Ketoprofen glucuronidation and bile excretion in carbon tetrachloride and alpha-naphthylisothiocyanate induced hepatic injury rats

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Abstract

A pharmacokinetic study was carried out in rats to investigate the effects of experimental hepatic injury on the liver glucuronidation and bile excretion of ketoprofen (KP) and its glucuronides (KPGs). In vivo, KP (20 mg/kg b.w.) was intravenously administered to carbon tetrachloride (CCl\textsubscript{4}) or alpha-naphthylisothiocyanate (ANIT) induced hepatic injury male rats. Concentrations of KP and its glucuronides (S-KPG and R-KPG) in plasma and bile were determined by RP-HPLC. It was observed that there was significant difference in the accumulative bile excretion of KPGs between the CCl\textsubscript{4} intoxicated rats and the normal rats (54 ± 18.3% versus 90 ± 6.9%), while it was extremely inhibited in ANIT intoxicated rats (2.0 ± 3.1% versus 90 ± 6.9%). As the result of reduction of KPGs excreted in bile, the area under the curve (AUC (0–\infty)) of KP and KPGs were higher in blood in CCl\textsubscript{4} and ANIT hepatic injury rats than those of the normal rats. Specifically, ANIT caused approximately 10-fold elevation of AUC\textsubscript{(0–\infty)} of plasma S-KPG. In microsomal incubations experiment, the glucuronyltransferase activity was impaired in CCl\textsubscript{4} and ANIT intoxicated rats. It suggested that the glucuronyltransferase activity was impaired in CCl\textsubscript{4} and ANIT intoxicated rats, while the bile excretion function was suppressed extremely in ANIT intoxicated rats.

Keywords: Hepatic injury; Carbon tetrachloride; Alpha-naphthylisothiocyanate; Glucuronidation; Bile excretion; Ketoprofen

1. Introduction

Rats treated with carbon tetrachloride (CCl\textsubscript{4}) or alpha-naphthylisothiocyanate (ANIT) are commonly used to study the mechanisms of hepatic injury and the therapeutic effects of medicines. CCl\textsubscript{4} induced hepatic injury is initiated by the production of an active metabolite, the carbon trichloride free radical (CCl\textsubscript{3}), by cytochrome P450. The metabolite attacks membrane lipids, and induces the forming of lipid peroxide...
molecules several hours after CCl₄ administration. It leads to the release of serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) from dying cells, and a rise in serum ALT and AST levels at 24–48 h. ANIT is known to cause cholestasis which is characterized by periportal bile duct epithelial degeneration and necrosis and a pronounced neutrophil infiltration. ANIT (100 mg/kg bw, i.g.) caused about 10-fold elevation of plasma ALT, a 6-fold elevation of total plasma total bilirubin (TBl), and a reduction by 80% in bile flow 24 h after administration (Xu and Xu, 2003; Taira et al., 2004; Kim et al., 2005).

Pharmacokinetic study is a valuable tool to evaluate the liver metabolisms (Taira et al., 2004). Glucuronidation, a phase II metabolic pathway, is an important metabolic process of xenobiotics, endobiotics and/or their phase I metabolites to more water-soluble compounds which can be readily excreted from the body. However, little pharmacokinetic information is available in the literature concerning drug glucuronidation and bile excretion in experimental hepatic injury rats.

A nonsteroidal anti-inflammatory drug, ketoprofen (KP), is mainly eliminated by glucuronidation. In addition, its glucuronides (KPGs), are mainly excreted via the bile (Meunier and Verbeeck, 1999; Soars et al., 2001; Sakaguchi et al., 2004; Jamali and Brocks, 1990). In this investigation, we selected KP as a model drug to study the effects of experimental hepatic injury on the glucuronidation and the bile excretion of glucuronide conjugate metabolite.

2. Materials and methods

2.1. Chemicals and animals

KP was obtained from Sigma (St. Louis, MO, USA). KP (8 mg/mL) was dissolved in saline by adding adequate 2 mol/L sodium hydroxide solution and then adjusted the pH to 7–8 with 0.5 mol/L hydrochloric acid. KPGs were isolated from human urine and purified by semipreparative high-performance liquid chromatography (HPLC) and used to prepare standards for calibration curves. The identity of the isolated material was confirmed by mass spectrometry (fast atom bombardment) and by alkaline hydrolysis to KP (Meunier and Verbeeck, 1999). CCl₄ (20%, v/v) and ANIT (10 mg/mL) were dissolved in peanut oil, respectively. Uridine diphosphate glucuronic acid (UDPGA) was purchased from Sigma. Acetonitrile was HPLC grade; tetrahydroammonium bromide and potassium dihydrogen phosphate were high-purified grade. All other reagents were of the highest purity and commercially available.

