Simultaneous Evaluation of Inhibition on Human CYP450 of Asarum Volatile Oil by a Cocktail Approach

Lei ZHANGa, Li-Fang ZHOUb, Tong ZHANGc, Ai-Hong YANGd,*
School of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine, Tianjin, P.R. China.
a75712595@qq.com, b867954905@qq.com, cjiuji03@qq.com, dyah408@163.com
*Corresponding author

Keywords: Cocktail, Asarum volatile oil, CYP450, Probe substrate.

Abstract. In this study, the cocktail method was used to study the inhibitory effect of the volatile oil components including asarinin, methyl eugenol, asarone, safrole and myristicin on the CYP450 enzymes. Five kinds of probe substrates metabolized by different CYP450 enzymes were administered simultaneously in the system of co-incubation of the drug with human liver microsomes. The effect of the drug on the metabolism of the probe substrate was determined by the change of the metabolites of the probe substrate using UPLC/MS, and the inhibition of the enzyme was determined. The results showed that four drugs have an inhibitory effect on the metabolism of CYP450 enzymes except methyl eugenol, and myristicin has the strongest inhibitory effect on CYP1A2.

Introduction

Cytochrome P450 (CYP450) enzymes play a critical role in major phase I metabolism. CYP450 can metabolize 75% of drugs and are involved in the biotransformation of xenobiotics [1]. Five major CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4) can metabolize approximately 90% of the drugs on the market, including synthetic and plant-derived compounds, these five CYP450 are critical to the oxidative metabolism of humans [2]. A new strategy for the study of drug oxidative metabolism by "cocktail" probe method was first proposed in 1990 [3]. Cocktail is a method of giving two or more probe drugs at a time. Modern instrument analysis technique is used to measure metabolism rate or metabolic typing index of probe drugs in biological samples and to obtain phenotypic information of multiple CYP450 isozymes [4]. Cocktail approach is a fast, sensitive and high-throughput approach to determine CYP450 enzymes activity. It has been widely used to screen early drug development, to analyze drug metabolism types and confirm the metabolism pathways [5]. And it is useful to optimize clinical regimen, evaluate post marketing drugs and help liver / kidney pathological studies.

Cocktail probe method can be used for rapid screening and investigating the characteristics of hepatic drug enzyme metabolism of Chinese traditional medicine active components, which may play a certain role in the metabolic characteristics of drugs, the mechanism of toxic side effects and the potential drug-drug interaction [6]. Some reports have shown the effect of compound prescription of traditional Chinese medicine on CYP450 enzyme by cocktail method. Zheng et al. [7] studied the effect of polygonum capitatum on CYP450 in rats by cocktail method. It showed that it can increase the rate of drug appreciation with its compound. In western medicine, this method is also used in many fields, such as confirmation
of drug metabolic pathway and investigation of the effect of CYP enzyme activity etc [8]. Y.L. Han et al. studied Erigeron breviscapus injection and incubated Asarum injection with rat liver microsomal CYP3A. By measuring the IC$_{50}$ value and $K_i$ value of Asarum injection, it was found that Asarum had inhibitory effect on liver microsomal CYP3A. Asarinin, methyl eugenol, asarone, safrole and myristicin were main constituents of Asarum volatile oil, and Fig. 1 shows the structures of five compounds. In this study, the cocktail method was used to study the inhibitory effect of the volatile oil components on the CYP450 enzymes.

![Chemical structures](image)

Fig. 1 The structures of five compounds in Asarum volatile oil. (a) safrole, (b) methyleugenol, (c) asarone, (d) myristicin, (e) asarinin.

**Materials**

**Chemicals and reagents.** Asarinin, methyl eugenol, asarone, safrole and myristicin were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Phenacetin, midazolam, dextromethorphan, (s)-mephenytoin, chlorzoxazone and NADPH ($\beta$-nicotinamide adenine dinucleotide 20-phosphate reduced tetrassodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pooled human liver microsomes (HLMs) were purchased from the Research Institute for Liver Diseases Co., Ltd. (Shanghai, China), which were prepared from human liver tissues under Chinese organ donation regulations with the full consent of the patients. Methanol, acetonitrile (HPLC pure) was purchased from Tianjin Concord science and Technology Development Co Ltd. DMSO (99.7%) was purchased from Sigma Chemical Co. The other reagents were of high-performance liquid chromatography grade.

