Original Article

Effect of diphenoxylate on CYP450 isoforms activity in rats

Peiwu Geng1*, Jinzhang Cai2*, Shuanghu Wang1, Suping Yang3, Zezheng Liu3, Yingying Lin3, Congcong Wen3, Xianqin Wang4*, Yunfang Zhou1, Meiling Zhang3,4

1Laboratory of Clinical Pharmacy, The People’s Hospital of Lishui, Wenzhou Medical University, Lishui 323000, China; 2The Second Affiliated Hospital & Yuying Children’s Hospital, Wenzhou Medical University, Wenzhou 325000, China; 3Analytical and Testing Center of Wenzhou Medical University, Wenzhou 325035, China; 4Shanghai Key Laboratory of Forensic Medicine, Institute of Forensic Science, Ministry of Justice, Shanghai 200063, China. *Equal contributors.

Received August 21, 2015; Accepted October 13, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: In order to investigate the effects of diphenoxylate on the metabolic capacity of cytochrome P450 (CYP) enzymes, a cocktail method was employed to evaluate the activities of CYP2B6, CYP2D6, CYP2C19, CYP1A2, CYP3A4, CYP2C9. The rats were randomly divided into diphenoxylate group (Low, Medium, High) and control group. The diphenoxylate group rats were given 12, 24, 48 mg/kg (Low, Medium, High) diphenoxylate by continuous intragastric administration for 7 days. Six probe drugs bupropion, metropolol omeprazole, phenacetin, testosterone and tolbutamide were given to rats through intragastric administration, and the plasma concentrations were determined by UPLC-MS/MS. Statistical pharmacokinetics difference for omeprazole, phenacetin and tolbutamide in rats were observed by comparing diphenoxylate group with control group. Continuous 7 days-intragastric administration of diphenoxylate induces the activities of CYP2C19, CYP1A2 and CYP2C9 of rats. Induction of drug metabolizing enzyme by diphenoxylate would reduce the efficacy of other drug. Additionally, high dosage diphenoxylate may cause hepatotoxicity.

Keywords: CYP450, diphenoxylate, cocktail, UPLC-MS/MS, rat

Introduction

Diphenoxylate, a weak opioid agonist has been considered as a drug of low abuse potential. There have been however, only case reports of diphenoxylate being abused by patients with medical illnesses and substance users [1, 2]. It has also been tried during detoxification from opioids and has been shown to decrease withdrawals [3, 4]. Diphenoxylate is useful in the treatment of protracted diarrhea in children and disorders in which rapid gastrointestinal transit is the main cause of diarrhea. However, it is not recommended for acute infective diarrhea in childhood. Diphenoxylate has also been found to provide substantial relief from detoxification symptoms in opioid-dependent patients with no drug-associated adverse effects [1].

Cytochrome P450 (CYP) enzymes are responsible for most biotransformation steps of xenobiotics and endogenous molecules [5]. Variations of their activity by inhibition or induction can influence the pharmacokinetics and thereby the effect of drugs (of abuse). Enzyme inhibition by co-administered drugs (of abuse) and/or genetic variations of their expression can increase the risk of adverse reactions [6] or reduce the desired effect [7]. Such drug-drug interactions were described as a major reason for hospitalization or even death [8].

So far, no study on the effects of diphenoxylate on the metabolic capacity of CYP enzyme was reported. Therefore, in this study, six probe drugs were employed to evaluate effect of diphenoxylate on the metabolic capacity of CYP2B6, CYP2D6, CYP2C19, CYP1A2, CYP3A4, CYP2C9. The effects of diphenoxylate on rat CYP enzyme activity will be evaluated according to the pharmacokinetic parameters changes of
Effect of diphenoxylate on CYP450 isoforms activity of rats

