Published in final edited form as:

J Liq Chromatogr Relat Technol. 2015 January 1; 38(5): 647–653. doi:10.1080/10826076.2014.936606.

SEPARATION AND PURIFICATION OF TWO MINOR COMPOUNDS FROM *RADIX ISATIDIS* BY INTEGRATIVE MPLC AND HSCCC WITH PREPARATIVE HPLC

Zhenjie Liang^a, Bin Li^b, Yong Liang^a, Yaping Su^a, and Yoichiro Ito^c

^aInstitute of Analytical Chemistry, School of Chemistry and Environment, South China Normal University, Guangzhou, China

^bShenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen 518067, China

^cLaboratory of Bioseparation Technology, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract

Radix isatidis has been widely used as a Chinese traditional medicine for its anti-virus and anticancer activities where the minor components may contribute to these beneficial pharmaceutical effects. In order to enrich the target minor compounds effectively and rapidly, extraction, medium-pressure liquid chromatography (MPLC), high-speed countercurrent chromatography (HSCCC) and preparative high-performance liquid chromatography (pre-HPLC) were integratively used for separation and purification of two target minor compounds indole-3-acetonitrile-6-O- β -D-glucopyranoside (target 1) and clemastanin B (target 2) in the present study. *Radix isatidis* was dried, pulverized and extracted with 50% methanol at room temperature, then concentrated and subjected to pretreatment with D-101 macroporous resin chromatography and extraction by MPLC. The first target compound was separated by MPLC at the purity raised to 70–80%, but without the second minor compounds which were irreversibly adsorbed by C18 solid support. Therefore, the second target compound in the crude extract was directly separated by HSCCC at purity of 80–90%. Finally these refined samples were further separated by pre-HPLC to obtain a high purity at 98–99%. The chemical structure identification of each target compound was carried out by IR, ESI-MS and ¹H NMR.

Keywords

Radix isatidis; Macroporous resin; MPLC; HSCCC; HPLC

INTRODUCTION

Radix isatidis (Cruciferae), a traditional Chinese medicinal herb, has been used for the treatment of seasonal febrile, pestilence, mumps, eruptive inflammatory redness of skin, and sore throat.^[1–3] Pharmacological research indicated that it has anti-virus, anti-bacterial, anti-endotoxic, antitumor, anti-inflammatory and immune regulatory activities.^[4–5] The main functional components in *Radix isatidis* include alkaloids, sucrose, organic acids, glycosides, nucleosides, lignanoids and phenylpropanoids. Development of an effective and

rapid separation method for minor compounds^[6] will promote the *Radix isatidis*' pharmaceutical value. In this paper^[7] two minor components, indole-3-acetonitrile-6-O- β -D-glucopyranoside ^[8–9] (target 1) and clemastanin B of phenylpropanoids (target 2) were separated (chemical structures are given in Figure 1)^[10–12]. These two target compounds have been reported to be responsible for anti-virus, anticancer, anti-inflammatory, anti-bacterial, and abirritative properties as well as suppression of capillary permeability. Nevertheless, they are present at a low level in *Radix isatidis* at 0.1–5% of the total peak area of ultraviolet absorption. Although the first target compound was purified with silica gel, macroporous resin,^[13] polyamide and preparative HPLC^[14] from various plant materials, these chromatographic methods are tedious and result in sample loss due to irreversible adsorption onto the solid support. In the present study D-101 macroporous resin LC, MPLC, HSCCC and pre-HPLC were successively used to obtain available quantities of the two targets in *Radix isatidis*.

Resin-based column chromatography has large capacities for effective preparative separation of the plant extracts, which can adsorb analytes according to their polarities. Polar D-101 macroporous resin chromatography^[15] is an effective method for the separation and purification in Chinese herbal medicine. According to different polarities of the materials, different proportions of alcohol-water are used to elute the compounds. In the study, D-101 macroporous resin column chromatography and organic solvent extraction are compared in their ability to concentrate the target compounds from the ethanol aqueous extract. It proved that they both can be a suitable technique for separation of natural products directly at purities of 30–40%. Operation of organic solvent extraction is easier than D-101 macroporous resin, but D-101 macroporous resin yields better separation than organic solvent extraction.

