**Regular Article**

*Evaluation of Dextromethorphan Metabolism Using Hepatocytes from CYP2D6 Poor and Extensive Metabolizers*

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**Summary:** It is important to estimate the defective metabolism caused by genetic polymorphism of drug metabolizing enzymes before the clinical stage. We evaluated the utility of cryopreserved human hepatocytes of CYP2D6 poor metabolizer (PM) for the estimation of the metabolism in PM using dextromethorphan (DEX) as the probe drug for CYP2D6 substrate.

The results of low formations of dextrorphan (DXO) and 3-hydroxymorphinan (3-HM) in CYP2D6 PM hepatocytes incubated with dextromethorphan reflected the clinical data. Formation of 3-methoxymorphinan (3-MEM) normalized by CYP3A4 activity in the PM hepatocytes reached about 2.8-fold higher than that in CYP2D6 extensive metabolizer (EM) hepatocytes, which clearly showed the compensatory metabolic pathway of O-demethylation catalyzed by CYP2D6 as seen in clinical study. On the contrary, in the condition of the EM hepatocytes with CYP2D6 inhibitors, the enhancement of 3-MEM formation was not observed. In phase II reaction, the glucuronide formation rate of DXO in the PM hepatocytes was lower than that in the EM hepatocytes, which was consistent with clinical data of DXO-glucuronide (DXO-glu) concentration. These results would suggest that CYP2D6 PM hepatocytes could be a good *in vitro* tool for estimating CYP2D6 PM pharmacokinetics.

**Key words:** CYP2D6 poor metabolizer; CYP2D6 extensive metabolizer; cryopreserved human hepatocytes; dextromethorphan; 3-methoxymorphinan; alternative metabolic pathways

**Introduction**

Genetic polymorphisms of cytochrome P450 isoforms have been shown to have clinical consequences resulting in toxicity of some drugs in the affected individual, and may alter any efficacy of other drugs. The defect of CYP2D6 gene is known to be responsible for poor metabolism of debrisoquine or sparteine. It has been reported that CYP2D6 oxidizes more than 120 drugs and plays a clinically significant role in the metabolism of over 50 drugs. Those who have homozygous alteration in this recessive gene, are so-called “poor metabolizers (PM)”, and can suffer from severe side effects due to defective metabolism of drugs. Such genetic alterations occur at rates of 1-30% in different ethnic populations. Therefore, it is important to estimate the defective metabolism of drugs caused by genetic polymorphism of drug metabolizing enzymes before the clinical stage, especially at the drug discovery stage.

*In vitro* experimental models using human liver materials are the good tools for the estimation of the metabolism of human pharmaceuticals. Especially, the present consensus is that hepatocytes, which contain full complements of enzymes and cofactors at physiological levels and natural orientations, could be more representative of the liver *in vivo* than liver slices or cell free system such as S9 fractions, microsomes and cytosol fractions. Recently screening assays using cryopreserved human hepatocytes with average metabolic properties have been developed in drug discovery stage. However, studies using PM hepatocytes have never reported yet and usefulness of PM hepatocytes has been little discussed. Under these circumstances, when we have an *in vitro* assay method for estimating drug metabolism in PM, that would be a great tool for drug discovery. Therefore we report the new method using PM hepatocytes for estimating drug metabolism in PM.

Dextromethorphan (DEX), which is widely used for the treatment of pain and cough, is suggested as a kind of ideal probe for phenotyping study of polymorphic drug metabolism by CYP2D6 because of its safety and
As shown in Fig. 1, it has been reported that CYP2D6 is responsible for O-demethylation of DEX to dextrorphan (DXO), and CYP3A4 is involved in N-demethylation of DEX to 3-methoxymorphinan (3-MEM). In fact, DXO and 3-MEM concentrations against DEX concentration provide reliable index of CYP2D6 and CYP3A4 activity in the phenotyping study, respectively. The clinical data have been reported that significant differences are observed in plasma concentration, AUC and half-lives of DEX and its metabolites (DXO, 3-MEM, 3-hydroxymorphinan (3-HM) and DXO-conjugates) between CYP2D6 extensive metabolizer (EM) and CYP2D6 PM. Especially, the result that 3-MEM concentrations in CYP2D6 PM is higher than those in EM is often observed in several reports. Namely, it is considered that the enhancement of the alternative metabolic pathway of DXO metabolism to 3-MEM is observed in CYP2D6 PM.