**Instruments.** Agilent ZORBXXDB C$_{18}$ column (3.5 $\mu$m, 2.1 mm $\times$ 50 mm) was used. High-speed centrifugal machine (ALLEGRA-64R, BECKMAN, USA); miniature vortex mixe (WH-3, West Analytical instrument Factory, Ltd., Shanghai); Pipette; Super constant temperature tank (DKB-501A, Jinhong Experimental equipment Co., Ltd. Shanghai); Ultra-pure water machine (Millipore Milli-Q/30 L, France).

**Methods.** CYP cocktail assays were employed to analyze and screen the drug-mediated inhibition of five CYP isoforms. Phenacetin (CYP1A2), dextromethorphan (CYP2D6), chlorzoxazone (CYP2E1), midazolam (CYP3A4), and (s)-mephenytoin (CYP2C19) were chosen as probe substrates for each corresponding CYP. Firstly, all incubating reactions were
carried out in a 37 °C shaking bath. The total volume of the preincubation system is 200 μL. It contains 0.1 mol/L phosphate buffer (pH 7.4), 2 mg/mL human liver microsomal protein, tested drugs (5 μM or 50 μM) was preincubated for 30 minutes with or without adding 1 mM NADPH (Replace with PBS). Then the sample preincubated for 30 min was diluted 10 times, in other words, 20 μL of preincubated sample was added to the system containing 100 μL NADPH (1 mM) and 80 μL mixed probe substrate (10/2.5/20/5/20 μmol/L), and incubated for 15 min. Adding 400 μL of ice methanol (containing 50 ng/mL carbamazepine) to stop reaction. Finally, the samples were centrifuged (4°C, 5 min, 10000 g). The supernatant was used to determine the five metabolites by LC-MS. The assays were performed in triplicate for all test specimens.

Liquid-mass spectrometry condition. Flow rate: 0.45 mL/min; sample volume 10 μL; detection wavelength: 280 nm. The mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B) with a linear-gradient elution at a flow rate of 0.2 mL/min. The HPLC gradient program used was as follows: mobile phase A was maintained at 98% for 2 min and then a linearly programed gradient dropped mobile phase A from 98 to 2% and from 2 to 3.5 min. Next, mobile phase A was ramped to 98% again in 0.01 min toward the end of the analysis. The parameters of ion source are as follows: curtain gas is 20 psi; IonSpray Voltage 5000V (ESI+), -4200V (ESI-), Ion source gas1 and ion source gas2 are 55 psi and 50 psi respectively, and the ion source temperature is 550°C. The parameters of mass spectrometry for components to be tested and internal standards are shown in Table 1.

Table 1. Mass Spectrometry conditions of five metabolites.

<table>
<thead>
<tr>
<th>Target compound</th>
<th>Mode</th>
<th>Prec ion(m/z)</th>
<th>Prod ion(m/z)</th>
<th>DP (V)</th>
<th>CE(eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>ESI+</td>
<td>152.1</td>
<td>110.1</td>
<td>61</td>
<td>23</td>
</tr>
<tr>
<td>Demethylated dextromethorphan</td>
<td>ESI-</td>
<td>258.1</td>
<td>199.1</td>
<td>70</td>
<td>38</td>
</tr>
<tr>
<td>1-hydroxymidazolam</td>
<td>ESI-</td>
<td>342.1</td>
<td>203.1</td>
<td>83.3</td>
<td>40</td>
</tr>
<tr>
<td>4-hydroxymefantoin</td>
<td>ESI-</td>
<td>235.1</td>
<td>150.1</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>6-hydroxycclozoxazone</td>
<td>ESI-</td>
<td>184.2</td>
<td>120</td>
<td>-73</td>
<td>-26</td>
</tr>
</tbody>
</table>

Results and Discussion

The inhibition of Asarum volatile oil on human CYP450 can be evaluated simultaneously by a cocktail approach. The inhibitory effects can be seen through the relative activity (%) of corresponding metabolites to specific substrates. Relative activity (%) stands for the ratio of the amount of metabolites to specific substrates in drug group and control group.