MPLC is used for isolation of natural products and considered as an efficient tool^[16] to separate the target compounds. MPLC has been mostly used for crude separation of samples, and the method has proved in the present study that it is a suitable technique directly used for separation of natural products at high purity of 70–80%. The fractions collected by MPLC were further purified by preparative HPLC.

HSCCC is a form of liquid–liquid partition chromatography.^[17] where solute separation is based on partitioning between the two immiscible liquid phases: the liquid stationary phase is retained in the column free of solid support by the aid of a centrifugal force provided by the planetary motion of the column. Therefore, the system eliminates irreversible adsorption of samples onto the solid support, and is very suitable for separation of active components from traditional Chinese medicinal herbs and other natural products. In virtue of superexcellent efficiency, pre-HPLC is a powerful tool to achieve high purity.^[18–19]

The objectives of the present study, therefore, are to isolate and purify the minor target compounds with the combined use of D-101 macroporous resin chromatography, MPLC, HSCCC and pre-HPLC. In order to track experimental procedure, analytical HPLC was used throughout this study. The results are significant and effective, and the method would be applied for the preparative isolation and purification of flavone C-glucosides from other plants.

EXPERIMENTAL

Apparatus and Reagents

The analytical high-performance liquid chromatography (HPLC) system used throughout this study consisted of LC-20AT pump (Shimadzu, Kyoto, Japan), an SPD-20A UV-VIS the diode array detector (DAD, Shimadzu), an LC-solution workstation (Shimadzu), and a 20 μ l sample loop. The column used was a reversed-phase C18 (250 $\times$ 4.6 mm i.d., sinochrom ODS-BP, 5 μ).

MPLC, HSCCC and pre-HPLC systems used in the present study are as follows: LC3000 semi-preparative gradient HPLC system consisted of P3000 HPLC pump, a UV3000 UV-VIS detector, a CXTH-3000 Chromatography workstation. The chromatographic column of MPLC was C18 column (390 $\times$ 29 mm i.d., 40–60 μ). The HSCCC instrument was a GS10A-2 high-speed counter-current chromatograph (Beijing Institute of New Technology Application, Beijing, China). The chromatographic column was a set of multilayer coils connected in series (i.d. of the tubing = 1.6 mm, total volume = 82 mL). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. The chromatographic column of pre-HPLC applied in this work was a YMC-Pack ODS-A column (250mm $\times$ 10 mm, i.d., 5 μ , YMC Co. Ltd., Japan).

The D-101 macroporous resin (Nankai University Chemical Plant, Tianjin, China), which is milky white spherical granules of cinnamene and hydrophobic sorbent, has stronger adsorption capability and is more easily activated than most of other sorbents.

Ethanol, petroleum ether, ethyl acetate, n-butanol, acetic acid were analytical reagents and purchased from commercial sources and used as received. Acetonitrile and methanol were of HPLC grade. Deionized water was passed through a 0.45 μ m filter. The standards of the indole-3-acetonitrile-6-O- β -D-glucosid and clemastanin B were 99% purity.

Preparation of plant extracts

A 10 kg amount of *Radix isatidis* powder was boiled twice in water for 24 h and filtered. The filtrate was concentrated by rotary evaporation in vacuum at 50°C and then added with ethanol to precipitate for 24 h. The mixture was filtered and the filtrate was concentrated again. The standards of the indole-3-acetonitrile-6-O- β -D-glucosid and clemastanin B were dissolved in 50% methanol-water with the concentration of 1 mg/ml. The target compounds in the concentrated solution were analyzed by HPLC. Complexity of chemical constituents in Chinese medicinal herb is the present problem. A preliminary analysis of ultraviolet absorption is needed for the chromatographic analysis for the crude extract and the standards of the indole-3-acetonitrile-6-O- β -D-glucosid and clemastanin B. With the distribution of ultraviolet absorption peak chromatograms and retention time, target 1 was indole-3-acetonitrile-6-O- β -D-glucosid, and target 2 was clemastanin B.^[20–21]

