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Original Article

Detection of lithospermate B in rat plasma at the nanogram level by LC/MS in multi reaction monitoring mode

Tse-Yu Chung [a], Nan-Hei Lin [a], Yue-Chiun Li [a], Tzu-Yin Chen [a], Ping-Chung Kuo [b], Wen-Ying Chen [c,**], Jason T.C. Tzen [a,*]

[a] Graduate Institute of Biotechnology, National Chung-Hsing University, Taichung, Taiwan, ROC
[b] School of Pharmacy, National Cheng Kung University, Tainan, Taiwan, ROC
[c] Department of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan, ROC

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ABSTRACT

Low bioavailability and high binding affinity to plasma proteins led to the difficulty for the quantitative detection of lithospermate B (LSB) in plasma. This study aimed to develop a protocol for detecting LSB in plasma. A method was employed to quantitatively detect LSB of 5–500 ng/mL by LC/MS spectrometry in multi reaction monitoring mode via monitoring two major fragments with m/z values of 519 and 321 in the MS2 spectrum. To set up an adequate extraction solution to release LSB captured by plasma proteins, recovery yields of LSB extracted from rat plasma acidified by formic acid or HCl in the presence or absence of EDTA and caffeic acid were detected and compared using the above quantitative method. High recovery yield (~90%) was achieved when LSB (5–500 ng/mL) mixed in rat plasma was acidified by HCl (5 M) in the presence of EDTA (0.5 M) and caffeic acid (400 μg/mL). The lower limit of detection and the lower limit of quantification for LSB in the spiked plasma were calculated to be 1.8 and 5.4 ng/mL, respectively. Good accuracy (within ±10%) and precision (less than 10%) of intra- and inter-day quality controlled samples were observed. Oral bioavailability of LSB in rat model was detected via this optimized extraction method, and the maximum plasma concentration (C_{max}) was found to be 1034.3 ± 510.5 μg/L at t_{max} around 10 min, and the area under the plasma concentration–time curve (AUC) was 1414.1 ± 851.2 μg·h/L.

1. Introduction

Danshen, the dried roots of *Salvia miltiorrhiza*, is a well-known Chinese herb found in many medicinal formulates traditionally used for promoting blood circulation, eliminating blood stasis, relieving pain, stimulating menstrual discharge, and relaxing the mind [1]. On the basis of its therapeutic effects, danshen has been widely prepared not only alone but

* *Corresponding author.*
** *Corresponding author.* Department of Veterinary Medicine, National Chung-Hsing University, 145 Xingda Rd., South Dist., Taichung 402, Taiwan, ROC.
E-mail address: tctzen@dragon.nchu.edu.tw (J.T.C. Tzen).

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also in combination of other herbs for the treatments of coronary heart disease, heart stroke, myocardial infarction, menstrual disorder, and other cerebrovascular diseases [2–7]. Nowadays, many commercial danshen-containing products were popularly consumed by people in several Asia countries [8,9]. Notably, danshen was the first traditional Chinese herb subjected to phase 2 and 3 clinical trials in the USA in 1997 [10].

Lithospermate B (LSB), a tetramer of caffeic acid, is the most abundant polyphenolic constituent in the soluble extract of danshen [9]. LSB extracted from danshen was mainly found as a salt form bound with Mg^{2+} termed magnesium lithospermate B (MLB) [11]. MLB was shown to possess inhibitory potency on Na^+/K^+-ATPase, and thus suggested to be possibly responsible for promoting the blood circulation of danshen [1,12,13]. The magnesium ion of MLB could be replaced with some transition metal ions; the complexation of LSB with transition metal ions was demonstrated to enhance the inhibitory potency on Na^+/K^+-ATPase [14]. In addition, obesity, hyperlipidemia, hyperglycemia, glucose intolerance, insulin resistance, and hepatic steatosis were demonstrated to be improved via daily supplementation of MLB in a rodent model of metabolic syndrome [15,16].

Although LSB as well as MLB exhibited several effective bioactivities, the low bioavailability of LSB was observed in rat, dog, and rabbit experiments [2,17–20]. After oral administration by gastric intubation at 100 mg/kg, LSB in plasma was monitored up to 180 min and the highest concentration was reached at 0.5 h in the rat model; however, C_{max} (~40 ng/mL) was observed to be relatively low [20]. The low concentration of LSB in plasma (far below μg/mL) led to a difficulty for the quantitative detection by commonly available methods, such as high performance liquid chromatography (HPLC).

