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The exposure of highly toxic aconitine does not significantly impact the activity and expression of cytochrome P450 3A in rats determined by a novel ultra performance liquid chromatography–tandem mass spectrometric method of a specific probe buspirone

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ABSTRACT

Aconitum species are widely used to treat rheumatism, cardiovascular diseases, and tumors in China and other Asian countries. The herbs are always used with drugs such as paclitaxel. Aconitine (AC) is one of the main bioactive/high-toxic alkaloids of *Aconitum* roots. AC is metabolized by cytochrome P450 (CYP) 3A. However, whether AC inhibits/induces CYP3A, which causes drug–drug interaction (DDI) is unclear. Our study aims to explore the potent effects of AC, as a marker component of *Aconitum*, on CYP3A using the probe buspirone in rats. The effects of oral AC on pharmacokinetics of buspirone were evaluated. CYP3A activity and protein levels in rat liver microsomes pretreated with oral AC were also measured using *in vitro* buspirone metabolism and Western blot. Buspirone and its major metabolites 1-(2-pyrimidinyl)piperazine and 6′-hydroxybuspirone were determined using a newly validated UPLC–MS/MS method. Single dose and 7-day AC administration at 0.125 mg/kg had no effect on CYP3A activity since no change in the formation of 1-(2-pyrimidinyl)piperazine and 6′-hydroxybuspirone. CYP3A activity and protein levels in liver microsomes were also not affected by 7-day AC pretreatment at 0.125 mg/kg. Therefore, AC neither inhibits nor induces CYP3A in rats, indicating AC does not cause CYP3A-related DDI in the liver.

1. Introduction

Aconitine (AC) (Fig. 1) is one of the main bioactive diterpenoid alkaloids present in the roots of *Aconitum* species (Ranunculaceae family), which have been widely used in China and other Asian countries for treating polyarthralgia such as rheumatoid arthritis, as well as cardiovascular diseases, tumors, skin wounds, depression, and diarrhea (Singhuber et al., 2009). It was reported that AC has analgesic, antipyretic, and local anesthesia functions as well as the roots of *Aconitum* (Song et al., 2001; Zhou et al., 1984). However, AC has a narrow therapeutic index and is well known for its acute, high toxicity (Wada et al., 2005). The median lethal dose (LD_{50}), absolute lethal dose, and arrhythmia dose of AC are 0.270 mg/kg ± 0.002 mg/kg (mice, i.v.), 0.102 mg/kg ± 0.008 mg/kg (rats, i.v.), and 0.034 mg/kg ± 0.004 mg/kg (rats, i.v.), respectively (Zhou et al., 1984). AC metabolism is mainly carried out by esterases. AC is converted into benzoylaconine through hydrolysis in the C-8 position, and into aconine in the C-8 and C-14 positions (Mizugaki et al., 1998; Tang et al., 2011).

Aconitum species have been used in China as an essential drug in Traditional Chinese Medicines (TCM) for 2000 years. *Aconitum* is included in up to 600 formulations from both the historical literature and modern clinical reports (Singhuber et al., 2009). The most commonly used *Aconitum*-containing herbal products in the market are "Fuzi Lizhong Borus," "Xiao Huoluo Dan," "Jingui Shenqi Borus," and "Shenfu Injection" (Wang and Lu, 1999). The annual consumption of *Aconitum* species in China exceeds 2,000,000 kg (Li et al., 2002). However, *Aconitum*-related poisoning occurs frequently. Thus, *Aconitum* is commonly used combined with other herbal medicine, including ginseng, *Rhizoma zingiberis*, *Glycyrrhiza*, *Rheum officinale*, and *Radix poeoniae* Alba, because these herbal medicines help relieve *Aconitum*-related poisoning through herb–herb interaction (Gu et al., 2008; Wang and Lu, 1999). *Aconitum*

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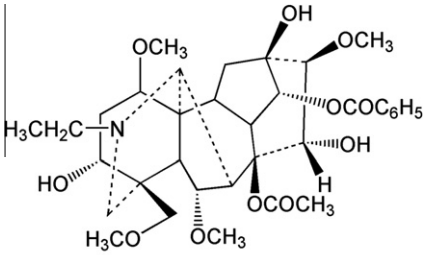

