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ORIGINAL ARTICLE

Chiral separation of bavachinin in *Fructus Psoraleae* and rat plasma by liquid chromatography using permethylated-β-CD as a chiral selector

Jing-Jing Liu, Juan Zhang, Zi-Lin Chen[*]

Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Wuhan University, Ministry of Education, Wuhan University School of Pharmaceutical Sciences, Wuhan 430071, China

Abstract A simple, sensitive and selective method of high-performance liquid chromatography (HPLC) has been successfully developed for separation of bavachinin enantiomers in *Fructus Psoraleae* and rat plasma. The separation and detection conditions of HPLC were optimized. Chiral bavachinin were separated with the mobile phase of methanol and water (70:30, v/v) at a flow rate of 1.0 mL/min. The linear ranges were in the range of 20–1000 µg/mL. The detection limits were tested as 4 ng/mL and 6 ng/mL for (+)-bavachinin and (−)-bavachinin, respectively. The method has been applied to analyze chiral bavachinin in rat plasma. HPLC–MS method was used to test the accuracy.

1. Introduction

Chirality is a ubiquitous feature in nature. Most of the agrochemicals and drugs are chiral compounds. Though the enantiomers of chiral compounds have similar physical and chemical properties, the enantiomers often exhibit different pharmacological and toxicological properties [1]. Generally, only one enantiomer has desirable therapeutic activity, while the other exhibits no therapeutic activity, antagonistic effects, or even toxic effects. Meanwhile, the absorption, distribution, metabolism and excretion of enantiomers in vivo is also quite different [2]. During the late 1950s and early 1960s, thalidomide, a sedative and hypnotic drug, which was administered by pregnant women, caused the birth of babies with malformations. Researches have revealed that the teratogenic effect results from the *S*-(-)-isomer [3]. As thalidomide is unstable in the body and easily experiences racemization, even though the administration of the pure *R*-(+)-isomer could not prevent the disaster [4,5]. Furthermore, more and more new drugs tend to appear on the market as single enantiomers with

*Corresponding author at: Institute of Pharmaceutical Analysis, School of Pharmaceutical Sciences, Wuhan University, Wuhan 430071, China. Tel.: +86 27 68759893; fax: +86 27 68759850.
E-mail address: chenzl@whu.edu.cn (Z.-L. Chen)

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the desired therapeutic activity. Chiral separation plays an important role in the agrochemical and pharmaceutical fields.

Several chromatographic separation methods, such as gas chromatography (GC), high performance liquid chromatography (HPLC), supercritical fluidic chromatography (SFC), and capillary electrophoresis (CE), have been used for enantioseparation. These analytical techniques can be ascribed to indirect and direct separation methods. Indirect method is mainly based on the transformation of enantiomers into diastereoisomers with chiral derivatization reagents. The enantioseparation can be performed on an achiral column [6]. The direct method is based on the interactions between enantiomers and chiral stationary phases (CSP). The mechanism of chiral recognition using CSPs is based on difference of stability on the formed temporary diastereoisomers. In fact, direct chiral separation using chiral stationary phases for HPLC is the most popular tool for the analysis of enantiomer and the preparation of pure enantiomers. It also has been widely applied to the determination of Traditional Chinese Medicine (TCM) at low concentration levels.

Fructus Psoraleae (Buguzhi in Chinese), the dried ripe fruit of *Psoralea corylifolia* L. (Fabaceae), is a well-known TCM which is usually used to alleviate diarrhea and asthma and also to treat vitiligo and alopecia areata in some East Asian countries [7]. It mainly contains four kinds of active compounds, such as coumarins, flavonoids, benzofuran glycosides and meroterpene [8]. These compounds have many pharmacological activities. For example, they are able to stimulate bone formation [9], enhance cardiac contractility [10], inhibit skin tumor [11], induce apoptosis in cancer cells [12] and so on. Bavachinin (Fig. 1), as one of its main active ingredients, possess some special effects including anti-bacterial [13], anti-oxidative activities [14,15], anti-inflammatory [16], inhibition to α-glucosidase activities [17], and inhibition the accumulation of nitric oxide (NO) [18].

Although many methods have been reported to separate and identify active components in the TCM, such as micellar electrokinetic chromatography (MEKC) [19], thin-layer chromatography (TLC) [20], high-speed counter-current chromatography (HSCCC) [21], high-performance liquid chromatography (HPLC) [22,23], liquid chromatography–mass spectrometry (LC/MS) [24,25], high-performance liquid chromatography–electrochemical detection (HPLC/ECD) [26], there is no report to describe the chiral separation of the active components in *F. Psoraleae*. In this paper, an HPLC method using permethylated-β-CD as a chiral selector was developed to determine the enantiomers of bavachinin in *F. Psoraleae* and rat plasma. Several separation conditions were systematically optimized. Furthermore, HPLC–MS method was used to identify and confirm the racemic bavachinin in *F. Psoraleae*.
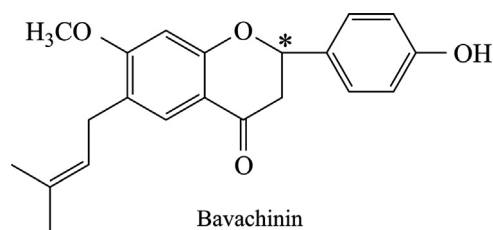

Fig. 1 Chemical structure of bavachinin.