Some in vitro methods such as a method of EM hepatocyte assay with CYP2D6 inhibitor might be a plausible assay system for the estimation of the metabolism in PM. Here in this report, the metabolic studies were conducted in CYP2D6 PM hepatocytes using DEX as a chemical probe, and then the results of CYP2D6 PM hepatocyte studies are compared to other in vitro system.

**Materials and Methods**

**Reagents:** DEX (HBr salt), DXO (D-tartrate salt), 3-MEM (HCl salt), levallorphan (D-tartrate salt) was purchased from Sigma/RBI (Natick, MA). 3-HM (HBr salt), midazolam (MDZ) HCl and 4-hydroxymidazolam (4OH-MDZ) were purchased from Ultrafine Chemicals and Research Ltd. (Manchester, UK). Dextrorphan-O-glucuronide (DXO-glu) was obtained from Toronto Research Chemicals Inc. (Ontario, Canada). Krebs Henseleit buffer and L-15 Medium LEIBOVITZ were purchased from Sigma Chemicals (St. Louis, MO). Solvents and the other reagents were from common sources and were of HPLC grade or better.

**Materials:** Cryopreserved human hepatocytes were purchased from In Vitro Technologies, Inc. (Baltimore, MD). Lot ETR was PM hepatocytes of Caucasian having CYP2D6 *4/*4 alleles. Lot VTA and 64 were PM hepatocytes of Caucasian having CYP2D6 *3/*4 alleles. Lot ENR, IZG and 87 were CYP2D6 EM hepatocytes (wt/wt).

**Metabolic incubations in cryopreserved human hepatocytes:** On the day of an experiment cryopreserved hepatocytes were thawed rapidly in a water bath at 37°C, then diluted (1:50) with ice-cold thawing medium (L-15 Medium LEIBOVITZ and 0.5 g/L glucose). Cells were spun down for 3 minutes at 50 g then resuspended in ice-cold assay buffer (Krebs Henseleit buffer) containing 0.373 g/L calcium chloride dihydrate, 2.1 g/L sodium bicarbonate and 1.5 g/L HEPES. Cell viability was measured using trypan-blue exclusion. Cell viabilities were between 60 and 84%. Incubation reactions for the determination of the enzymatic kinetic parameters were carried at a cell concentration of 1.0 million cells/mL. Cells were preincubated for 30 minutes at 37°C before addition of an equal volume of assay medium containing several concentrations of substrates (final conc.: 2, 10, 50 μM DEX and 10 μM MDZ), and then were incubated unshaken at 37°C in a 95% air/5% CO2 atmosphere. Incubations were stopped at 0, 30 and 60 minutes by the
addition of an equal volume of acetonitrile, and then a part of these incubates were diluted by the 4-fold of 10% acetonitrile containing an LC-MS-MS internal standard (levallorphan at 100 ng/mL final conc.). The mixtures were centrifuged for 10 min at 770 g, and supernatants were used for LC-MS-MS analyses to determine the production rates of DXO, 3-MEM, 3-HM, DXO-glu and 4OH-MDZ.

Metabolic incubations in cryopreserved human hepatocytes under the condition of CYP2D6 inhibition: After preparing cell suspensions as described above, cells were preincubated for 30 minutes before addition of an equal volume of assay medium containing several concentrations of substrates (final conc.: 2, 10, 50 μM DEX and 10 μM MDZ) containing 10 μM quinidine. Subsequently, cells were incubated unshaken at 37°C in a 95% air/5% CO₂ atmosphere. Incubations and sample preparation was conducted as described above.

