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Short communication

Isolated perfused lung extraction and HPLC–ESI–MS^n analysis for predicting bioactive components of Saposhnikoviae Radix

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ABSTRACT

A novel strategy for predicting bioactive components in traditional Chinese material herb was proposed, using isolated perfused rat lung (IPL) extraction and high performance liquid chromatography\tandem mass spectrometry (HPLC–MS^n) analysis. The hypothesis is that when the IPL is perfused with the extract of Saposhnikoviae Radix (ESR), the potential bioactive components in the ESR should selectively combine with the receptor or channel of lung, by changing the pH of perfused liquid, the combining components would be eluated and then detected by HPLC–ESI–MS^n. Five compounds were detected in the desorption eluate of IPL; among these compounds, two potential bioactive compounds, prim-O-glucosylcimifugin (**2**) and 4′-O-β-D-glucosyl-5-O-methylvisamminol (**4**) were identified by comparing with the chromatography of the standard sample, and three other compounds, i.e. cimifugin (**1**), 5-O-methylvisamminol (**3**) and sec-O-glucosylhamaudol (**5**) were determined by analysis of the structure clearage characterization of mass spectrometry. The application of IPL extraction coupled with HPLC–ESI–MS^n for predicting potential bioactive components of TCMs is rapid, convenient, operational, economic and reliable.

1. Introduction

Saposhnikoviae Radix, called “Fang feng” in China, is the root of *Saposhnikocia divaricata* (Turcz.) Schischk, one of the most famous and important Traditional Chinese Medicines (TCMs). It has been widely used for the treatment of pyrexia, rheumatism, headache and convulsion for thousands of years in Chinese clinical practices [1,2]. Phytochemical studies showed abundant compounds in Saposhnikoviae Radix, such as coumarins, chromones, alkapolyalkynes and polysaccharides, among which coumarins and chromones are the main active components [2,3].

The traditional procedures for finding bioactive components of TCM are extraction and purification of constituents from TCM followed by *in vitro* or *in vivo* pharmacological screening of the purified compounds. Although possibly, only a limited number of compounds are responsible for pharmaceutical effects of TCM, the bioactive compounds are usually among complex mixtures containing up to hundreds or even thousands of different components, which makes the purification and screening extremely difficult.

Modern pharmacological studies have demonstrated that the ability of a drug to interact with receptors or channels on cell or membranes is very important as the first step in determining the behavior of the drug in the organism. Therefore, the interaction of compounds with cell membranes, cells or organ has been successfully used as a basis for hypothesis of bioactive components in Chinese medicines [4–6]. Recently, IPL extraction which has the ethical advantage of markedly reducing animal usage and also avoids the problem of inter-animal differences in drug administration has been successfully designed to address specific issues such as pharmacological effect [7], physiological phenomena [8], drug dissolution and absorption [9], mechanisms of absorption [10], metabolism [11–13], tissue disposition [14,15] and retention [15,16]. In this study, IPL was creatively used for predicting bioactive components of Saposhnikoviae Radix. Then the combined compounds were identified by HPLC–ESI–MS^n, which is a powerful technique for the identification of molecular structure and more and more used in the modern pharmaceutical analysis of TCM [17–20]. Recently, high performance liquid chromatography coupled with mass spectrometry technologies was frequently applied for the analysis and identification of constituents and metabolites from Saposhnikoviae Radix [22,25–26]. Five compounds were detected in the desorption eluate of IPL; among these compounds, two potential

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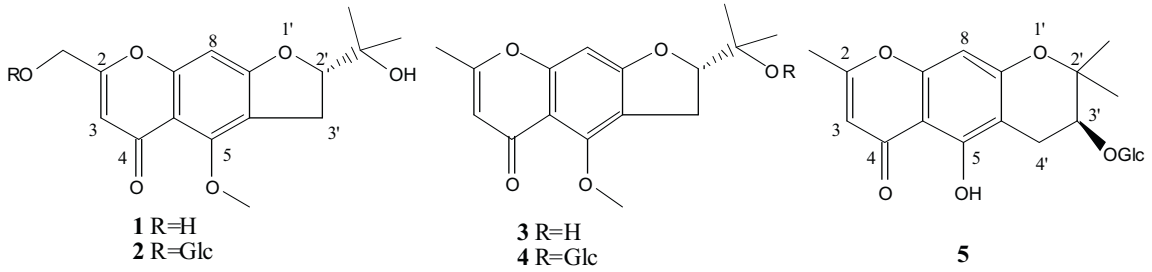