Male Sprague–Dawley rats weighted 230 ± 20 g were obtained from Southeast University (Nanjing, China), and were housed in plastic cages on aspen-chip bedding under conditions of controlled temperature (18–21 °C) and humidity (55 ± 5%) with a 12-h light/12-h dark cycle. Rats were allowed free access to rat chow and tap water.

2.2. Experimental protocols

Rats were randomly assigned to two experimental hepatic injury model groups (n = 12 rats each group). Rats, as CCl₄ induced hepatic injury group, were injected intraperitoneally with CCl₄ (0.1 mL/100 g bw). Rats, as ANIT induced hepatic injury group, were fasted for a night (12 h) prior to receiving ANIT (5 mg/100 g bw, i.g.). At 36 h after rats were intoxicated (Xu and Xu, 2003; Taira et al., 2004), the pharmacokinetics procedures (n = 4), bile excretion experiments (n = 4) and microsomal incubations in vitro (n = 4) were carried out in each group, respectively. At the same time, healthy male rats were used as control.

2.3. Pharmacokinetic procedures

Approximate 0.15–0.2 mL of blood was collected at 5, 10, 20, 40, 60, 90, 120, 180 min from the eyeground veniplex using a heparinized glass capillary after KP (20 mg/kg) was administered. To avoid conversion of the metabolites of glucuronide conjugates, blood specimens immediately were centrifuged at 4 °C after harvesting, then 50 μL plasma was separated and transferred to centrifuge tubes containing 10 μL potassium dihydrogen phosphate (1 mol/L). And the quantity of KP and KPGs was analyzed by RP-HPLC immediately.

2.4. Bile excretion experiments

The rats were anesthetized with urethane (1 g/kg i.p.). The bile duct was cannulated with PE10 polyethylene tubing (inner diameter, 0.28 mm; outer diameter, 0.61 mm; Becton Dickinson). After the operation, KP (20 mg/kg) was injected via the jugular vein. Bile specimens were collected into preweighed Eppendorf tubes containing 100 μL potassium dihydrogen phosphate (1 mol/L) at 0–30, 30–60, 60–90, 90–120, 120–180, 180–240, and 240–360 min. The sampling tubes were kept on ice during sampling. And the quantity of KP and KPGs was analyzed by RP-HPLC immediately.

2.5. In vitro study

2.5.1. Preparation of microsomes

The livers were homogenized initially in a 0.12 M KCl–0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 9000 × g for 20 min. The supernatant was then centrifuged at 100,000 × g for 1 h. Following the first high-speed spin, the supernatant was discarded and the pellet was resuspended in a 0.12 M KC1–0.1 M potassium buffer and spun again at 100,000 × g for 1 h. The pellet was then resuspended in a 0.12 M KC1–0.1 M phosphate buffer–glycerol (25%) (pH 7.4) for a final protein concentration of 20 mg/mL (Easterbrook
et al., 2001). The microsomes were stored at −70 °C until use.

2.5.2. Microsomal incubations
KP was prepared at concentrations to span a range (1.667, 3.334, 8.154, 16.67, 81.54 mM), which were suitable to determine accurately the \( K_m \) and \( V_m \) values. The 1 mL reaction system includes 4% Triton X-100 50 \( \mu \)L, 1 M Tris–HCl 100 \( \mu \)L, 0.5 M MgCl\(_2\) 20 \( \mu \)L, 0.1 M phenylmethyl sulfonylfluoride (PMSF) alcoholic solution 20 \( \mu \)L, 0.4 M 1,4-glucurolactone 50 \( \mu \)L and water 100 \( \mu \)L. Microsomal protein was added to a final concentration of 5 mg/mL. The samples were pre-warmed to 37 °C and the reactions were initiated with 10 mg/mL UDPGA 100 \( \mu \)L and KP of different concentrations 60 \( \mu \)L (Boase and Miners, 2002; Ilett et al., 2002). The reactions were stopped after 20 min with an equal volume of acetonitrile. And the quantity of KP and KPGs was analyzed by RP-HPLC immediately.