Aconitine ($C_{34}H_{47}NO_{11}$: 645.74)

Fig. 1. Chemical structure of aconitine.

will likely be administered concomitantly with other chemical drugs because patients with rheumatoid arthritis, cardiovascular diseases, and tumors need multiple drug therapy. The active ingredients of *Aconitum* and prescription drugs that are used to treat these complex diseases, such as diclofenac, meloxicam, lornoxicam, lidocaine, felodipine, enalapril, paclitaxel, docetaxel, and vincristine are primarily metabolized by cytochrome P450 enzymes (CYPs) (Dong and Wang, 2009; Hu et al., 2002; Li et al., 2006). As describe above, the safety assessment related to drug–drug interaction (DDI) between *Aconitum* and conventional drugs is essential and urgent because the incidence of DDI will increase sharply with the increasing number of drugs used in co-therapy (Weaver, 2001).

DDI usually occurs because of the inhibition/induction of CYPs. CYPs inhibition results in undesirable elevations in plasma concentrations of co-administered drugs, with adverse effects and toxicologically unsafe consequences (Sugiyama et al., 2011). However, CYPs induction results in ineffective pharmacotherapy. Herbal medicines such as St. John's wort (*Hypericum perforatum*), garlic (*Allium sativa*), piperine (from *Piper* sp.), ginseng (*Ginseng* sp.), gingko (*Gingko biloba*), soya beans (*Glycine max*), alfalfa (*Medicago sativa*), and grape fruit juice have clinically been shown to interact when co-administered with drugs because of alterations in CYP activity caused by these herbal medicines (Izzo and Ernst, 2009; Saxena et al., 2008). Our previous studies have shown that CYP3A was the major enzyme involved in the metabolic pathways of AC (Tang et al., 2011; Wang et al., 2006). CYP3A is the most abundant CYPs protein in the liver. It involved in the metabolism of 45–60% of all currently used drugs (Burk and Wojnowski, 2004). Unfortunately, CYP3A activity is frequently affected by its own substrates in clinical practice and is recognized by authorities as one of the most important causes of DDI (Martignoni et al., 2006). Four-day AC pretreatment reportedly decreases testosterone (CYP3A probe substrate) 6β-hydroxylation activity in rat liver microsomes (RLM), although the effect was not significant (Yamada et al., 1998). However, no studies are available on the effects of AC on the *in vivo* pharmacokinetics of CYP3A probe substrates and CYP3A protein expression. Thus, to understand the effects of *Aconitum* on CYPs and to predict the risk of DDI between *Aconitum* and other drugs, AC was adopted as a marker for *Aconitum* to investigate the effects of AC on CYP3A.

CYPs probe substrates are commonly used to evaluate the effects of target drugs on CYPs. Buspirone (BP), a sensitive CYP3A substrate, has been accepted as a specific probe substrate for CYP3A activity *in vivo* by the U.S. Food and Drug Administration (FDA) (FDA, 2006). The major BP metabolic pathways are mediated by CYP3A, including *N*-dealkylation to 1-(2-pyrimidinyl)piperazine (1-PP) and 6′-hydroxylation to 6′-hydroxybuspirone (6′-OH-BP) (Zhu et al., 2005) (Fig. 2). In the present study, the pharmacokinetics of BP in rats after a single dose and after 7-day AC pretreatments were investigated. The plasma concentrations of BP, 1-PP, and 6′-OH-BP were determined using a new ultra performance liquid chromatography–tandem mass spectrometric (UPLC–MS/MS) method. The CYP3A activity in RLM after 7 days of AC pretreatment was also examined by determining the formation rate of two main BP metabolites, namely, 1-PP and 6′-OH-BP. The hepatic CYP3A protein levels in the RLM were also analyzed via Western blot analysis.