Fig. 1. Chemical structures of potential bioactive compounds from Saposhnikoviae Radix: **1** cimifugin; **2** prim-O-glucosylcimifugin; **3** 5-O-methylvisamminol; **4** 4′-O-β-D-glucosyl-5-O-methylvisamminol; **5** sec-O-glucosylhamaudol.

bioactive compounds, prim-O-glucosylcimifugin (**2**) and 4′-O-β-D-glucosyl-5-O-methylvisamminol (**4**) were identified by comparing with the chromatography of the standard sample, and three other compounds, i.e. cimifugin (**1**), 5-O-methylvisamminol (**3**) and sec-O-glucosylhamaudol (**5**) were identified by analysis of the fragmentation behavior characterization of mass spectrometry. The structures of the five bioactive compounds are shown in Fig. 1.

2. Experimental

2.1. Herbal materials and chemicals

Saposhnikoviae Radix was bought from Guangzhou Nepstar Chain Drugstore and authenticated by Professor Quan Zhu. A voucher specimen (No. 20090910) was deposited at the Consun pharmaceutical Co. LTD. 4′-O-β-D-glucosyl-5-O-methylvisamminol (No. 11523-200405) and prim-O-glucosylcimifugin (No. 11522-200406) were obtained from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Krebs–Henseleit (K–H, pH 7.4) buffer and citric acid–disodium hydrogen phosphate buffer (pH 4.0) were prepared in our laboratory. HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany). HPLC-grade formic acid was purchased from Tedia Company Inc. (Fairfield, USA). Pentobarbital was purchased from Sigma China Co., Ltd. Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA). The perfused apparatus for IPL extraction was purchased from Kent Science Company. Wistar rats (200 ± 20 g) were purchased from experimental animal center of South Medical University. All other chemicals were of analytical grade. All solvent and samples were filtered through 0.45 μm nylon membranes before use.

2.2. Preparation of ESR

Saposhnikoviae Radix was dried and ground to powders; then 1 g was immersed in 30 ml of 70% ethanol for 0.5 h and refluxed for 1 h twice. The extract was concentrated to dryness at 40 °C under vacuum, then dissolved in 100 ml K–H buffer to make the 10 mg/ml (material drug) solution, filtered through a 0.22 μm membrane; the filtrate was used as a sample for HPLC–ESI–MS^n analysis and IPL perfusion and extraction.

2.3. Preparation of standard samples

Weigh accurately appropriate amount of prim-O-glucosylcimifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol, dissolved in initial mobile phase to prepare the 58 μg/ml of prim-O-glucosylcimifug-in and 73 μg/ml of 4′-O-β-D-glucosyl-5-O-methylvisamminol solution.

2.4. Lung perfusion and extraction

Rats were adaptive for 1 week followed by forbidden to take food but free to take water for one day before the experiment began. Then the rat was anesthetized with pentobarbital and the lung was removed from the rat. The trachea of the lung was cannulated according to the procedure described by Law and So [21]. After the lung was attached to the perfusion apparatus, K–H buffer was perfused with a equilibration period for about 20 min, then 50 ml of the 10 mg/ml ESR was perfused with a constant rate of 10 ml/min, and then washed with K–H buffer for 5 min and the last 5 ml K–H buffer was collected, at last, eluated with 5 ml citric acid–disodium hydrogen phosphate buffer and collected as the extraction of IPL.

2.5. Samples preparation

From 5 ml of the last eluate of H–K buffer, extraction of IPL in Section 2.4, and ESR, standard sample solution were extracted with the same volume of EtoAC for 3 times, respectively. The extract was concentrated to dryness at 40 °C under vacuum. Then the residue was dissolved in 250 μl initial mobile phase and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to an autosampler vial and stored at −20 °C until use.

2.6. Chromatographic conditions

Separation was performed by a Zorbax SB-C18 reserved-phase column (100 mm × 2.1 mm i.d., 3.5 μm) with a mobile phase consisting of acetonitrile (A) and water–0.1% formic acid (B). The gradient elution conditions were: 0–5 min, 5–15% A; 5–15 min, 15–25% A; 15–30 min, 25–50%; 30–60 min, 50–60% A; 60–70 min, 60–85% A. The flow rate was 0.2 ml/min. The column temperature was set at 30, and the injection volume was 30 μl.