Preparation of the Crude Extract

The concentrated solution was dissolved in hot water (50°C, 100:1 g per L) and filtered to obtain two fractions, the filtrate and the precipitation. Analytical HPLC showed that the

filtrate had targets, and it was submitted to D-101 macroporous resin column chromatography and organic solvent extraction.^[22–23]

Preparation by macroporous resin

The pre-purification of the crude sample has been applied by two methods: The one is D-101 macroporous resin, which was pretreated and filled in a low-pressure glass chromatographic column (150 cm × 100 mm). The filtrate liquor was loaded onto the pretreated column and adsorbed statically overnight. Subsequently, the column was eluted with 24 L of deionized water, 5% ethanol in water, 20% ethanol in water and 50% ethanol in water in this order each at a flow rate of 30 mL/min. The fractions, with tracking by HPLC, were carefully collected and concentrated by a rotary evaporator.

Sample preparation by extraction

The second method was extracting with an organic solvent. The concentrated solution was added with water and partitioned with petroleum ether, ethyl acetate, and n-butanol in sequence. All of the organic solvent extracts were then concentrated by a rotary evaporator. Finally, these crude extracts of the target compounds obtained by the two methods were submitted for purification by MPLC.

Purification of target 1 by MPLC

The medium-pressure C18 chromatographic column (390 × 29 mm i.d. 40–60 μ) was conditioned by first eluting with 100% methanol for 10 min at a flow rate of 30 mL/min, and then equilibrating with 5% methanol aqueous water at the same elution time and flow rate. After dissolving the concentrated extract in methanol completely, the solution was loaded in the pre-column and separated in the following gradient elution program: The mobile phase was water with 0.1% acetic acid (solvent A) and methanol (solvent B). The elution was started with 5% B (0–10 min), 5–25% B (10–15 min), 25% B (15–150 min), 72% B (150–240 min). The flow rate was from 5 mL/min to 20 mL/min while the single detection wavelength was 254 nm. The peak fractions were collected manually by observing the elution profile of the CXTH-3000 chromatography workstation.

The purity of each collected fraction was determined by analytical HPLC with the mobile phase of 0.1% acetic acid (solvent A) and methanol (solvent B) with the gradient elution (5-25-25% B, 0-20-40 min). When the purities of the monomers were 80–90%, the fractions were submitted for further purification by preparative HPLC.

Purification of target 2 by HSCCC

The second target compound was directly separated from crude extract by HSCCC. The first step, a two-phase solvent system composed of ethyl acetate–n-butanol–water (1: 4: 5, v/v) was optimized to use. The coil column was first cleaned by purging N₂, and then entirely filled with the upper phase. Then the lower mobile phase was eluted through the column at a flow rate of 1 mL/min while the apparatus was rotated at 400 rpm. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the sample solution, 1.42 g of crude extracts dissolved in the 10 mL of the solvent system with 5 mL upper phase and 5 mL lower phase, was injected through the injection valve. The effluent

from the outlet of the column was continuously monitored with a UV–vis detector at 254 nm. Peak fractions were automatically collected with a fraction collector. When the separation procedure was finished, the column contents were collected to measure the retention of the stationary phase in the column. The fractions containing the target compounds were evaporated to dryness by a rotary evaporator. The yielded sample was separated by the next run of pre-HPLC.

Further purification by Preparative HPLC

Preparative high-performance C18 chromatography was performed similarly to medium-pressure C18 chromatography, except that the flow rate was 10 ml/min with the different time program. The maximum detection wavelengths for the two target compounds 1 and 2 were 265 nm and 280 nm, respectively. The purity of each collected fraction of the first target compound was determined by analytical HPLC with the same mobile phase in MPLC, while the second target compound was determined by analytical HPLC with the same mobile phase in HSCCC. The final product was obtained after freeze-drying. The process of the isolation and purification was shown in Figure 2.

