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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.

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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 addresses: wychen@dragon.nchu.edu.tw (W.-Y. Chen), 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 $\mu\text{g/mL}$) led to a difficulty for the quantitative detection by commonly available methods, such as high performance liquid chromatography (HPLC).

Several techniques, such as immunoassaying, radio-labeling 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 (Hsinchu, 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 quadrupole mass spectroscopy with an electrospray ionization (ESI) interface. Ionization voltage was -4.5 kV, and source temperature was 250 $^{\circ}\text{C}$. 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 $^{\circ}\text{C}$. After centrifugation, plasma was collected from the upper layer and stored at -80 $^{\circ}\text{C}$. 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 $\mu\text{g/mL}$) 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 $\mu\text{g/mL}$), 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 vacTM 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

procedure, rat plasma samples (150 μL) mixed with 15 μL of LSB (100, 500 and 5000 ng/mL) were used as intra-day quality controlled (QC) samples. Inter-day QC samples were prepared by mixing LSB (100, 500 and 5000 ng/mL) and EDTA (0.5 M) with rat plasma, and frozen at $-80\text{ }^{\circ}\text{C}$ for one day. The relative standard deviations of intra-day and inter-day QC samples were calculated.

2.4. Detection of protein denaturation by fluorescence spectrometry

Fluorescence spectra were monitored by Infinite[®] M200 pro microplate readers (TECAN, Austria, Switzerland). The samples were prepared as follows: 15 μL of rat plasma and 4 μL of EDTA (0.5 M) in company with 23 μL of formic acid ($\sim 100\%$) or HCl (5 M) were added with water to a final volume of 250 μL . To denature proteins, urea was added to the samples with a final concentration of 7M. After shaking for 30 min at room temperature, 200 μL of the samples were transferred to a 96-well UV plate (Corning, Corning, USA). Trp and Tyr residues of plasma proteins were excited at the wavelength of 280 nm, and the corresponding emission spectra between 310 and 400 nm were recorded.

2.5. Calibration of LSB detection

A series of LSB solutions were prepared at concentrations of 50, 100, 200, 500, 2000, and 5000 ng/mL. Following the same extraction method, the signals of LSB in the spiked rat plasma of the six solutions were monitored by LC/MS. The area of peak analyzed in the MRM mode versus the concentration of LSB spiked in the rat plasma was plotted, and the values of slope, intercept and R squared were calculated by fitting with a linear curve. Assayed concentrations and accuracies of intra- and inter-day QC samples were calculated by the linear equation of the calibration curve. The lower limit of detection (LOD) and the lower limit of quantification (LOQ) were calculated as following equations:

$$\text{LOD} = \frac{3.3\sigma}{S}$$

$$\text{LOQ} = \frac{10\sigma}{S}$$

σ is the standard derivation of peak area at concentrations of 5, 10 and 20 ng/mL; and S is the slope of the linear curve plotted in the range of 5–20 ng/mL of LSB.

2.6. Animal pharmacokinetic study

Male Sprague–Dawley rats with body weight of 400–500 g were maintained in an environmentally controlled breeding room (12:12 h light–dark cycle with humidity at $60\pm 5\%$ and temperature at $22\pm 2\text{ }^{\circ}\text{C}$) The rats were fasted for at least 12 h with free access to drinking water prior to the oral administration. Rats were anesthetized with Zoletil 50 (40 mg/kg, IP; Virbac Laboratories, Carros, France), and blood samples were collected from femoral artery of the rats before and after administration of LSB (100 mg/kg) by oral gavage. Approximately, 0.25 mL of blood was collected in heparinized tubes

after administration of LSB for 10, 20, 40, 60, 90, 150, and 240 min. Bloods samples were centrifuged at $3000\times g$ for 10 min, and then 120 μL of plasma was collected immediately and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

2.7. Pharmacokinetic analysis

At first, the plasma concentration of LSB monitored by LC/MS versus time was plotted. Maximum plasma concentration (C_{max}) of LSB and the time to reach C_{max} (t_{max}) were recorded directly from the plot. The area under this profile (AUC) was calculated by the linear trapezoid rule. Mean residence time (MRT) was calculated by area under the first moment curve (AUMC)/AUC. Apparent total body clearance from plasma (CL/F) was calculated by dose ($\mu\text{g}/\text{kg}$)/AUC ($\mu\text{g}\cdot\text{h}/\text{L}$). All parameters were calculated in excel 2013, and presented as mean \pm SEM.

