vmax/Km) requires testing at a low substrate concentration. Despite the high sensitivity of the 1-OHP UGT assay, only slight inductive effects could be observed in catechol-pretreated rats, whereas in the PCB-treated rats, the glucuronidation rate rose drastically (Table 2).

The glucuronidation kinetic constants estimated from the Michaelis-Menten and Eadie-Hofstee plots gave very similar values for Km and Vmax. An exception was the 1-OHP kinetic data analyzed in PCB-induced microsomes (Figs. 4 and 5). The enzyme kinetic data of both 1-OHP and entacapone glucuronidation agree with the above findings (Table 2; Fig. 3), which indicate that catechols are poor inducers of hepatic UGT activities in the rat. In the light of the kinetic data, we can conclude that PCB induced several UGT activities and, in particular, high-affinity enzyme forms, probably efficient catalysts of phenolic compounds, including catecholic compounds.

Entacapone (hUGT1A9), 1-naphthol (hUGT1A6), and 1-OHP (hUGT1A6/1A9) are preferentially conjugated by these two human isoforms, which are, in most cases, recognized as the major catalysts of catechol glucuronidation. Large differences are, however, observed in the glucuronidation rates among individual catechols as found here and in previous studies (Antonio et al., 2002; Taskinen et al., 2003). For this reason, it is important to study in each case, also, the glucuronidation by the catechol of interest. Although it is unclear by which UGTs entacapone, 1-naphthol, and 1-OHP are catalyzed in rat liver, they may be used as sensitive tools for screening ratUGT activity that may also catalyze the glucuronidation of many catechols. Compared with the assay methods based on entacapone and 1-naphthol, the great advantage provided by the 1-OHP glucuronidation assay is its extraordinarily high sensitivity. By using 1-OHP as a surrogate substrate, it is possible to quantify UGT activity (that is not readily detected by catechol glucuronidation methods) in micro-size samples such as human liver microsomes (E. Elovaara, J. Mikkola, and O. Pelkonen, unpublished results) or very-low-activity samples such as human blood lymphocytes (E. Elovaara and J. Mikkola, unpublished results). Our findings suggest that induction of UGTs by catecholic compounds is very low in comparison with an effect displayed by potent inducers such as the Aroclor 1254-PCB mixture. Based on this and previous work, it appears that PAH-type enzyme inducers are the most powerful agents, which may greatly enhance the activity of catechol-metabolizing UGTs in the liver.

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References

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