Confirmation

Target compounds were dissolved in methanol and passed through a 0.45 µm filter. The purities of target 1 were determined by HPLC using a gradient elution with the mobile phase composed of 0.1% acetic acid water (solvent A) and methanol (solvent B): 0–8 min, 0% B, 8–25 min, 9% B, 25–35 min, 9–12% B, 35–45 min, 12–16% B, 45–55 min, 16–18% B, 55–100 min, 18–30% B. The flow rate was 0.4 mL/min at 25°C with the UV detection wavelength of 265 nm. And the target compound 2 was determined by HPLC in the same mobile phase at a flow rate of 0.4 mL/min at 25°C with the UV detection wavelength at 277 nm. The chemical structure identification of each target compound was carried out by IR, ESI-MS and ¹H NMR.

RESULTS and DISCUSSION

HPLC analysis

The analysis of extract performed with a gradient elution with 0.1% acetic acid water (solvent A) and methanol (solvent B): 0–8 min, 0% B, 8–25 min, 9% B, 25–35 min, 9–12% B, 35–45 min, 12–16% B, 45–55 min, 16–18% B, 55–100 min, 18–30% B. The flow rate was 0.4 mL/min and the detection wavelength was 254 nm. The obtained chromatogram is shown in Figure 3. The retention time was between 60 min for peak 1 and 63 min for peak 2. The chemical property was medium polarity. Peak 1 had two maximum wavelengths at 219 nm and 265 nm, while peak 2 had two maximum wavelengths at 225 nm and 277 nm.

Preparation of the crude extract

D101 macroporous resin was initially eluted with deionized water to remove phenolic acids, minerals and sugars, and subsequently eluted with 5%, 20%, and 50% (v/v) ethanol solution in succession. Comparing the analytical HPLC of the four fractions, 20% ethanol solution contained the small amount of the target compounds. All of the 50% ethanol solution was

concentrated by a rotary evaporator at 50°C. The 20% ethanol fraction concentrate was further purified by MPLC.

The filtrate (Figure 2) was extracted using petroleum ether, ethyl acetate, n-butanol in sequence. After tracking by HPLC in the mobile phase of acetonitrile-water, the target components were mainly enriched in ethyl acetate and n-butanol fractions. Since the extract of ethyl acetate had many impurities, it was removed out of the gathering. The entire n-butanol fraction was then concentrated by a rotary evaporator at 50°C and submitted to purification by MPLC.

Purification by MPLC

Fraction 1 obtained from the crude extract was further purified by MPLC. Four fractions (I–V) were collected manually by observing the minor target compounds in MPLC chromatogram, as shown in Figure 4A. The purity of each collected fraction was determined by analytical HPLC (Figure 4B). The peaks in the MPLC chromatogram were identified according to their retention times and UV spectra from the analytical HPLC system. Consulting the DAD spectra, the first minor flavone C-glucosides (first target compound) in fraction II in Figure 4B was enriched and concentrated by a rotary evaporator for further purification. The left analytical HPLC chromatograms had nothing but the main components. In the present study, therefore, MPLC was mainly used to isolate the first target monomer. Since the second target monomer was not enriched, it was purified by another method.