2. Materials and methods

2.1. Chemicals and reagents

Aconitine (purity > 98%, HPLC grade, confirmed by UPLC–MS/MS) was purchased from Chengdu Mansite Pharmaceutical Co. Ltd. (Chengdu, China). NADPH regenerating system, pooled male RLM (20 mg/mL) was purchased from BD Gentest Corp. (Woburn, MA, USA). Buspirone hydrochloride (BP·HCl), and testosterone were purchased form Sigma–Aldrich (St. Louis, MO, USA). The metabolites standards 1-(2-pyrimidinyl)piperazine and 6′-hydroxybuspirone were obtained from Toronto Research Chemicals Inc. (Toronto, CA). Acetonitrile, dichloromethane, and formic acid were high-performance liquid chromatography (HPLC) grade. All other chemicals were analytical reagent grade or better.

2.2. Animals

Male Sprague–Dawley rats (250–300 g) were supplied by the Medicine Laboratory Animal Center of Guangdong province and housed in animal holding room under standard conditions with 12 h light–dark cycle, with free access to food and water. All the experimental procedures had been approved by the Animal Ethics Committee in accordance to the Department of Health guidelines in Care and Use of Animals of Southern Medical University.

2.3. UPLC–MS/MS method development and validation

2.3.1. UPLC–MS/MS assay for BP and its metabolites

UPLC–MS/MS system including a binary solvent manager and a triple quadrupole Quattro Premier XE from Waters (Boston, MA, USA) was used for sample separation and analysis. Sample separation was performed in a Waters ACQUITY HSS T3 column (2.1 mm × 100 mm, 1.8 μm) with flow rate of 0.2 mL/min at 40 °C. The mobile phase consisted of 5% acetonitrile in water with 0.01% formic acid (A) and 5% water in acetonitrile with 0.01% formic acid (B). Typical conditions for elution were as follows: 2% B (0–1.0 min); 2–50% B (1.0–3.0 min); 50–75% B (3.0–3.5 min); 75% B (3.5–6.0 min); 75–2% B (6.0–6.2 min); 2% B (6.2–6.5 min). Samples analysis was carried out in positive electrospray ionization mode under multiple reaction monitoring (MRM) mode. The capillary voltage was set at 3000 V. The source temperature was 120 °C and the desolvation temperature was 350 °C. Nitrogen was used as a drying gas with a flow rate of 600 L/h and the core gas flow was set to 50 L/h. BP, 1-PP and 6′-OH-BP were monitored at *m*/*z* 386 > 122, 165 > 122 and 402 > 122 transitions. Testosterone was used as internal standard (IS) and its transition *m*/*z* was monitored at 289 > 97.

2.3.2. Preparation of plasma samples

Blood samples were collected into heparinized tubes and plasma was separated at 6000*g* for 8 min immediately after collection. Aliquot (10 μL) of testosterone solution was added to 100 μL plasma. Following protein precipitation by 200 μL of acetonitrile, the mixture was shaken for 1 min, and then centrifugation for 30 min at 18,000*g*, the supernatant was transferred to another tube and dried with a gentle stream of nitrogen gas. The residue was redissolved with 300 μL of 5% acetonitrile in water, and an aliquot (10 μL) of the supernatant was analyzed by UPLC–MS/MS.

2.3.3. Recovery and matrix effect

Blank rat plasma samples were spiked with three concentrations (7.8, 62.5 and 500.0 ng/mL) of BP, 1-PP, and 6′-OH-BP. After preparation of the plasma samples, fixed amounts of testosterone (IS) were added to the plasma for normalization. The extraction recovery for BP, 1-PP, and 6′-OH-BP were determined by comparing the peak area ratios of the analyte to IS obtained from plasma samples with the analytes spiked before extraction to those that were spiked after the extraction. The matrix effect caused by plasma endogenous materials was evaluated by comparing the peak areas of the samples that were extracted from plasma to those that were extracted from deionized water.

2.3.4. Precision and accuracy

The intra-daily and inter-daily precision and accuracy levels of the assay were assessed via replicate analyses of quality control samples at the nominal concentrations of 2.4, 39.1, and 625.0 ng/mL. For the determination of intra-daily precision, five replicates of plasma samples at each concentration were processed according to Section 2.3.3 and analysed within 1 day. For the determination of inter-daily precision, the same procedure was repeated for 3 consecutive days.