2.7. Mass spectrometric conditions

An agilent 1200 LC system (Agilent company, USA) and a thermofisher LCQ-Fleet ion trap mass spectrometer system (Thermofisher company, USA) were employed for the sample analysis. Ionization was achieved using electrospray in the positive mode. Ultra-high purity helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. The mass detector was optimized to obtain maximum yields of $[M+H]^+$ or $[M+Na]^+$ ions of the compounds. The mass parameters in the positive ion mode were optimized as follows: ion spray voltage, 4.5 kV; sheath gas (N_2) pressure: 40 arbitrary units; auxiliary gas (N_2) pressure: 10 arbitrary units; capillary temperature, 300 °C; capillary voltage: 5 V. For full-scan MS analysis, the spectra were recorded in the range of m/z 80–1200. A data-dependent program was used in the liquid chromatography/tandem mass spectrometric analysis so that the two most abundant ions in each scan were selected and subjected to MS^2 and MS^n ($n = 3, 4$) analyses. The collision energy for MS^n was

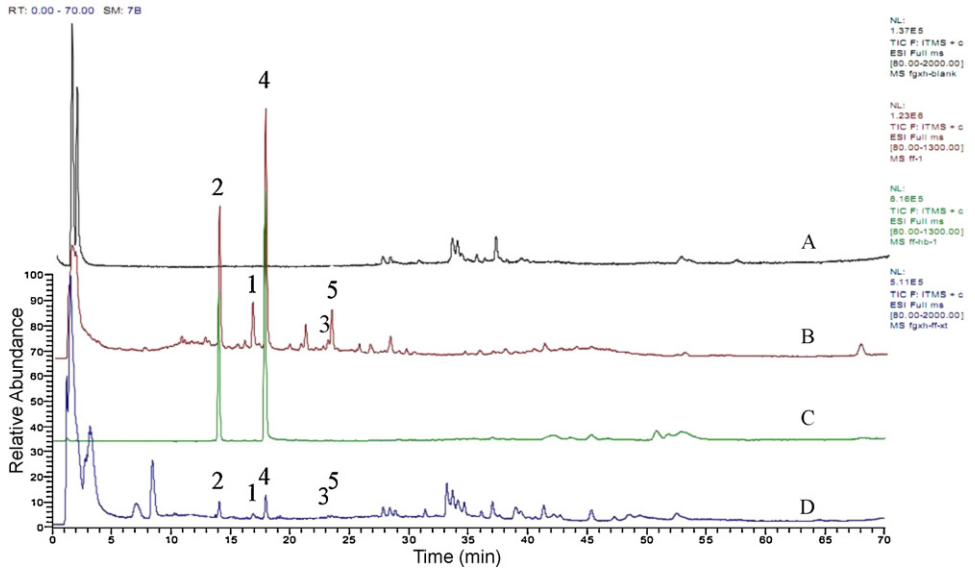


Fig. 2. Total ion chromatography of Saposhnikoviae Radix: (A) the last eluate of K–H buffer; (B) ESR; (C) standard sample solution; (D) extraction of IPL; **1** cimifugin; **2** prim-O-glucosylcimifugin; **3** 5-O-methylvisamminol; **4** 4′-O-*β*-D-glucosyl-5-O-methylvisamminol; **5** sec-O-glucosylhamaudol.

adjusted to 45% in LC/MS analysis, and the isolation width of the precursor ions was 1.5 Th.

3. Results and conclusions

*3.1. Bioactive compounds extracted by IPL extraction and the identification of compounds **2** and **4** using standard samples*

In Fig. 2, 5 peaks were found in the extraction of IPL (D) by comparing the HPLC–ESI–MS^n fragmentation behavior and retention time of with ESR (B), while these peaks were not detected in the last eluate of K–H buffer (A). So it was no doubt that these five compounds were extracted by some receptors or channels of IPL in the analogical physiological condition. When the IPL extract was eluated with citric acid–disodium hydrogen phosphate buffer (pH 4.0), it was denatured and the compounds would drop out of the receptors and channels. Compounds 2 and 4 can easily be identified as prim-O-glucosylcimifugin (**2**) and 4′-O-*β*-D-glucosyl-5-O-methylvisamminol (**4**) by comparing the HPLC–ESI fragmentation behavior and retention time with the standard sample solution, while the compounds **1**, **3**, **5** would need the further analysis of tandem mass spectrum.