Purification by HSCCC

A successful separation of the target compounds using HSCCC requires a careful search for a suitable two-phase solvent system to provide an ideal range of partition coefficients (K). Optimized selections of the two-phase solvent systems were based on good K values close to unity. The K value was defined as the peak area of the target compound in the upper phase divided by that in the lower phase. In order to achieve efficient separation of target compounds, various two-phase systems were tested. The two-phase solvent systems n-hexane–ethyl acetate–methanol–water (5: 5: 5: 5, v/v), n-hexane–n-butanol–methanol–water (2: 3: 1: 4, 1: 3: 1: 4, 1.5: 3: 1: 4, v/v) were excluded because of the little K values of target compounds. And the two-phase solvent systems ethyl acetate–n-butanol–water (2: 7: 9, 3: 2: 5, 2: 3: 5, 1: 4: 5, v/v) were further evaluated. When the two-phase solvent system ethyl acetate–n-butanol–water (1: 4: 5, v/v) was applied, the second target compound clemastanin B was eluted without any other compounds. Therefore, this solvent system was selected for HSCCC with a flow rate of 1.0 mL/min, revolution speed of 750 rpm and temperature of 30°C, and 1.6 g of crude extracts dissolved in 20 ml (10 mL of each phase) was injected through the injection valve. The total separation time was about 4 h (Figure 4C) and the retention of the stationary phase was 76%. Obviously, the method is very effective for such a large amount of sample containing a minute amount of the second target compound without being lost due to irreversible adsorption. Under the optimized optimum HSCCC conditions, a small amount of the target compound in the crude extracts was separated and purified. The typical HSCCC chromatogram is shown in Figure 4C, and the typical HPLC analytical chromatogram is shown in Figure 4D. The second target compound was enriched

in the peak fractions III at the purity of was 82%, while fractions I and II were the mixture of the strongly polar components without much monomers. Fraction IV and V contained the second target compound with the lower polar components, which could be evaporated and injected to the HSCCC again. Then the fraction III was evaporated to dryness. This refined sample was then further separated by pre-HPLC.

Further purification by pre-HPLC

The C18 column temperature was kept at room temperature and detection wavelength was 265 nm. The applied flow rate was from 5 mL/min to 8 mL/min and the mobile phase of gradient elution was methanol - 0.1% acetic acid water (5 - 5 - 16 - 16 - 25 - 25-100%, 0 - 10 -20 - 60 - 100 - 120 min). The total separation time was about 2 h (Figure 5A), and the target compound 1 was sectionalized by the UV absorption for fractions I–III. As shown in the analytical HPLC chromatograms of fractions I–III (Figure 5B), the first target compound was separated. Determined by area normalization method, the purity of fraction III in chromatograms reached to 99.64%. Fraction III was dried by lyophilization and was submitted for further confirmation. Finally, 80 mg of refined sample was obtained

The target Compound 2 with the purity as 82%, was submitted to further purification by pre-HPLC. The C18 column temperature was also kept at room temperature and the detection wavelength was 277 nm. At a flow rate of 1.5 mL/min the mobile phase of gradient elution was applied: methanol - 0.1% acetic acid water (5 - 20 - 20 %, 0 - 30 -100 min). The target compound 2 was sectionalized by the UV absorption for fractions I–II, which were shadowed in Figure 5C. At the same time, Figure 5D shows the analytical HPLC chromatograms of fractions I–II. The purity was greatly improved to reach 98.4% in fraction II, and 95% in fraction I. Then, the fraction II was dried by lyophilization. The result of separation of the pre-HPLC was effective as a direct method for isolation and purification of the target compounds. These two target compounds were submitted for further confirmation as described below. Finally, 50 mg of refined sample was obtained.

HPLC Analysis and Chemical Structure Identification

The purities of the two compounds were assessed by analytical HPLC, using 0.1% acetic acid (solvent A)–acetonitrile (solvent B) water as the mobile phase with the gradient elution (0–8 min, 0% B, 8–25 min, 9% B, 25–35 min, 9–12% B, 35–45 min, 12–16% B, 45–55 min, 16–18% B, 55–100 min, 18–30% B). The flow rate was 0.4 ml/min and the detection wavelength was 265 nm for target 1 and 277 nm for target 2. HPLC chromatograms and UV spectra are shown in Figure 6. The purity of the first target was 99.6% and that of isoscoparin was 98.4%.

Identification of indole-3-acetonitrile-6-O- β -D-glucopyranoside and clemastaninB was carried out according to its ESI-MS and ^1H NMR data.