2.6. Measuring the concentration of KP and KPGs by RP-HPLC method

2.6.1. Apparatus and chromatographic conditions
The HPLC system consisted of a Model LC-10ATvp pump, a Model SPD-10A UV detector, and a Model SCL-10Avp system controller (Shimadzu, Japan). The chromatographic separation was performed using an ODS analytical column (200 mm × 4.6 mm i.d. 5 \( \mu \)m). The mobile phase consisted of acetonitrile and 0.05 mol/L phosphate buffer (pH 5.6) including 5 mmol/L tetrabutylammonium bromide (35:65), and the solution was filtered using 0.45 mm nylon membrane prior to use and delivered at a flow rate of 1 mL/min. The detector wavelength was 232 nm, the volume of injection was 20 \( \mu \)L, and the oven temperature is 30 °C.

2.6.2. Pretreatment and measuring of the samples
For 50 \( \mu \)L plasma, 50 \( \mu \)L internal standard solution (5 \( \mu \)g/mL naproxen dissolved in methanol) and 100 \( \mu \)L acetonitrile were added, then, mixed with a vortex mixer for 1 min and centrifuged at 14,000 rpm for 4 min, 20 \( \mu \)L supernatant was injected to the HPLC column. For 20 \( \mu \)L bile samples, 80 \( \mu \)L mobile phase and 20 \( \mu \)L internal standard solution were added, and 20 \( \mu \)L mixture was injected for HPLC analysis. Fifty microlitres microsomal incubations mixture was diluted in three times volume of mobile phase then centrifuged at 12,000 rpm for 5 min to remove the protein. One hundred microlitres supernatant and 20 \( \mu \)L internal standard solution were mixed, and 20 \( \mu \)L mixture was injected for HPLC analysis.

2.7. Serology determination
Serum total bilirubin (TBil), ALT and blood urea nitrogen (BUN) levels were determined using a commercially available clinical test kit with a chemistry analyzer system (Beckman coulter synchron clinical system Lx 20).

Table 1
The concentration of serum ALT, TBil and BUN in CCl4, ANIT induced hepatic injury rats and control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ALT (IU/L)</th>
<th>TBil (( \mu )mol/L)</th>
<th>BUN (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.8 ± 18.36</td>
<td>8.1 ± 0.94</td>
<td>15.3 ± 1.74</td>
</tr>
<tr>
<td>CCl4</td>
<td>248.7 ± 70.24**</td>
<td>9.2 ± 3.70</td>
<td>18.8 ± 4.11</td>
</tr>
<tr>
<td>ANIT</td>
<td>231.0 ± 84.36**</td>
<td>35.1 ± 14.72*</td>
<td>22.5 ± 9.49</td>
</tr>
</tbody>
</table>

Data are showed as mean ± S.D.M. of 8 rats.

\* \( p < 0.05 \) vs. control.

\** \( p < 0.01 \) vs. control.

2.8. Histological assessment of liver damage

Liver portions of rats were excised and fixed in 10% neutral formalin. Three or four paraffin sections (4–5 \( \mu \)m thick) per liver were prepared and stained with hematoxylin and eosin. Section preparation and assessment were conducted by professional pathologists (AiFeng Zhang, Department of Pathology, Southeast University, Nanjing, China).

2.9. Analysis of data

All pharmacokinetics models were performed using WinNoline. All values are expressed as mean ± S.D. The statistical significance was evaluated by Non-parametric test (Test for Sever Independent Samples and Two-Independent-Samples Test) in SPSS version 10.0. Statistical significance was defined as \( p < 0.05 \).

3. Results

3.1. Acute hepatic injury modeling

The concentration of serum ALT (but not TBil) was increased significantly (\( p < 0.05 \)), after the administration of CCl4 (0.1 mL/100 g b.w i.p.). The pathological changes of liver cells presented hydropic, acidophily or fatty degeneration and focus necrosis. Both the concentration of serum ALT and TBil were increased significantly (\( p < 0.05 \)), after the administration of ANIT (50 mg/kg b.w. i.g.). ANIT induced hepatic injury in rats was characterized by cholangiolic hepatitis, which presented periportal bile duct epithelial degeneration and necrosis and a pronounced neutrophil infiltration. For both of hepatic injury models, the concentration of serum BUN did not change significantly (Table 1).