Several techniques, such as immunoassaying, radiolabeling and mass spectrometric methods might be employed to detect compounds at low concentrations. Among these techniques, mass spectrometric detection could monitor compounds directly without preparation of antibody or radio-labeling [21–23]. For quantification of several known compounds in one experiment, selection ion monitoring (SIM) and multi reaction monitoring (MRM) seemed to effectively expand the utilization of mass spectrometry [24]. In this study, we aimed to develop a protocol to detect LSB in plasma at the nanogram level. Through the combination of an optimized extraction method and LC/MS analysis in MRM mode, low concentrations of LSB in plasma could be adequately analyzed.

2. Methods

2.1. Chemicals and materials

Purified LSB was obtained from KO DA Pharmaceutical Company (Taiwan). Caffeic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). EDTA and urea were purchased from Amresco (Solon, OH, USA). Formic acid was bought from Choneye Pure Chemicals (Taipei, Taiwan). Hydrochloric acid was purchased from Union Chemical Works Ltd (Xinzhu, Taiwan). HPLC-grade ethyl acetate was purchased from Fisher Scientific (Waltham, MA, USA). HPLC-grade methanol and acetonitrile were bought from Aencore (Surrey Hills, Australia).

2.2. LC/MS analysis

The quantification of LSB and caffeic acid was analyzed by Shimadzu LC-MS 8040 (Shimadzu Cooperation, Kyoto, Japan) using a Shim-pack XR-ODS II column (2.0 × 100 mm, inner diameter 2.2 μm, Shimadzu Cooperation, Japan). The column was maintained at room temperature, and 5 μL of sample was injected to the LC system at a flow rate of 0.2 mL/min. The mobile phase was composed of 0.2% formic acid in (A) water and (B) acetonitrile. The gradient was as follows: 0–12 min, linear gradient from 6% to 70% B; 12–13 min, maintained 70% B; 13–16 min, linear gradient from 70% to 6% B. For continual sample analysis, the column was equilibrated with 6% B for 4 min before the next sample injection. Two wavelengths of UV absorbance were monitored at 286 and 329 nm.

The MS spectra (negative ion mode) were obtained on the Shimadzu LC-MS 8040 triple quadruple mass spectroscopy with an electrospray ionization (ESI) interface. Ionization voltage was −4.5 kV, and source temperature was 250 °C. The flow rates of nebulizer and drying gas offered by nitrogen gas were 3 and 15 L/min, respectively. Helium gas was used as collision gas for tandem mass spectrometric experiment. The MRM mode was used to quantify LSB; the optimized parameters were as follows: m/z value of the precursor ion was set at 717, and those of two product ions were set at 519 and 321; the collision energies for product ions with m/z 519 and 321 were 19 and 38 V; Dwell time was 100 msec [25]. All of MS spectra and data were collected and processed by the LabSolutions software (Shimadzu).

2.3. Extraction and analysis of LSB mixed in rat plasma

Male Sprague–Dawley rats of weighting approximately 400 g were purchased from BioLASCO, Taiwan Co., Ltd. (Taipei, Taiwan). Rat blood was withdrawn into heparinized tubes and centrifuged at 3000 g for 10 min at 4 °C. After centrifugation, plasma was collected from the upper layer and stored at −80 °C. The animal experiments were approved by the Institutional Animal Care and Use Committee of the National Chung-Hsing University (IACUC Approval Number: 105–69). Stock solutions of LSB (5000 ng/mL) and caffeic acid (400 μg/mL) were prepared in methanol. Rat plasma of 150 μL was mixed with 15 μL of LSB (500 ng/mL) and 15 μL of caffeic acid (40 or 400 μg/mL), and then added with 23 μL of 100% formic acid or 5 M HCl with or without the addition of 4 μL of EDTA (0.5 M). The mixture was spiked with 0.7 mL of ethyl acetate by vortexing for 5 min, and centrifuged at 4200 g for 5 min. The extraction step with ethyl acetate was repeated for four times. The upper layer was transferred to a clean tube and evaporated to dryness in the speed vac™ concentrator (Thermo Fisher Scientific, WA, USA). The dried residue was dissolved with 150 μL of methanol, and 5 μL of this solution was subjected to LC/MS analysis. The extraction recovery was calculated by peak area ratios of LSB and caffeic acid in rat plasma samples. In the LC/MS system, LSB was monitored in the MRM mode as described above, and caffeic acid was detected by the absorbance at the wavelength of 329 nm. Following the same

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