Target 1: $\text{IR}_{\text{max}}(\text{KBr}) \text{ cm}^{-1}$: 3400(OH), 3297 (NH), 2224($\text{C}\equiv\text{N}$). ESI-MS (–) m/z: 293.29 [M-H]. ^1H -NMR(DMSO) δ : 7.52(1H, s, 3-H), 7.25 (1H, d, J = 7.2 Hz, 7-H), 7.05 (1H, s, 5-H), 4.64 (1H, d, J = 8.1 Hz, β -Glc-1'), 3.26 ~ 3.60(6H, m, 2' ~ 6'-H). Comparing the data by the IR, MS, ^1H -NMR with the literature, [8, 9] target 1 was identified as indole-3-acetonitrile-6-O- β -D-glucopyranoside.

Target 2: IR_{max}(KBr) cm⁻¹: 3400 (OH), 2928, 1583, 1555, 1234, 1074. ESI-MS (–) m/z: 707 [M+Na]⁺, ¹H NMR(CDCl₃, 300 M)δ: 6.72(1H, s, 2-H), 6.48 (1H, s, 5'-H), 6.44 (1H, s, 6'-H), 6.35(1H, s, 2'-H), 6.31(1H, s, 5-H), 4.29-4.10 (2H, d, J = 10.5 Hz, 9'-H), 3.76 (1H, dd, J = 3.6, 3.9 Hz, 7'-H), 3.41-3.38(3H, s, 3'-OCH₃), 3.18 ~ 3.13(3H, s, 3-OCH₃), 3.03(1H, m, 8'-H). Comparing the data the IR, MS, ¹H-NMR with the literatures, [10, 11] target 2 was identified as clemastanin B.

CONCLUSIONS

Radix isatidis is a widely used traditional Chinese medicine. The development of effective and rapid separation methods and techniques for minor compounds would benefit the *Radix isatidis*' pharmaceutical value. In the present study the two minor target compounds of indole-3-acetonitrile-6-O-β-D-glucopyranoside and clemastanin B were effectively isolated from an ethanol aqueous extract of *Radix isatidis* by the integration of MPLC, HSCCC and pre-HPLC at high purity. The chemical structure of these target compounds was identified by IR, ESI-MS and ¹H-NMR. Overall results of our studies indicate that the integration of MPLC, HSCCC and pre-HPLC would be suitable for preparing available quantities of highly polar minor compounds in traditional Chinese medicine. Since HSCCC could use pH-zone-refining method to separate alkaloids, further studies using this method should be considered.

References

1. Liu Z, Yang ZQ, Xiao H. Antiviral activity of the effective monomers from *Folium isatidis* against influenza virus in vivo. *J Virol sinica*. 2010; 25(6):445–451.
2. Deng XY, Gao GH, Zheng SN, Li FM. Qualitative and quantitative analysis of flavonoids in the leaves of *Isatis indigatica* fort. by ultra-performance liquid chromatography with PDA and electrospray ionization tandem mass spectrometry detection. *J Pharm Biomed Anal*. 2008; 48:562–567. [PubMed: 18585883]
3. Liao BC, Jong TT, Lee MR, Chen SS. LC-APCI-MS method for detection and analysis of tryptanthrin, indigo, and indirubin in daqingye and banlangen. *J Pharm Biomed Anal*. 2007; 43:346–35. [PubMed: 16884885]
4. Fang J-G, Liu Y-H, WANG W-Q, Xie W, Fang S-X, Han H-G. The anti-endotoxic effect of o-aminobenzoic acid from *Radix Isatidis*. *J Acta Pharm Sinica*. 2005; 26(5):593–597.
5. Shin EK, Kim DH, Lim H, Shin H-K, Kim J-K. The Anti-Inflammatory Effects of a Methanolic Extract from *Radix Isatidis* in Murine Macrophages and Mice. *J Inflammation*. 2010; 2(33):110–118.
6. Li W, Cheng J, Yuan L, Li HB, SUN Q, Luo Y. Methodological research on the quality evaluation of *Radix Isatidis* based on antibacterial potency. *J Mode Tradit Chin Med Mater Med*. 2008; 10(2): 33–36.
7. Zhang XL, et al. The herbalome—an attempt to globalize Chinese herbal medicine. *J Anal Bioanal Chem*. 2012; 402:573–581.
8. Li X, Chen A-J, Li C. Studies on Water-soluble Chemical Constituent in *Radix Isatidis*. *J Chin Exp Trad Med Formulae*. 2010; 5(16):64–67.
9. Li, Bing; Chen, WS.; Zheng, SQ.; Yang, GJ.; Qiao, CZ. Two new alkaloids isolated from *Tetraploidy banlangen*. *J Acta Pharm Sinica*. 2000; 7(35):508–510.
10. Fu YY, Zu YG, Liu W, Hou CL, Chen LY, Li SM, Shi XG, Tong MH. Preparative separation of vitexin and isovitexin from pigeonpea extracts with macroporous resins. *J Chromatogr A*. 2007; 1139:206–213. [PubMed: 17140590]