LC-MS-MS conditions: Substrates and metabolites were measured by LC-MS-MS using the HP-1100 HPLC (Agilent Technologies Inc., Germany) and API-4000 (PerkinElmerSCIEX Instruments, Boston, MA) with a turbo ionspray interface. Multiple reactions monitoring of positive-ion mode were used for all analyses. Analytical conditions including the selection of ions and parameters for multiple reaction monitoring were automatically obtained for each compound by the application software, Automaton (PerkinElmerSciex Instruments). Mass number (m/z) of precursor ions and product ions for each compound was identified as follows (precursor ion to product ion): m/z 272.2 to 215.1 for DEX, m/z 258.2 to 156.9 for DXO, m/z 258.3 to 215.0 for 3-MEM, m/z 244.2 to 156.9 for 3-HM, m/z 434.3 to 258.3 for DXO-glu, m/z 326.1 to 291.1 for MDZ, m/z 342.0 to 296.9 for 4OH-MDZ and m/z 284.1 to 156.7 for levallorphan. A fast-gradient condition using two switching valves and pumps was used as an analysis for the samples. For DEX and its metabolites, YMC J’sphere ODS-L80 3 μm, 2.0 mm × 35 mm column was used as an analytical column, and the flow rate of 0.50 mL/min of acetonitrile/water (10:90) containing 10 mM ammonium acetate was the initial condition used. After 0.5 min of the sample injection, the ratio of acetonitrile/water was changed to 80:20 linearly for 1.0 min and maintained for the next 1.0 min. The column was then washed with acetonitrile/water (10:90) containing 10 mM ammonium acetate. For MDZ and 4OH-MDZ samples, Phenomenex C18 (2) Luca 3 μm, 2.0 mm × 50 mm column was used as an analytical column, and the flow rate of 0.35 mL/min of acetonitrile/water (14:86) containing 20 mM acetic acid (pH 4.0) was the initial condition used. After the injection of a sample, the ratio of acetonitrile/water was changed to 50:50 linearly for 4 min and maintained for the next 0.5 min. The column was then washed with acetonitrile/water (14:86) containing 20 mM acetic acid (pH 4.0).

### Table 1. Correlations between metabolite formation rates of DEX in cryopreserved human hepatocytes.

<table>
<thead>
<tr>
<th>CYP2D6 genotype</th>
<th>Lot#</th>
<th>DEX conc. (μM)</th>
<th>Metabolite formation rate (pmol/min/million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DXO</td>
</tr>
<tr>
<td>EM (wt/wt)</td>
<td>ENR</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>IZG</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>PM (*3/*4)</td>
<td>64</td>
<td>2</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>VTA</td>
<td>2</td>
<td>&lt;0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>1.8</td>
</tr>
<tr>
<td>PM (*4/*4)</td>
<td>ETR</td>
<td>2</td>
<td>&lt;0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Results

Metabolite formation from DEX in CYP2D6 PM/EM hepatocytes: In order to compare the metabolic properties of CYP2D6 PM and EM hepatocytes, the metabolite formation rates were used as indices of metabolic activity. Formation rates of DEX metabolites (DXO, 3-MEM, 3-HM and DXO-glu) in the PM and EM hepatocytes are summarized in Table 1. DXO formation in the PM hepatocytes increased proportionally as increasing DEX concentration, whereas DXO formation was saturated from 2 μM DEX incubation in the EM hepatocytes (Table 1). DXO formation showed by far the highest activity of all tested metabolites in the EM hepatocytes, resulting in the main clearance route of DEX in the EM hepatocytes. In contrast with those results, the metabolic pattern in the PM hepatocytes was quite different from that in the EM hepatocytes; namely 3-MEM formation reached the similar rates to DXO formation. DXO formation rates in the EM hepatocytes were at least 2.5 times higher than those in the PM hepatocytes (Table 1). These differences of DXO formation between the PM and EM hepatocytes were consistent with the clinical results.19,20 DXO-glu formation followed by DXO production from DEX in the PM hepatocytes were lower than those in the EM hepatocytes at the concentration of 2 and 10 μM DEX (Table 1). These differences in DXO-glu formation between the EM and PM hepatocytes were thought to be dependent on the differences in DXO formation rates.
even in considering the differences of glucuronyl transferase activity among hepatocyte batches (data not shown). DXO-glu formations in the PM hepatocytes increased proportionally as increasing DEX concentration, whereas DXO-glu formations decreased from 10 to 50 μM DEX incubation in the EM hepatocytes (Table 1). In the clinical data, plasma concentrations of DXO-conjugates were 4–12 fold higher than those of DXO.19,20) In contrast, DXO-glu formations in any tested hepatocytes were low, corresponding to maximally a 20% of DXO formations.