3.2. Pharmacokinetic behaviors of KP and its glucuronides in rats

3.2.1. Plasma concentration and pharmacokinetics of KP

Clearance was significantly lower, while \( t_{1/2\beta} \) and log AUC(0–\( \infty \)) were increased significantly in CCl4 and
Table 2
The effect of experimental hepatic injury on pharmacokinetics parameters of KP after 20 mg/kg of KP was administered intravenously

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CCl₄</th>
<th>ANIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>V₅ (L)</td>
<td>0.023 ± 0.0014</td>
<td>0.022 ± 0.0011</td>
<td>0.019 ± 0.0037</td>
</tr>
<tr>
<td>t₁/₂B (min)</td>
<td>67.8 ± 21.67</td>
<td>284.2 ± 149.99*</td>
<td>129.0 ± 37.00*</td>
</tr>
<tr>
<td>CL (mL/min)</td>
<td>0.169 ± 0.0263</td>
<td>0.046 ± 0.0194**</td>
<td>0.080 ± 0.0112**</td>
</tr>
<tr>
<td>log AUCᵢ₋∞</td>
<td>4.69 ± 0.072</td>
<td>5.26 ± 0.187**</td>
<td>5.05 ± 0.099**</td>
</tr>
</tbody>
</table>

Data are shown as mean ± S.D.M. of 4 rats.
* p < 0.05 vs. control.
** p < 0.01 vs. control.

Table 3
The effect of experimental hepatic injury on pharmacokinetics parameters of KPGs after 20 mg/kg of KP was administrated (data are showed as mean ± S.D.M. of 4 rats)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CCl₄</th>
<th>ANIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>log AUCᵢ₋∞</td>
<td>3.4 ± 0.197</td>
<td>3.9 ± 0.144*</td>
<td>4.9 ± 0.172**</td>
</tr>
<tr>
<td>log Cₘₐₓ</td>
<td>1.31 ± 0.152</td>
<td>1.62 ± 0.131</td>
<td>2.38 ± 0.092**</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>45.1 ± 3.23</td>
<td>95.9 ± 5.22**</td>
<td>92.9 ± 6.64**</td>
</tr>
</tbody>
</table>

R-KPG was below the detection limit in CCl₄ induced hepatic injury rats and control.
* p < 0.05 vs. control.
** p < 0.01 vs. control.

ANIT hepatic injury models compared with healthy rats (Fig. 1 Table 2).

### 3.2.2. Plasma Concentration and pharmacokinetics of KPGs

Compared with healthy male rats, ANIT and CCl₄ induced hepatic injury rats had larger AUCᵢ₋∞ of S-KPG. The plasma concentrations of R-KPG measured from ANIT intoxicated rats were higher than 50 nmol/mL from 10 to 180 min after the administration of KP, while it was below the detection limit in CCl₄ induced hepatic injury rats and control (Fig. 2, Table 3).

### 3.3. Bile excretion of KP and KPG

For all experimental rats, the accumulative bile excretion of KP is less than 2% of the dose administrated, and the amount of S-KPG excreted in bile was more than that of R-KPG. The bile excretion of KPGs was obviously suppressed in CCl₄ induced hepatic injury rats. The cumulative amounts of S- and R-KPG excreted in bile were 64 ± 9.6% and 26 ± 8.3% of the dose in control rats, respectively, while they were 41 ± 26.0% and 13 ± 7.9% in CCl₄ induced hepatic injury rats, respectively (Fig. 3, Table 4). However, the bile excretion of KPGs was extremely inhibited in ANIT intoxicated rats (2.04 ± 3.10% versus 90 ± 6.9%, p < 0.01).

Fig. 1. The effect of experimental hepatic injury on drug plasma concentration–time cures of KP after 20 mg/kg of KP was administered intravenously (data are showed as mean ± S.D.M. of 4 rats).

Fig. 2. The effect of experimental hepatic injury on drug plasma concentration–time curves of S-KPG and R-KPG after 20 mg/kg of KP was administered intravenously. R-KPG is not detected in CCl₄ intoxicated and healthy rats (data are showed as mean ± S.D.M. of 4 rats).
The effect of experimental hepatic injury on the accumulative bile extraction of S-KPG and R-KPG after 20 mg/kg of KP was administered (data are showed as mean ± S.D.M. of 4 rats). For ANIT intoxicated rats, the accumulative bile excretion of KPGs is less than 0.03 of the dose administrated (data not shown).