11. Peng JR, Fan GR, Wu YT. Isolation and purification of clemastanin B and indigoticoside A from *Radix Isatidis* by high-speed counter-current chromatography. *J Chromatogr A*. 2005; 1091:89–93. [PubMed: 16395796]
12. Xiao, S-Sn; Jin, Y.; Sun, Y-Q. Recent progress in the studies of chemical constituents, pharmacological effects and quality control methods on the roots of *Isatis indigotica*. *J Shenyang Pharmaceutical Univer*. 2003; 6(20):455–459.
13. An YQ, Jia XB, Chang LL, Shi F. Comparison of content of clemastanin B of *Radix Isatidis* in different growing areas. *J Chin Mater Med*. 2009; 4(34):1823–1825.
14. Zhang Y, Jiao JJ, Liu CM, Wu XQ, Zhang Y. Isolation and purification of four flavone C-glycosides from antioxidant of bamboo leaves by macroporous resin column chromatography and preparative high-performance liquid chromatography. *J Food Chem*. 2008; 107:1326–1336.
15. Zhang Y, Jiao JJ, Liu CM, Wu XQ, Zhang Y. Isolation and purification of four flavone C-glycosides from antioxidant of bamboo leaves by macroporous resin column chromatography and preparative high-performance liquid chromatography. *J Food Chem*. 2008; 107:1326–1336.
16. Wang XH, Liang Y, Zhu LC, Xie HC, Li H, He JT, Pan M, Zhang TY, Ito Y. Preparative Isolation and purification of flavone c-glycosides from the leaves of *Ficus Microcarpa* L.f by Medium-Pressure Liquid Chromatography, High-speed Countercurrent Chromatography, and Preparative Liquid Chromatography. *J Liq Chromatogr Relat Technol*. 2010; 4(33):462–480.
17. Ito Y. Golden rules and pitfalls in selecting optimum conditions for highspeed counter-current chromatography. *Journal of Chromatogr A*. 2005; 1065:145–468.
18. Wang XH, Liang Y, Peng CL, Xie HC, Pan M, Zhang TY, Ito Y. Preparative Isolation and Purification of Chemical Constituents of *Belamcanda* by MPLC, HSCCC, and prep-HPLC. *J Liq Chromatogr Rel Technol*. 2011; 4(34):241–257.
19. Wang C, Pan YJ, Fan GR, Chai YF, Wu YT. Application of an efficient strategy based on MAE, HPLC-DAD-MS/MS and HSCCC for the rapid extraction, identification, separation and purification of flavonoids from *Fructus aurantii immaturus*. *J Biomed Chromatogr*. 2010; 24:235–244.
20. Zhou W, Zhang X-Y, Lva Y-P, Duan G-L. Rapid Sample Pretreatment, Identification and Determination of Active Constituents in Water-soluble *Radix isatidis* Extract via MAE, ESI-MS and RODWs-HPLC. *J Chin Chem Soc*. 2012; 59:51–59.
21. Zhou W, Xie M-F, Zhang X-Y, Liu T-T, Yu Y-J, Duan G-L. Improved liquid chromatography fingerprint of fat-soluble *Radix isatidis* extract using multi-wavelength combination technique. *J Sep Sci*. 2011; 34:1123–1132. [PubMed: 21442749]
22. Kong W-J, Zhao Y-L, Shan L-M, Xiao X-H, Guo W-Y. Investigation on the spectrum-effect relationships of EtOAc extract from *Radix Isatidis* based on HPLC fingerprints and microcalorimetry. *J Chromatogr B*. 2008; 871:109–114.
23. Zhao YL, Cao L, Wang JB, Jin C, Xiao XH. Study on the n-BuOH Extract's Fingerprint Chromatography and Bacteriostatic Activity of *Radix Isatis* and Correlation Analysis. *J Chin Med Mater*. 2005; 12(18):1079–1082.