*3.2. Identification of compounds **1**, **3** and **5** by analysis of the fragmentation behavior characterization of mass spectrometry*

From the HPLC–ESI–MS^2 cleavage characterization, compound **2** seems to lose a 162 Da (–glc) to generate the base peak 307, which indicated that compound **1** ([M+H]$^+$ at *m/z* 307) may be the aglycone of compound **2**. Similarly, compound **3** may be the aglycone of compound **4**, so all the five compounds including compounds **2** and **4** were applied to the HPLC–ESI–MS^n analysis (Table 1) for their structure identification. The protonated molecule [M+H]$^+$ at *m/z* 307 of **1** was subjected to MS/MS and produced diagnostic product ions at *m/z* 289, 259, 235 and 221, which was similar to the [M−glc]$^+$ at *m/z* 307 cleavage of **2**. The ESI–MS^3 fragmentation behaviors at *m/z* 289 from **1** and **2** were almost the same, so **1** was identified as cimifugin. The relationship between **3** and **4** also can be proved by the fragmentation cleavage characterizations, so **3** was identified as 5-O-methylvisamminol. Kang et al. [22] had reported the cleavage characterization of chromones from Saposhnikoviae Radix as follows: the linear dihydrofurochromones, characteristic eliminations of 18, 48 and 72 Da were observed, the loss of 18 Da could arise from two different fragmentation pathways, and the observed ion was composed of a mixture of two different structural ions. For the linear dihydropyranochromones, it was found that the dihydropyran ring was converted into the pyran ring by the elimination of the C-3' substituting group. This fragmentation was followed by the diagnostic losses of 18, 28, 42 and 54 Da in tandem mass spectrometry. According to the cleavage rule, compounds **1**–**4** should be linear dihydrofurochromones while **5** is linear dihydropyranochromones with diagnostic losses of 18, 28, 42 and 54 Da in tandem mass spectrometry (Fig. 3). By comparing the fragmentation behaviors, the structures of compounds **1** and **3** could also be affirmed as cimifugin and 5-O-methylvisamminol. The protonated molecule [M+H]$^+$ at *m/z* 439 of **5** was easy to lose 162 Da to pro-

Table 1
Characterization of potential bioactive compounds from ESR by HPLC–ESI–MS^n.

Compound	Retention time (min)	ESI–MS	ESI–MS^2	ESI–MS^3
1	16.99	307 [M+H]	289, 259, 235, 221	(307 → 289): 274, 259, 257 (307 → 259): 203, 177 (307 → 235): 217, 207
2	14.15	469 [M+H]	307, 289, 235	(469 → 307): 289, 259, 235, 257, 221 (469 → 289): 274, 259, 235
3	23.11	291 [M+H]	273, 243, 219, 205	(291 → 273): 258, 243, 241 (291 → 219): 202, 191, 177
4	18.11	453 [M+H]	291, 273	(453 → 291): 273, 243, 219, 205 (453 → 273): 258, 243, 231, 205
5	23.88	439 [M+H]	277, 217	(439 → −277): 259, 217, 205, 241, 231