Table 4
The accumulative bile extraction of KPGs after 20 mg/kg of KP was administrated

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>CCl4</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.25 ± 0.065</td>
<td>0.05 ± 0.019**</td>
</tr>
<tr>
<td>60</td>
<td>0.51 ± 0.084</td>
<td>0.12 ± 0.063**</td>
</tr>
<tr>
<td>90</td>
<td>0.65 ± 0.077</td>
<td>0.17 ± 0.091**</td>
</tr>
<tr>
<td>120</td>
<td>0.75 ± 0.079</td>
<td>0.22 ± 0.119**</td>
</tr>
<tr>
<td>180</td>
<td>0.85 ± 0.067</td>
<td>0.34 ± 0.195**</td>
</tr>
<tr>
<td>240</td>
<td>0.88 ± 0.066</td>
<td>0.40 ± 0.232**</td>
</tr>
<tr>
<td>360</td>
<td>0.90 ± 0.069</td>
<td>0.54 ± 0.283*</td>
</tr>
</tbody>
</table>

Data are showed as mean ± S.D.M. of 4 rats. For ANIT intoxicated rats, the accumulative bile excretion of KPGs is less than 0.03 of the dose administrated (data not shown).

* p<0.05 vs. control.
** p<0.01 vs. control.

3.4. Microsomal incubations

Enzymatic parameters of KP glucuronidation showed that the glucuronyltransferase activity was impaired in CCl4 and ANIT intoxicated rats. And the impairments were similar in CCl4 and ANIT intoxicated rats (Table 5, Fig. 4).

4. Discussion

Approximate 90% of the dose administrated is glucuronided and excreted into bile in control rats. The result of our studies is consistent with previous reports (Meunier and Verbeeck, 1999) about the pharmacokinetics of KP. In addition, it is easy to directly measure the biliary excretion of the KPGs, and the disposition character of KP would greatly simplify the pharmacokinetic interpretation of the data. KP is therefore a reasonable model drug to characterize the intrinsic liver capacity of glucuronidation and elimination of the phase II metabolites.

In the present study, the accumulative bile excretion of KPGs was lower in the CCl4 intoxicated rats, while it was nearly completely suppressed in ANIT intoxicated rats. As the result of reduction of KPGs excreted in bile, the concentration of KP and KPGs was higher in blood in CCl4 and ANIT hepatic injury model rats. Prominently, ANIT caused about 10-fold elevation of AUC of S-KPG. In microsomal incubations experiment, enzymatic parameters of KP glucuronidation showed that the glucuronyltransferase activity was impaired in CCl4 and ANIT intoxicated rats. And the impairment was similar in both kinds of acute hepatic injury model rats. So it can be speculated that the glucuronyltransferase activity was impaired in CCl4 and ANIT intoxicated rats, while the bile excretion function was suppressed extremely in ANIT intoxicated rats. In CCl4 induced hepatic injury rats, the pathological changes presented liver cells degeneration and necrosis,

Fig. 3. The effect of experimental hepatic injury on the accumulative bile extraction of S-KPG and R-KPG after 20 mg/kg of KP was administered (data are showed as mean ± S.D.M. of 4 rats). For ANIT intoxicated rats, the accumulative bile excretion of KPGs is less than 0.03 of the dose administrated (data not shown).

Table 5
Enzymatic parameters of KP glucuronidation in vitro

<table>
<thead>
<tr>
<th>Group</th>
<th>$V_m$ (mmol/mg/min)</th>
<th>$K_m$ (mmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.86 ± 0.912</td>
<td>13.65 ± 1.172</td>
</tr>
<tr>
<td>CCl4</td>
<td>0.89 ± 0.535*</td>
<td>6.79 ± 1.575*</td>
</tr>
<tr>
<td>ANIT</td>
<td>1.09 ± 0.569*</td>
<td>5.39 ± 1.464*</td>
</tr>
</tbody>
</table>

Data are showed as mean ± S.D.M. of 4 rats.

* p<0.05 vs. control.
while ANIT induced hepatic injury was characterized by cholangiolic hepatitis. The findings of pharmacokinetics and the bile excretion of KP and its glucuronides were consistent with the changes of pathology and serology, so it can be inferred that KP is therefore a reasonable model drug to evaluate the intrinsic liver capacity of glucuronidation and elimination of the phase II metabolites in hepatic injury.

Today, various medicines have been expected to be effective against acute and chronic experimental hepatic injury (Kiso et al., 1984; Lao, 2005). The pharmacokinetics of KP therefore could be used as a tool to evaluate the therapeautic effects of medicines used for liver disease.

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References