Indole-3-acetonitrile-6-O-β-D-glucopyranoside

ClemastinB

Figure 1.
Chemical structures of indole-3-acetonitrile-6-O-β-D- glucopyranoside and clemastanin B.

Figure 2.
Separation procedure of target compounds from crude sample of *Radix isatidis*.

Figure 3.

HPLC chromatogram of the crude extraction of *Radix isatidis* by 50% methanol. Mobile phase: 0.1% acetic acid water (solvent A) - acetonitrile (solvent B), gradient elution: 0–8 min, 0% B, 8–25 min, 9% B, 25–35 min, 9–12% B, 35–45 min, 12–16% B, 45–55 min, 16–18% B, 55–100 min, 18–30% B, flow rate: 0.4mL/min⁻¹; detection wavelength: 254 nm.

Figure 4.

The Chromatograms of the target 1 by MPLC (A), the analysis of different fractions (I–V) (B), the target 2 by preparative HSCCC(C), and the analysis of different fractions (I– V) (D). The C18 MPLC column (390 * 29 mm I.D. 40–60 μ m). Mobile phase: 0.1% acetic acid water (solvent A) - acetonitrile (solvent B), gradient elution: 0–10 min, 5–5% B, 10–15 min 5–25% B, 15–150 min 25–25% B, 150–240 min 72–72% B. flow rate: 5–30 mL/min; detection wavelength: 254 nm. The HSCCC column was with multilayer coil separation columns connected in series (i.d. of the tubing = 1.6 mm, total volume = 82 mL). Solvent system: ethyl acetate–n-butanol–water at a volume ratio of 1:4:5. Mobile phase: the lower phase; flow rate: 1.0 mL/min; revolution speed: 750 rpm; detection wavelength: 254 nm.

Figure 5.

The Chromatograms of the target 1 purified by preparative HPLC (A), the analysis of different fractions (I–III) (B), the target 2 purified by preparative HPLC (C), and the analysis of different fractions (I– II) (D). YMC-Pack ODS-A column (250mm * 10mm, i.d., 5 μ m). Mobile phase: 0.1% acetic acid water (solvent A) -methanol (solvent B), gradient elution: (5 - 5 - 16 - 16 - 25 - 25-100% B, 0 - 10 - 20 -60 - 100 - 120 min), flow rate: 5–8mL/min; detection wavelength: 254 nm.

Figure 6.

HPLC chromatogram and UV-vis of the target compounds. The black was the target 1 and the red was the target 2, and the blue was for mixture of the target compounds. Mobile phase: 0.1% acetic acid water (solvent A) - acetonitrile (solvent B), gradient elution: 0–8 min, 0% B, 8–25 min, 9% B, 25–35 min, 9–12% B, 35–45 min, 12–16% B, 45–55 min, 16–18% B, 55–100 min, 18–30% B, flow rate: 0.4 L/min; detection wavelength: 265 nm for target 1, 277 nm for target 2, 254 nm for the mixture of target compounds.