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Screening for *in vitro* metabolites of kakkalide and irisolidone in human and rat intestinal bacteria by ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry

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ABSTRACT

Kakkalide and irisolidone, the main isoflavones of *Flos Puerariae*, exhibit a wide spectrum of bioactivities. Intestinal bacteria biotransformation plays an important role in the metabolic pathways of flavones, and is directly related to the bioactivities of the prodrugs after oral administration. To the best of our knowledge, the metabolic pathways of kakkalide and irisolidone *in vitro* have not been comprehensively studied yet. This paper describes the strategy using ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF MS) for the rapid analysis of the metabolic profiles of kakkalide and irisolidone after incubated with human and rat intestinal bacteria. Bacteria incubated samples were prepared and analyzed after incubated under anaerobic conditions for 48 h. A total of 17 metabolites, including parent compounds, were detected in human and rat intestinal bacteria incubated samples. The results obtained indicate that hydrolysis, dehydroxylation, demethoxylation, demethylation, hydroxylation, decarbonylation, and reduction were the detected metabolic pathways of kakkalide and irisolidone *in vitro*. The conversion rate of irisolidone in human and rat bacteria was 8.57% and 6.51%, respectively. Biochanin A was the relatively main metabolite of irisolidone, and the content of biochanin A in human and rat bacteria was 3.68% and 4.25%, respectively. The conversion rate of kakkalide in human and rat bacteria was 99.92% and 98.58%, respectively. Irisolidone was the main metabolite of kakkalide, and the content of irisolidone in human and rat bacteria was 89.58% and 89.38%, respectively. This work not only provides the evidence of kakkalide and irisolidone metabolites *in vivo*, but also demonstrates a simple, fast, sensitive, and inexpensive method for identification of metabolites of other compounds transformed by intestinal bacteria.

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1. Introduction

Traditional Chinese medicines are mainly administered orally and most of their components are transformed by intestinal bacteria before being absorbed into blood [1–7]. Usually, the pharmacological activity produced by intraperitoneal administration of medicines may be different from that produced after its oral administration. For example, kakkalide (KA, irisolidone-7-*O*-xylosylglucoside, Fig. 1), the main isoflavone of *Flos Puerariae*, can reduce alcohol toxicity mortality to 40% when administered intraperitoneally at a dose of 50 mg/kg, while it reduced mortality only by 10% when taken orally [8]. Intestinal bacteria has been used extensively for the metabolic study of natural products *in vitro* [9,10], and microbial transformation frequently influences the biological activities of natural products, such as soy isoflavones [11,12]. Therefore, it is important to study the metabolism of drugs by intestinal bacteria especially if these drugs are natural compounds.

Recently, in China, Korea and Japan, phytochemicals containing *Pueraria thomsonii* flower become one of the most popular herbal medicines for the treatment of diseases such as alcohol intoxication, liver injury or diabetes. Irisolidone (Ir, Fig. 1), as the aglycone of KA, exhibits similar bioactivities to KA, such as hepatoprotection [8,13,14], estrogenic [15], anti-inflammation [16], and antioxidation effects [17]. Earlier studies indicate that KA can be transformed into Ir both *in vivo* and *in vitro* [12,15]. Also, KA can be metabolically converted *in vitro* by intestinal bacteria into Ir, 6-hydroxybiochanin A (6-OH-BiA) and Ir-7-*O*-glucoside [18] (Fig. 1). Bai et al. have investigated the metabolism of KA in rat plasma, and found

Abbreviations: BiA, biochanin A; ESI, electro spray ionization; CLog *P*, calculated 1-octanol/water partition coefficient; Glc, glucose; Ir, irisolidone; KA, kakkalide; Te, tectorigenin; UHPLC/Q-TOF MS, ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry; Xyl, xylose.

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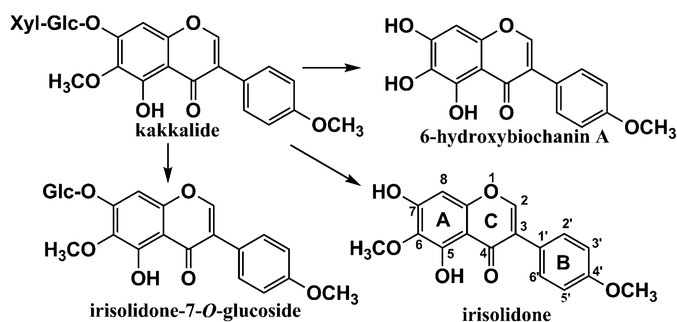

Fig. 1. Chemical structures of kakkalide and irisolidone, and their metabolites reported in the published literature.

that Ir-7-*O*-glucuronide (Ir-7G), 6-OH-BiA-*O*-glucuronide (6-OH-BiA-G) and tectorigenin-7-*O*-glucuronide (Te-7G) were the major plasma metabolites, indicating that intestinal bacteria could play an important role in KA metabolism *in vivo* [19]. Thus, the major metabolic pathways of KA and Ir *in vitro* need to be evaluated.

Nowadays, UHPLC is introduced as commercially available instrument, which has been applied for the pharmaceutical, toxicological and biochemical analysis [20–24]. It has the advantages of fast analysis, high peak capacity, good sensitivity and low consumption of samples compared with conventional HPLC [25]. To keep up with the pace of data acquisition, time-of-flight (TOF) mass spectrometer was developed and suitable for non-targeted identification [26–29]. UHPLC/Q-TOF MS, in particular, offers rapid and efficient separation and detection methods with accurate mass measurement and tandem mass spectrometry (MS/MS) [30]. Metabolynx™ software, combined with mass defect filter (MDF), is capable of automatically processing LC/MS data and suitable for searching for expected (targeted) and unexpected (non-targeted) metabolites by comparing the chromatogram of the analytes with the controls. It has been designed specifically to detect and identify metabolites for drug metabolism studies [31].