Table 1. Pharmacokinetic parameters of bupropion and metoprolol from control group and diphenoxylate group rats (mean ± SD, n=8)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC_{(0-t)}</th>
<th>AUC_{(0-∞)}</th>
<th>t_{1/2}</th>
<th>CL</th>
<th>V</th>
<th>C_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/mL *h</td>
<td>ng/mL *h</td>
<td>h</td>
<td>L/h/kg</td>
<td>L/kg</td>
<td>ng/mL</td>
</tr>
<tr>
<td>Bupropion (CYP2B6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>974.9±1167.4</td>
<td>1024.4±1147.6</td>
<td>1.8±1.1</td>
<td>15.8±7.8</td>
<td>46.6±42.6</td>
<td>527.0±623.5</td>
</tr>
<tr>
<td>Low</td>
<td>1194.1±1025.4</td>
<td>1512.1±1817.8</td>
<td>1.8±1.6</td>
<td>12.6±7.6</td>
<td>24.5±15.1</td>
<td>348.3±238.1</td>
</tr>
<tr>
<td>Medium</td>
<td>1106.6±449.0</td>
<td>1156.5±455.7</td>
<td>1.7±0.5</td>
<td>9.8±3.5</td>
<td>25.5±17.9</td>
<td>333.8±147.1</td>
</tr>
<tr>
<td>High</td>
<td>1456.9±1014.0</td>
<td>2877.1±3298.5</td>
<td>5.6±5.6</td>
<td>10.2±11.2</td>
<td>48.8±43.0</td>
<td>369.7±285.1</td>
</tr>
<tr>
<td>Metroprolol (CYP2D6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>890.7±848.9</td>
<td>906.1±849.2</td>
<td>1.2±0.4</td>
<td>16.7±9.9</td>
<td>28.8±13.8</td>
<td>463.5±468.4</td>
</tr>
<tr>
<td>Low</td>
<td>1120.5±738.5</td>
<td>2045.7±3141.1</td>
<td>3.2±5.0</td>
<td>10.9±5.8</td>
<td>25.6±13.0</td>
<td>365.4±172.6</td>
</tr>
<tr>
<td>Medium</td>
<td>1184.1±558.6</td>
<td>1295.7±552.3</td>
<td>2.2±0.9*</td>
<td>8.9±3.2</td>
<td>30.4±19.0</td>
<td>337.4±183.9</td>
</tr>
<tr>
<td>High</td>
<td>1550.2±1010.5</td>
<td>2294.0±1697.1</td>
<td>3.9±2.2**</td>
<td>8.2±7.6</td>
<td>39.7±32.0</td>
<td>367.5±239.7</td>
</tr>
</tbody>
</table>

Diphenoxylate group was compared with the control group, *: P<0.05, **: P<0.01.

six specific probe drugs (bupropion, metoprolol, omeprazole, phenacetin, testosterone and tolbutamide).

Material and methods

Chemicals

Bupropion, metoprolol, omeprazole, phenacetin, testosterone and tolbutamide (all >98%) and the internal standard diazepam (IS) were obtained from Sigma-Aldrich Company (St. Louis, USA). Ultra-pure water was prepared by Millipore Milli-Q purification system (Bedford, USA). Methanol and acetonitrile (HPLC grade) were obtained from Merck Company (Darmstadt, Germany).

Animals

Sprague-Dawley rats (male, 220±20 g) purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Animals were housed under a natural light-dark cycle conditions with controlled temperature (22°C). All forty rats were housed at Laboratory Animal Research Center of Wenzhou Medical University. All experimental procedures were approved ethically by the Wenzhou Medical University Administration Committee of Experimental Animals.

Pharmacokinetics

Forty rats (220±20 g) were randomly divided into four different dosages of diphenoxylate groups (Low-group, Medium-group, High-group and control group with 8 rats in each group). Diphenoxylate was dissolved in corn oil as suspension at three different concentrations (12, 24, and 48 mg/mL). Three different diphenoxylate group (Low-group, Medium-group, High-group) were respectively give diphenoxylate 12, 24, 48 mg/kg one time by intragastric administration at every morning, and last for 7 days. Control group were give saline by same administration method. At 8 days morning, six probe drugs bupropion, metoprolol, omeprazole, phenacetin, testosterone and tolbutamide were respectively give to rats of three diphenoxylate groups and control group by intragastric administration at a single dosage 10 mg/kg for bupropion, metoprolol, omeprazole, phenacetin, testosterone, 1 mg/kg for tolbutamide.

Blood (0.3 mL) samples were collected into heparinized 1.5 mL polystyren tubes from the tail vein at 0.0833, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48 h after intragastric administration of six probe drugs. 100 μL of plasma was obtained from blood sample after centrifugation at 4000 g for 10 min. In a 1.5 mL centrifuge tube, 200 μL of acetonitrile (containing 50 ng/mL IS) was added into 100 μL of collected plasma sample. After vortex-mixing for 1.0 min, the sample was centrifuged at 13000 g for 15 min. Then supernatant (2 μL) was injected into the UPLC-MS/MS system for analysis.

Concentration of plasma probe drugs versus time was analyzed by Version 3.0 Data Analysis System (Wenzhou Medical University, China). The main pharmacokinetic parameters of the diphenoxylate group and control group were analyzed by SPSS 18.0 statistical software; statistical significance was assessed by t-test (P<0.05 was considered as statistically significant).
Effect of diphenoxylate on CYP450 isoforms activity of rats

UPLC-MS/MS determination of probe drugs

The concentration of bupropion, metoprolol omeprazole, phenacetin, testosterone and tolbutamide in rat plasma were simultaneously determined by a sensitive and simple UPLC-MS/MS method [9]. The compounds were analyzed by a UPLC-MS/MS with ACQUITY I-Class UPLC and a XEVO TQD triple quadrupole mass spectrometer that was equipped with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA). Data acquisition and instrument control were performed on the Masslynx 4.1 software (Waters Corp., Milford, MA, USA).