2.   Experimental

2.1.   Chemicals and materials

The ripe seed of *P. corylifolia* L. was obtained from Liuyouyu Drugstore (Wuhan, China). Bavachinin (purity ≥ 99%) was purchased from Shanghai Shunbo Bio-engineering Technology (Shanghai, China). HPLC grade methanol was purchased from Tedia Chemical (USA). Deionized water was obtained from a Milli-Q Purification System (Millipore, Bedford, MA, USA); all other chemicals and solvents in the experiments were analytical grade and obtained from commercial Chemical Reagent Co.

2.2.   Chromatographic conditions and instrumentation

The HPLC system was equipped with LC-20AT (Shimadzu, Japan), a sample injector equipped with a 20-μL loop and a Shimadzu SPD-20 A UV detector. Chromatographic separations were carried out on a NUCLEODEX Ô-PM column (100 Å, 5 μm, 200 mm × 4 mm, Germany) at room temperature. An isocratic elution was used with a mobile phase consisting of methanol and water (70:30, v/v) at the flow rate of 1.0 mL/min. The injection volume was 20 μL and the detection wavelength was set at 246 nm.

For HPLC–MS analysis, the Agilent 1100 HPLC system was coupled on-line to an LC/MSD Trap SL Plus spectrometer (Agilent Corp, Waldbronn, Germany) equipped with electrospray ionization (ESI) source. The Auto MS operation parameters are negative-ion mode (ESI⁻), nitrogen drying gas, 10 L/min; nebulizer, 50 psi; gas temperature, 350 °C; compound stability, 80%; mass range, 50–1000 m/z. Detection of bavachinin was performed in selected ion monitoring (SIM) mode at m/z 337 in negative mode.

2.3.   Preparation of standard solutions

The standard solutions of bavachinin were prepared by dissolving 5.0 mg in 5 mL methanol to yield a concentration of 1.00 mg/mL, and stored in the refrigerator at 4 °C. It was diluted with methanol to obtain calibration solutions ranging from 20 to 1000 μg/mL. All running solutions were filtered through a 0.45 μm nylon syringe filter.

2.4.   Extraction of F. Psoraleae

The powder sample (50 mg) *F. Psoraleae* was exactly weighed and mixed with 2 mL extraction solvent composed of methanol/concentrated hydrochloric acid (4/1, v/v) and extracted at 20 °C for 45 min by ultra-sonication. Then it was taken out of the ultrasonic bath and left at room temperature for 30 min. The extract liquid was centrifuged at 14,000 g for 20 min, and collected the supernatant and stored in a refrigerator at 4 °C.

2.5.   Preparation of rat plasma

Blood sample was obtained from a female rat weighing about 250 g. Then it was immediately centrifuged at 3500 rpm for 10 min, and collected the supernatant (plasma). Following that, the plasma was vortex-mixed (about 200 μL) with 1 mL methanol and 10 μL formic acid for 30 s and then centrifuged

at 14,000 rpm for 10 min. The supernatant was collected and dried with nitrogen gas. The residue was resolved with 100 μL methanol and filtered through a 0.45 μm of membrane filter. A volume of 20 μL of the clear filtrate was then injected into the HPLC-UV system.

2.6. Animal experiments

Healthy SD rats (250 g±20 g) were purchased from the Experimental Animal Center of Wuhan University. The rats were free to water and fed with a standard laboratory diet. Before the experiments, each animal was fasted overnight. And then 40 g/kg of racemic bavachinin in an aqueous solution containing 2% CMC-Na was orally administered. About 300 μL of blood samples were collected at 3 h post-dosing, and plasma was immediately separated from heparinized blood by centrifugation (3500 rpm, 10 min).