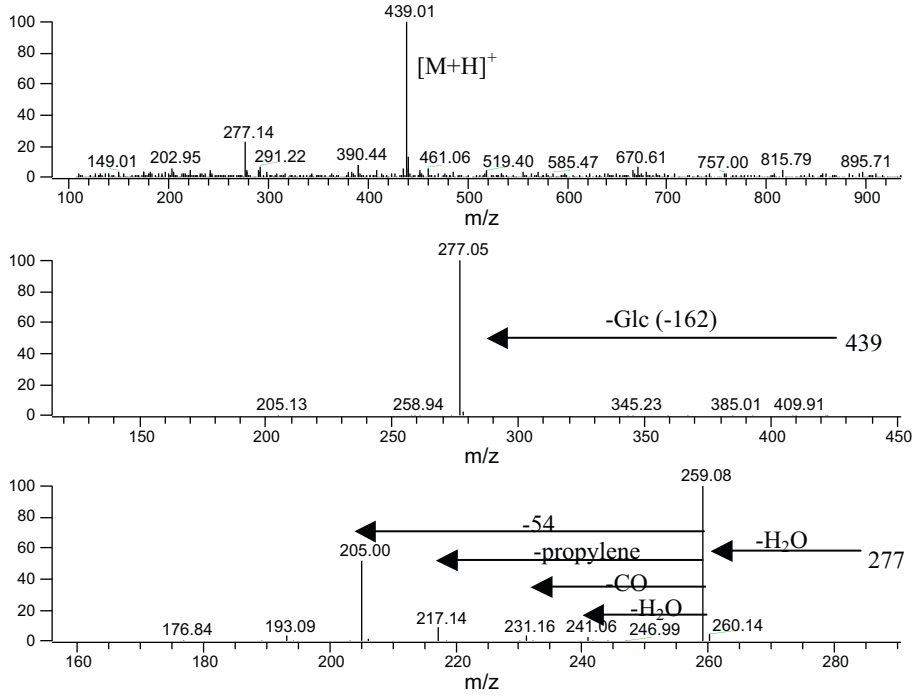
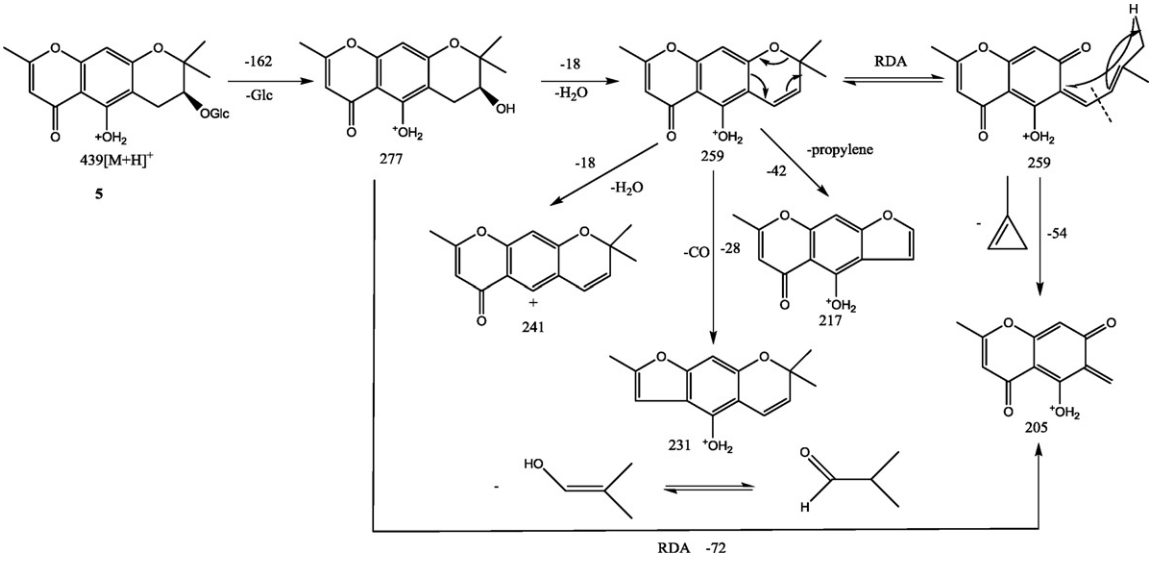


Fig. 3. Full-scan product ion spectra of $[M+H]^+$ ions and fragmentation pathways for compound **5**.



Scheme 1. Proposed fragmentation of sec-O-glucosylhamaudol cimifugin (**5**).

duce the base peak of *m/z* 277 in MS^2 spectra, which means that **5** was a dihydropyranchromone glucoside. Then in MS^3 spectra of *m/z* 277, the diagnostic losses of 18 ($-H_2O$), 28 (–CO), 42 (–propylene) and 54 Da were found and the probable fragmentation of **5** was proposed (Scheme 1), which provided the information that the glucose was combined to 3′-OH but not 5-OH. So **5** was identified as sec-O-glucosylhamaudol [22].

4. Discussions

Recently, more and more biological methods such as cells, cell membrane coupled with HPLC–MS have been applied for screening the bioactive components of TCMs.

IPL extraction, a ripe and reliable mode, has been frequently applied to pharmacological effect, physiological phenomena, drug dissolution and absorption, mechanisms of absorption, metabolism, tissue disposition and retention. Two different circulatory systems, the bronchial and the pulmonary, supply the lungs with blood. The bronchial circulation is a part of the systemic circulation and is under high pressure. It receives about 1% of the cardiac output and supplies the conducting airways, pulmonary blood vessels and lymph nodes. It is important for the distribution of systemically administered drugs to the airways and to the absorption of inhaled drugs from the airways [23]. In this study, bronchial was creatively used to extract and predict the potential bioactive components of Saposhnikoviae Radix and the structures of five chromones (cimifugin, prim-O-glucosylcimifugin, 5-O-methylvisamminol 4′-O-β-D-glucosyl-5-O-methylvisamminol and sec-O-glucosylhamaudol) were identified. According to the predecessor reported [24], coumarins and chromones are the main

bioactive components of Saposhnikoviae Radix, moreover, prim-O-glucosylcimifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol possess the bioactivity of antiinflammatory and anticoagulated blood, which is consistent with the results of our experiment.

IPL extraction for predicting bioactive components of TCMs possesses the advantages such as (1) it is more close to physiological state of animal; (2) reducing animal usage; and (3) taking less time to prepare the "extract material". So IPL extraction coupled with HPLC–ESI–MS^n analysis for predicting potential bioactive components of TCMs is easy, convenient, rapid, operational, economic and reliable, and it should be extensively applied to screen bioactive components of TCMs.

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