Bupropion, metoprolol omeprazole, phenacetin, testosterone, tolbutamide and diazepam (IS) were separated using a Waters BEH C18 column (2.1 mm×100 mm, 1.7 μm) at constant temperature 40°C. The initial mobile phase consisted of 0.1% formic acid and acetonitrile with gradient elution at a flow rate of 0.4 mL/min and an injection volume of 2 μL. Elution was in a linear gradient, with the acetonitrile changing from 30 to 60% in 0.3-1.8 min and increasing up to 95% over 0.2 min. The acetonitrile content was maintained at 95% for 0.5 min and decreased to 30% within 0.1 min, and then maintained at 30% for 0.4 min.

The mass spectrometric detection was performed in a positive mode. Nitrogen was used as the cone gas (50 L/h) and desolvation gas (1000 L/h). The mass conditions were set as follows: source temperature 150°C; capillary voltage 2.5 kV; desolvation temperature 500°C. The multiple reaction monitoring (MRM) mode with m/z 240.1→184.1 for bupropion, m/z 268.1→115.8 for metoprolol, m/z 345.1→197.8 for omeprazole, m/z 180.1→109.9 for phenacetin, m/z 289.0→97.0 for testosterone, m/z 271.2→155.1 for tolbutamide, and m/z 285.1→193.1 for IS was used for quantitative analysis.

The LLOQ for each probe drug in plasma was 2 ng/mL. The RSD of the six probe drugs were less than 15%. The calibration plot of the probe drugs is in the range of 2-2000 ng/mL (r>0.995). The intra-day and inter-day accuracy were less than 15%.
Effect of diphenoxylate on CYP450 isoforms activity of rats

Figure 1. The pharmacokinetic profiles of bupropion (A), metoprolol (B), omeprazole (C), phenacetin (D), testosterone (E), tolbutamide (F) in control group and diphenoxylate group (low, medium, high) rats (n=8).

ranged from 90% to 115%. The matrix effects were more than 82% or less than 113%. The extraction recoveries were better than 85%.

Histopathology

After pharmacokinetic properties analysis, rats were deeply anesthetized with 10% chloral hydrate (i.e., 20 mg/kg). The liver of control group and diphenoxylate treated groups were rapidly isolated and immersed in freshly prepared 4% w/v formaldehyde (0.1 M phosphate buffer, pH 7.2) for 48 h, and then embedded in paraffin. Then 5 µm-thick histologic sections were prepared and stained with routine HE method (hematoxylin and eosin). The morphological changes of liver were observed under light microscope.
Effect of diphenoxylate on CYP450 isoforms activity of rats

Figure 2. Morphological changes of liver in diphenoxylate treated group at low (A), medium (B), high (C) dosage and control group (D) (hematoxylin-eosin staining, ×100).

Results

Pharmacokinetics

The main pharmacokinetic parameters of bupropion, metoprolol, omeprazole, phenacetin, testosterone and tolbutamide calculated from non-compartment model analysis were summarized in Tables 1-3. The representative profiles of concentration of drugs (bupropion, metoprolol, omeprazole, phenacetin, testosterone and tolbutamide) vs. time were presented in Figure 1.

From the Table 1, compared with the control group, no difference in pharmacokinetic behaviors can be observed between low, medium dosage group and control group for bupropion and metoprolol. While from the Table 2, the pharmacokinetic behaviors of omeprazole in high dosage group compared with the control group, AUC$_{0-t}$ decreased ($P<0.01$), $C_{max}$ decreased ($P<0.05$), and no difference in pharmacokinetic behaviors can be observed between low, medium dosage group and control group. The pharmacokinetic behaviors of phenacetin in medium and high dosage group compared with the control group, AUC$_{0-t}$ decreased (medium, $P<0.05$; high, $P<0.01$), CL increased (medium, $P>0.05$; high, $P<0.01$), $C_{max}$ decreased (medium, $P<0.01$; high, $P<0.01$), and no difference in pharmacokinetic behaviors can be observed between low dosage group and control group. From the Table 3, compared with the control group, no difference in pharmacokinetic behaviors can be observed between low, medium dosage group and control group for testosterone. While for tolbutamide, compared with the control group, AUC$_{0-t}$ decreased (low, $P<0.05$; median and high, $P<0.01$), CL increased (Low and median, $P<0.05$; high, $P<0.01$), $C_{max}$ decreased (low, $P>0.05$; median, $P<0.05$; high, $P<0.01$).
**Morphological changes of liver**

There is no significant difference between control group and low dose group in the liver lobules at low magnification, Figure 2. Both of them are intact and liver cells were arranged tightly along with central veins. In middle dose group, the structure of liver lobule is still intact and can be recognized easily. There liver cells arranged regularly, and there was no swelling in them. The nucleus of liver cells is round, clear and fine luster. However, the cell gap between liver cells seems becoming larger than control group and low dose group.