3. Results and discussion

3.1. Separation mechanism of PM-β-CD

β-CD is cyclic oligosaccharides consisting of seven D-glucopyranose units bonded through α-(1,4) glycosidic linkages. The shape of its structure is like a truncated cone with a cavity. The secondary hydroxyl groups at the C-2 and C-3 positions were located on the wider edge of the ring, and the primary hydroxyl groups at the C-6 position were located on the narrower edge. This enables the external surface of the cavity to be hydrophilic, while the internal surface of the CD cavity consisting of glycosidic oxygen and methane protons is hydrophobic. Thus the separation interactions between β-CD and analytes are mainly through van der Waals interactions, hydrophobic interactions, π–π interactions, hydrogen bonding and dipole–dipole interactions. Meanwhile, the hydroxyl groups on the rim of CD can be easily modified by substituent groups to form various CD derivatives. Permethylated β-CD is one of its derivatives. Compared to the natural cyclodextrin and methylated cyclodextrin, PM-β-cyclodextrin not only has a great change in its cavity size and shape, but also the hydrophobic regions which moved from the center to the two ports of the cavity. Therefore, PM-β-CD shows a better inclusion behavior for certain analytes in the process of molecular recognition and chiral separation. As to the separation of bavachinin, both hydrophobic interaction and van der Waals probably play very important roles in the separation. The possible reasons are that the original hydrogen bond network of CD is damaged by the introduction of a large amount of methyl which results in hydrophobic cavity and strengthen the hydrophobic interactions between the hydrophobic groups of bavachinin and the cavity in the cyclodextrin.

3.2. Optimization of the mobile phase

Methanol and water were chosen as mobile phase in this work. The effect of methanol concentration in aqueous solution varied from 50% to 80% was investigated. With higher percent of methanol in the mobile phase, the retention time was too short to separate the enantiomers of bavachinin in *F. Psoraleae* well,

following with overlapped peaks. However, the two enantiomers were eluted very slowly and the peaks were too wide when methanol concentration was lower than 60%. Thus, 70% methanol in aqueous solution was selected as mobile phase.

3.3. Optimization of deproteinizing agent

Because some matrices in rat plasma could interfere the enantioseparation and were harmful to the chiral column, the pretreatment of the rat plasma was performed. Some of deproteinizing agents were tested, including methanol, acetonitrile, acetone and THF. Before the precondition, 10 μL (10 μg/mL) bavachinin was added into the blank rat plasma. The following step was the same as described in Section 2.5. Finally, 20 μL sample was injected for HPLC–UV analysis. There are no obviously significant differences between different deproteinizing agents. Considering the composition of mobile phase, methanol was chosen for deproteinization.

3.4. Linearity and LOD

Under the optimum chromatographic conditions, the linear relationships were obtained by plotting the peak areas versus the concentrations of ($\pm$)-bavachinin in standard samples at six different concentration levels in range of 20–1000 μg/mL. The linear regression equation was $y = 22.9x + 1882.7$ for (+)-bavachinin and $y = 19.8x + 1456.1$ for (−)-bavachinin, respectively. According to the equations, good linearity was obtained with the correlation coefficients of 0.9995 and 0.9993, respectively. The limits of detection (LODs) were determined by the ratio of signal to noise ($S/N = 3$), which was 4 ng/mL for (+)-bavachinin and 6 ng/mL for (−)-bavachinin. It suggests that this method has good sensitivity.
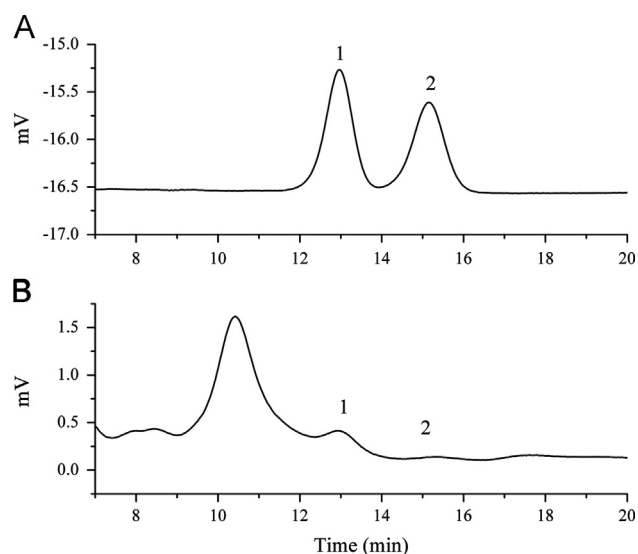

Fig. 2 Chromatograms of bavachinin enantioseparation in standard solution (A) and in real sample of *Fructus Psoraleae* (B). Mobile phase: methanol/water (7:3, v/v); Flow rate: 1.0 mL/min; Detection wavelength: 246 nm; Peak identification: 1, (+)-bavachinin; 2, (−)-bavachinin.

3.5. Application

The proposed method was applied to determine the bavachinin enantiomers in *F. Psoraleae*. Chromatograms of resolving bavachinin enantiomers in standard solution and in real sample of *F. Psoraleae* were shown in Fig. 2. The optical rotations of two peaks of bavachinin were testified by circular dichroism, in which (−)-bavachinin has longer retention time than (+)-bavachinin. The contents of the bavachinin enantiomers in *F. Psoraleae* were determined as 1.769 mg/g for (+)-bavachinin and 0.821 mg/g for (−)-bavachinin, respectively.