Cocktail screening results of methyl eugenol on CYP450 enzyme. The concentration of methyleugenol was 5 μm and 50 μm respectively. The five CYP450 enzymes were CYP1A2, CYP2D6, CYP2E1, CYP3A4 and CYP2C19 respectively. From the results in Fig. 2, it can be seen that methyleugenol with the high concentration inhibited the enzyme, while the effect of low concentration was not obvious. And there was no significant difference with/without
NADPH. The results showed that methyleugenol had no obvious inhibitory effect on human CYP450 enzyme.

**Cocktail screening results of CYP450 enzyme in asarum.** 5 μm and 50 μm of drug concentration were used in the screening of asarum. From the results in Fig. 3, it can be seen that asarum has an obvious effect on the metabolism of CYP450 enzyme especially when asarum had a high concentration. The activity of the five CYP450 enzymes decreased in the presence of NADPH, and the residual activities of CYP3A4 and CYP2E1 decreased more significantly than those of CYP2D6. Combined with the inhibition concentration and the results of adding NADPH, it was concluded that asarone had inhibitory effects on human CYP2D6, CYP3A4 and CYP2E1 enzymes.

**Cocktail screening results of asarone for CYP450 enzyme.** The concentration of asarone was 5 μm and 50 μm respectively. Fig. 4 showed that asarone with the high concentration has a greater effect on the metabolism of CYP450 than that of the low concentration. The activity of CYP3A4, CYP2C19 and CYP2E1 decreased more obviously in the presence of NADPH. It was found that asarone had inhibitory effects on human CYP1A2, CYP3A4, CYP2C19 and CYP2E1.

![Figure 2](image1.png)

Figure 2. Cocktail screening results of methyl eugenol on CYP450 enzyme.

![Figure 3](image2.png)

Figure 3. Cocktail screening results of asarum on CYP450 enzyme.
The inhibition effects of safrole and myristicin have also been studied [9,10] by our group. It reported that myristicin has inhibitory effects on human CYP2E1, CYP2C19 and CYP1A2, while has the most strongest inhibitory effect on CYP1A2 with the high and low relative activity values of 8.4 %, 28.8 %, respectively with NADPH. And the same to myristicin, safrole also has a very significant inhibition on CYP1A2.

Cocktail in vitro experiment has been widely used in the screening of new drugs and the study on drug interaction. However, as a clinical trial, there are still some controversies about it. Including problem in ethic, compliance of drug subjects after multiple probe drugs were given at the same time, whether the selected probe drug is reasonable, whether some other transporters or enzymes involved in phase metabolic reactions that affect drug metabolism process and so on. It shows that there are still many challenges and gaps in the research of cocktail probe drug method, but it is reasonable to believe that with the continuous improvement of the method, it will play a great potential and role. And it provides more detailed scientific basis for the research and development of new drugs, the second development of large varieties of traditional Chinese medicine and the guidance of rational drug use in clinic.

Conclusion

In summary, methyleugenol had no obvious inhibitory effect on human CYP450 enzyme. Asarone could inhibit human CYP2D6, CYP3A4 and CYP2E1 enzymes, while asarone could inhibit human CYP1A2, CYP3A4, CYP2C19 and CYP2E1. The inhibition of safrole and myristicin on CYP1A2 was stronger than that on the other four CYP450 enzymes (CYP2C19, CYP3A4, CYP2D6 and CYP2E1). In this work, the inhibition of Asarum volatile oil on human CYP450 can be evaluated simultaneously by the cocktail screening approach, so it is useful to improve the efficiency and reduce the cost of testing by using the cocktail screening approach.

Acknowledgments

This work was supported by National Natural Science Foundation of China (NSFC, No. 81503462).
References