Formation of 3-MEM in CYP2D6 EM and PM hepatocytes ranged from 0.24 to 12 and from 0.25 to 11 pmol/min/million cell, respectively (Table 1). Formation rate of 3-MEM in the EM hepatocytes was only 2 to 17% of DXO formation, whereas in the PM hepatocytes reached equivalent to DXO formation, which means 3-MEM formation against DXO formation (3-MEM/DXO ratio) in the PM hepatocytes was no less than 200 times as high as that in the EM. These large 3-MEM/DXO ratios in the PM hepatocytes suggest that 3-MEM formation would be one of the main clearance routes in the PM hepatocytes as a compensatory metabolic pathway. Based on the results of 3-MEM formation described above, further examination how 3-MEM formation increased in the PM hepatocytes was carried out. At first, as CYP3A4/5 activities were much different in each hepatocyte, 3-MEM formation was normalized by CYP3A4/5 activity. As the ratio of CYP3A5 to CYP3A4 on intrinsic clearance of 3-MEM formation (1.1) was very close to those of 4OH-MDZ (0.88), formation rate of 4OH-MDZ from MDZ were used for the normalization of CYP3A4/5 activity.16,21) Thus, mean 3-MEM/4OH-MDZ formation ratio in the PM hepatocytes reached no less than 2.8-fold higher than that of EM hepatocytes (Fig. 2). These data suggest that CYP3A4/5-catalyzed N-demethylation reaction would be proceeded in the PM hepatocytes as an alternative metabolic pathway of CYP2D6-catalyzed O-demethylation. This enhancement of alternative metabolic pathway from CYP2D6 to CYP3A4/5 was also observed in the clinical data, which 3-MEM concentrations in the PM plasma were at least 2.5 times higher than those in the EM.17,19) Formation of 3-HM, which was produced by both N- and O-demethylation reaction in DEX, was observed only in CYP2D6 EM hepatocytes at relatively low levels (less than 0.81 pmol/min/million cells shown in Table 1). Formation of 3-HM was hardly observed in CYP2D6 PM hepatocytes (Table 1). These data that small amount of 3-HM was detected in the case of the EM hepatocytes, whereas no 3-HM was observed in the PM hepatocytes reflected the clinical results.19)

**Table 1.** Metabolite formation rates of DEX in cryopreserved human hepatocytes under the condition of CYP2D6 inhibition by quinidine.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Lot #</th>
<th>DEX conc. (μM)</th>
<th>Metabolite formation rate (pmol/min/million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ENR</td>
<td>25</td>
<td>DXO: 0.53, 3-MEM: 0.39, 3-HM: 0.39, DXO-glu: 5.0</td>
</tr>
<tr>
<td>Quinidine</td>
<td>ENR</td>
<td>51</td>
<td>DXO: 2.8, 3-MEM: 0.87, 3-HM: 0.87, DXO-glu: 5.8</td>
</tr>
<tr>
<td>+ 10 μM</td>
<td>ENR</td>
<td>2.9</td>
<td>DXO: &lt;0.20, 3-MEM: &lt;0.20, 3-HM: 0.39, DXO-glu: &lt;0.20</td>
</tr>
</tbody>
</table>

**Table 2.** Metabolite formation rates of DEX in cryopreserved human hepatocytes under the condition of CYP2D6 inhibition by quinidine.
cells) were almost the same with those of the condition in the presence of quinidine (40 pmol/min/million cells), 3-MEM formation was not thought to be enhanced under the condition of CYP2D6 inhibition by quinidine.