Further, the developed HPLC method was applied to determine the bavachinin enantiomers in rat plasma. The chromatograms of bavachinin in blank plasma and rat plasma sample after oral administration of bavachinin are shown in Fig. 3. Moreover, an HPLC–MS method was also developed to confirm and identify the enantiomers. Fig. 4 illustrates the total ion chromatogram (TIC) of *F. Psoraleae* and standard solution of bavachinin by HPLC–MS.

4. Conclusions

In this work, the enantiomers of bavachinin in Traditional Chinese Medicine *F. Psoraleae* was separated and determined by HPLC-UV with permethylated β-CD as chiral stationary phase. The enantioseparation condition was optimized. Under
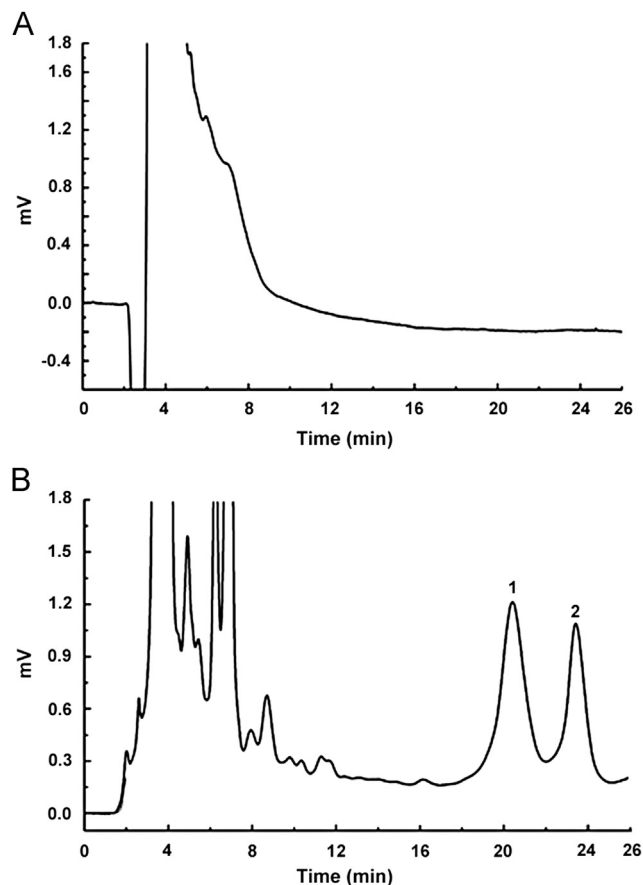

Fig. 4 Total ion chromatogram (TIC) of bavachinin enantiomers by HPLC–MS in *Fructus Psoraleae* (A) and standard solution (B). Flow rate: 0.5 mL/min; All other conditions were the same as in Fig. 2.

the optimum condition, the proposed method has good linearity and LOD. The method has been successfully applied to determine the bavachinin enantiomers in real sample of Chinese medicine of *F. Psoraleae* and in rat plasma after oral administration of bavachinin. In order to exclude the interference from impurity, different kinds of deproteinizing agents were discussed. In addition, MS detection has been further used to identify and confirm the peak. This method is promising for pharmacokinetic study of bavachinin enantiomers.

Fig. 3 Chromatograms of bavachinin enantioseparation in blank plasma (A) and rat plasma sample after oral administration (3 h) (B). All other conditions were the same as in Fig. 2.
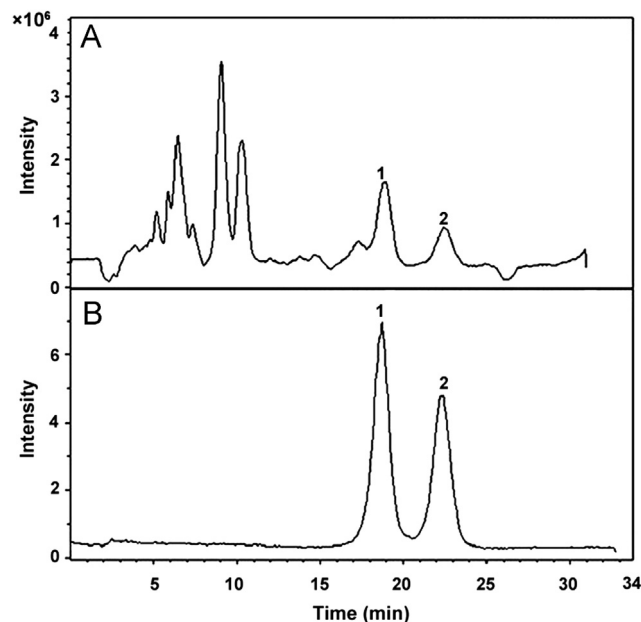
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