In the present study, UHPLC/Q-TOF MS method was used to characterize the metabolites of KA and Ir transformed by human and rat intestinal bacteria. Simple anaerobic incubation materials, MS^E data acquisition, and Metabolynx™ software allowed the identification of metabolites produced through anaerobic incubation *in vitro* a simple, sensitive, fast and low cost procedure. This method is also suitable for screening for *in vitro* metabolites of other natural compounds in human and rat intestinal bacteria.

2. Experimental

2.1. Chemicals and reagents

Kakkalide, irisolidone, 5,7-dihydroxy-8,4′-dimethoxyisoflavone, biochanin A and tectorigenin (purity > 95%) were separated in our laboratory. Their structures were elucidated using NMR, MS, UV and IR methods [32,33]. Anaerobic cultivation sealed plastic bags and pouch-anaero were purchased from Mitshbishi Gas Chemical Co. (Japan). Anaerobic broth was obtained from Land Bridge Co. (China) while acetonitrile (HPLC grade) and formic acid were purchased from Fisher Co. (USA). Ultra-pure water (18.2 MΩ) was prepared with a Milli-Q water purification system (France).

2.2. Preparation of general anaerobic medium broth

Anaerobic broth content (g/L): 15.0 g peptone, 5.0 g soya peptone, 5.0 g yeast extract, 5.0 g beef extract, 5.0 g glucose, 2.5 g KH_2PO_4, 5.0 g NaCl, 3.0 g soluble starch, 0.5 g L-cysteine hydrochloride, 0.005 g chlorhematin, 0.001 g Vitamin K1, and 4.6 g anaerobic broth powder, pH 7.1–7.5, were accurately weighted, then dissolved in 100 mL distilled water. The obtained anaerobic medium was then dispensed into conical flasks, autoclaved at 0.07 MPa at 115 °C for 30 min, and used immediately.

2.3. Preparation of human and rat intestinal bacterial mixture

Fresh feces (1 g) were obtained from two male Chinese volunteers (aged 27 and 34). These subjects were healthy, nonsmokers who had not taken alcohol or oral medications for at least one month, and had eaten soy-free diet for more than one week. Also, feces (1 g) were obtained from rats (four male Sprague-Dawley rats, 220–250 g, fed soy-free diet for more than a week) and transferred to two flasks, each of which contained 20 mL anaerobic medium. The feces were thoroughly dispersed in the medium, and then culture solutions of intestinal bacteria were prepared.

2.4. Intestinal bacteria culture and sample preparation

Firstly, 5 mg KA and Ir were weighed and dissolved in 50 μL methanol, respectively, and then 10 μL of the solutions of KA and Ir were added to 1 mL fresh human and rat intestinal bacteria solutions. At the same time, 10 μL methanol was added to 1 mL human and rat intestinal bacteria solutions as controls. Finally, the solutions were transferred to 2 mL Eppendorf tubes, and then put in a plastic bag and, after inserting a pouch-anaero into the plastic bag, the cultures were incubated at 37 °C for 48 h.

Addition of 2 mL methanol terminated the reaction, and samples were immediately vortex mixed for 30 s, and then centrifuged at 10,000 rpm for 10 min. The supernatants were transferred to other tubes and evaporated to dryness under a stream of nitrogen gas at room temperature. The residues were reconstituted in 2 mL methanol and centrifuged at 13,000 rpm for 10 min, and then 2 μL samples of the supernatants were used for UHPLC/Q-TOF MS analysis.

2.5. UHPLC/Q-TOF MS

Separations were performed on an Acquity UHPLC system (Waters) with an Acquity UHPLC column (HSS C_{18} 100 mm × 2.1 mm, 1.8 μm) at 40 °C. The flow rate was 0.45 mL/min and the Auto sampler was maintained at 4 °C. The mobile phase was (A) 0.2% formic acid in water (v/v) and (B) 0.2% formic acid in acetonitrile (v/v). Gradient elution was carried out as follows: 0–0.5 min, a linear gradient from 5–15% B; 0.5–4 min, 15–25% B; 4.1–5 min, 35–42% B; 5–7 min, 42–70% B; 7–7.1 min, 70–100% B; 7.1–9 min, and 100–5% B. The injection volume was 2 μL for both controls and samples. A Micromass-Q-Tof Premier mass spectrometer (Waters) coupled to an electro spray ionization (ESI) source was operated in positive ion mode. Data were acquired in sensitive mode and the MS tuning parameters were set as follows: the source and desolvation temperatures were set at 130 °C and 350 °C, respectively; the cone and desolvation gas flows were 50 L/h and 700 L/h, respectively; the capillary voltage was set at 3.0 kV, and the cone voltage was set at 40 eV; the micro-channel plates (MCPs) were operated at 1750 V with 3.6 GHz. MS^E mode was selected to acquire data with a low collision energy set at 6 eV in the first function and a collision energy ramp from 20 to 40 eV in the second function. Centroid mode data were collected over ranges of m/z 100–1000, and the scan time was 0.2 s with an interscan delay of 0.02 s. The solution of leucine-enkephalin generating an $[M+H]^+$ ion (m/z 556.2771) was infused through the Lock Spray probe at 10 μL/min.

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