Discussion

DEX metabolism of CYP2D6 PM and EM hepatocytes reflected the clinical results as reported in this study. Especially, the differences of DXO and DXO-glu formation were observed in the PM and EM hepatocytes. In addition, the alternative metabolic pathway regarding 3-MEM formation increased in the PM hepatocytes as shown in clinical results. However, inhibition studies using the EM hepatocytes didn’t show the enhancement of the alternative metabolic pathway.

The mechanism for the enhancement of the alternative metabolic pathway is unknown, but the increase of 3-MEM/4OH-MDZ ratio in CYP2D6 PM hepatocytes is thought to be independent of various factors derived from CYP3A4/5 such as the expression of CYP3A4/5. In addition, since CYP3A4/5 is primarily responsible for 3-MEM formation (more than 92% by relative activity factor) and the contribution of other CYPs (CYP2B6, 2D6, 2C9 and 2C19 etc.) seemed to be insignificant, the increase of 3-MEM/4OH-MDZ ratios in the PM hepatocytes was not responsible for any CYPs other than CYP3A4/5. CYP2D6 is mainly responsible for DXO formation (about 80%), whereas CYP2C9 and CYP3A4/5 also catalyze the reaction of DEX to DXO reaction with the contribution of 4 and 16% against whole metabolism, respectively, as reported previously. The saturation of DXO formation in CYP2D6 EM hepatocytes can be elucidated by the saturation of CYP2D6 reaction (K_m value in human liver microsomes: 5 μM). By contrast, concentration dependent manner of DEXO formation in CYP2D6 PM hepatocytes would be caused by some CYPs other than CYP2D6 or the insufficient CYP2D6 metabolism.

In the clinical data, DXO-conjugates are detected as some of final metabolites of DEX in plasma and urine. DXO-glu is considered to be one of the major conjugates of DXO, and its formation rates in hepatocytes are measured as the representative of conjugation reaction of DXO in this study. The results that DXO-glu formations in CYP2D6 PM hepatocytes were lower than those in CYP2D6 EM hepatocytes were consistent with the clinical results. However, the results of DXO-glu formation in hepatocytes were different from clinical results: the DXO-glu formations in the EM hepatocytes were maximally 20% of DXO formations, whereas plasma concentrations of DXO-conjugates were maximally 12 fold higher than those of DXO in the clinical data. As tested hepatocytes have enough activities of glucuronide conjugation such as naloxone metabolism (data not shown), the data of low DXO-glu formation are not due to the enzyme activity itself in hepatocytes, but might be caused by the other mechanisms such as transport of substrate into or out of hepatocytes. Meanwhile, DXO-glu formation rates decreased along with increasing DEX concentration from 10 to 50 μM in the EM hepatocytes. Further studies would be conducted to examine whether this result of DXO-glu formation is specific in hepatocyte assay or not.

In consequence, the study using CYP2D6 PM hepatocytes would be more useful for estimating the metabolism in PM. Especially, the enhancement of alternative metabolic pathway of CYP3A4/5 reaction could be observed in CYP2D6 PM hepatocytes. Furthermore, the differences in metabolite formation containing conjugates could be determined as well as oxidative metabolites.

Drug metabolizing enzyme cytochrome P450 is known to have several other genetic polymorphisms besides CYP2D6, examples of which include CYP3A4, CYP3A5, CYP3A7, CYP2C9, CYP2C19, CYP2A6, CYP1A1, CYP1A2 and CYP2E1. A lot of effort has been made toward addressing polymorphism issues for developing the new drug. PM hepatocytes of these genetic polymorphisms could also be used in the similar evaluation system as discussed in the present report, which provides the better solution for the issue. In addition, as the supply system of the human hepatocytes is recently getting better, it would be also possible to apply this PM hepatocyte studies to the evaluation of the drug candidates before clinical study, furthermore, to the high throughput screening in the early drug discovery stage.

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