While, compared with control group the intact structures of liver lobule are damaged obviously in high dose group. The regular and tight arrangement of liver cells along with central veins was disappeared. Most of liver cells are arranged in disorder. Beside, the some gaps of hepatic sinusoid decreased and disappeared, and the liver cells became edema and appeared to be vacuolization.

**Discussion**

In general, changes in pharmacokinetics are thought to be caused by drug-drug or drug-food interactions [10]. In pharmacokinetic interactions, approximately 65% of drug-drug interactions occur in metabolic sites, and drug metabolic enzymes are considered to be the most important interactive sites. A large number of drugs are metabolized by CYP enzymes in the liver, and more than 90% of drug-drug interactions occur at the CYP-catalyzed step [11, 12]. Similarly, supplement-drug interactions involving CYP activity are occasionally found to cause considerable adverse events. For these reasons, we evaluated the effects of acute paraquat poisoning on the activity of CYP enzymes in vivo. We selected CYP isoforms CYP1A2, CYP2D6, CYP3A4, CYP2C19, CYP2C9 and CYP2B6 because more than 90% of drugs are known to be metabolized by these 6 CYP enzymes [13, 14].

There no significant difference for AUC, CL and $C_{\text{max}}$ of bupropion, metoprolol and testosterone between the diphenoxylate group (low, median, high) and control group was observed. It suggested that the diphenoxylate was not able to induce or inhibit the activity of CYP2B6, CYP2D6 and CYP3A4 enzyme. The pharmacokinetic parameters of omeprazole experienced obvious change with decreased AUC$_{0-t}$ and increased CL after the dosage increase. This result indicates that the 7 days-intragastric administration of diphenoxylate with high dosage slightly induces the metabolism of omeprazole (CYP2C19) in rat. The pharmacokinetic parameters of phenacetin experienced obvious change with decreased AUC$_{0-t}$ and increased CL after the dosage increase. This result indicates that the 7 days-intragastric administration of diphenoxylate with medium dosage slightly induces the metabolism of phenacetin (CYP1A2) in rat, and the high dosage group induces the metabolism of phenacetin (CYP1A2), this results was consistent with Figure 1D. The pharmacokinetic parameters of tolbutamide experienced obvious change with decreased AUC$_{0-t}$ and increased CL after the dosage increase. This result indicates that the 7 days-intragastric administration of diphenoxylate with low dosage slightly induces the metabolism of tolbutamide (CYP2C9) in rat, the median and high dosage group induces the metabolism of tolbutamide (CYP2C9), this results was consistent with Figure 1F.

As diphenoxylate is always administrated in combination with other drugs, interactions between diphenoxylate and other drugs would increase the risk of either diminished efficacy or adverse effects. In our study, we found that 7 days-intragastric administration of diphenoxylate slightly induce the metabolism of omeprazole (CYP2C19), and induce phenacetin (CYP1A2) and tolbutamide (CYP2C9). Therefore, the metabolism and elimination of drugs would change if they are administrated in combination with diphenoxylate.

After the pharmacokinetic profiles evaluation by cocktail method, we also investigated the hepatotoxicity of diphenoxylate by observing the pathological changes of liver after diphenoxylate administration. The pathological changes of liver were observed at three difference dosages with small changes in high dosage and no change in low dosage, Figure 2. Therefore, high dosage diphenoxylate may cause hepatotoxicity. A more systematic and comprehensive study to investigate the hepatotoxicity of diphenoxylate will be carried out.

In conclusion, the results observed in this study would provide us valuable information regard-
Effect of diphenoxylate on CYP450 isoforms activity of rats

The interactions of diphenoxylate with other drugs. Induction of drug metabolizing enzyme CYP2C19, CYP1A2 and CYP2C9 by diphenoxylate would reduce the efficacy of other drug. Additionally, high dosage diphenoxylate may cause hepatotoxicity.

Acknowledgements

This study was supported by grants from the Science and Technology Committee of Shanghai Municipality, China, No. KF1504.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yunfang Zhou, The Laboratory of Clinical Pharmacy, The People’s Hospital of Lishui, Wenzhou Medical University, Lishui 323000, China. Tel: (86) 578 2780158; E-mail: zyf2808@126.com; Dr. Meiling Zhang, Analytical and Testing Center of Wenzhou Medical University, Wenzhou 325035, China. Tel: (86) 57786699156; E-mail: meiling308@163.com

References