3. Results

3.1. Detection of LSB at the nanogram level by LC/MS

To monitor LSB at a low concentration, an analytical method was employed to detect 5–500 ng/mL of LSB by using LC/MS spectrometry in MRM mode. To achieve high sensitivity, a relatively low flow rate (0.2 mL/min) was used to obtain stronger signals in the optimized LC/MS system as described in the Method (Section 3.2). After collision of the LSB precursor ion (m/z value 717), two major fragments with m/z values of 519 and 321 were monitored in the MS2 spectrum (Fig. 1A). These two fragments were putatively generated via the loss of one or two molecules of protocatechuic acid from LSB configuration (Fig. 1B). The signal intensity of 717 to 519 and that of 717 to 321 were used for the qualification and quantification of LSB. After the optimization of MRM parameters, 5 ng/mL of LSB could be obviously detected at the retention time of 9 min (Fig. 2A). The plot of peak area versus the concentration of LSB showed a linear response in the range of 5–500 ng/mL (Fig. 2B). Through the signal filtration of MRM model, peaks of LSB were predominantly detected in the LC/MS system; the interference, such as the background noise and the signals of other compounds from matrix, seemed to be nearly eliminated.

3.2. Extraction of LSB in rat plasma

Although LSB of 5–500 ng/mL could be detected by the LC/MS system in MRM mode, the extraction efficiency of LSB from rat plasma was found to be extremely low due to its binding to plasma proteins as reported previously [17]. To enhance the extraction efficiency of LSB from plasma, LSB of 50 ng/mL mixed in rat plasma was added with formic acid or HCl in the presence or absence of EDTA. The results showed that the MRM signal of LSB was undetected by the acidification of plasma with formic acid, but weakly detected by the acidification of plasma with HCl (Fig. 3). Moreover, the extraction recovery of LSB in spiked plasma acidified with HCl was enhanced from 27.2% to 37.7% by adding with EDTA.

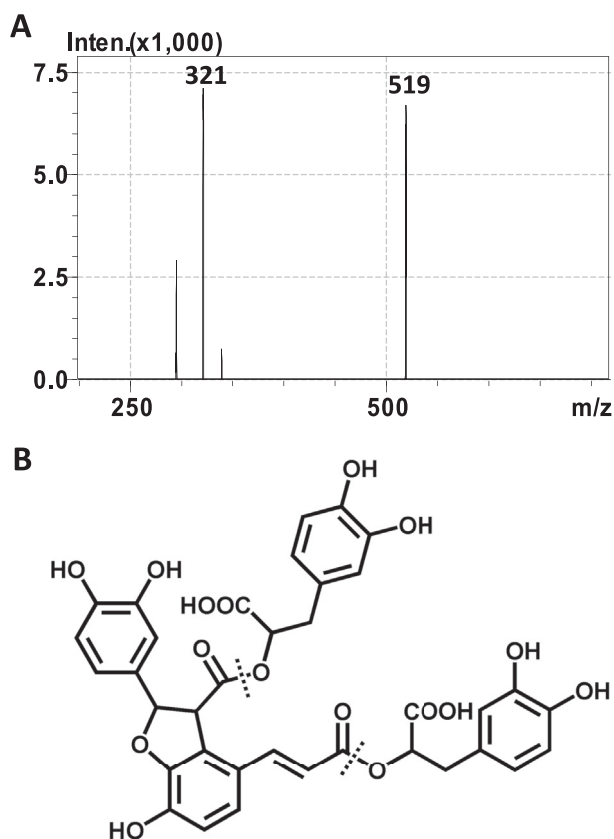


Fig. 1 – (A) MS2 spectrum of LSB. The *m/z* values (519 and 321) of the two major fragments in the MS2 spectrum were labeled. **(B)** Chemical structure of LSB. Dash lines showed the covalent bonds putatively broken down by collision in MS2 spectrum.

3.3. Efficiency of denaturation of plasma proteins acidified with formic acid or HCl

To examine if the low recovery yield of LSB from acidified plasma was resulted from the incomplete denaturation of plasma proteins by formic acid or HCl, changes in microenvironments of Trp and Tyr were monitored by fluorescence emission in the range of 310–400 nm to investigate the conformational alteration of plasma proteins under acidification. Trp and Tyr (non-polar amino acids) buried in the hydrophobic core of native plasma proteins seemed to expose to the aqueous environment after the protein denaturation by 7M of urea as observed by the shift of the maximum emission of Trp and Tyr from wavelength of 344 nm–356 nm (Fig. 4A). Similar shift of the maximum emission of Trp and Tyr from wavelength of 338 nm–350 or 352 nm was observed for plasma proteins acidified by formic acid or HCl after adding with 7 M of urea (Fig. 4B and C). The results suggested that plasma proteins were not completely unfolded by formic acid or HCl, and thus might be also able to capture LSB though not as tightly as their native forms did.

3.4. Enhancing the recovery yield of LSB from plasma by adding caffeic acid

Although urea or guanidine hydrochloride might be able to denature plasma proteins completely, they are non-volatile

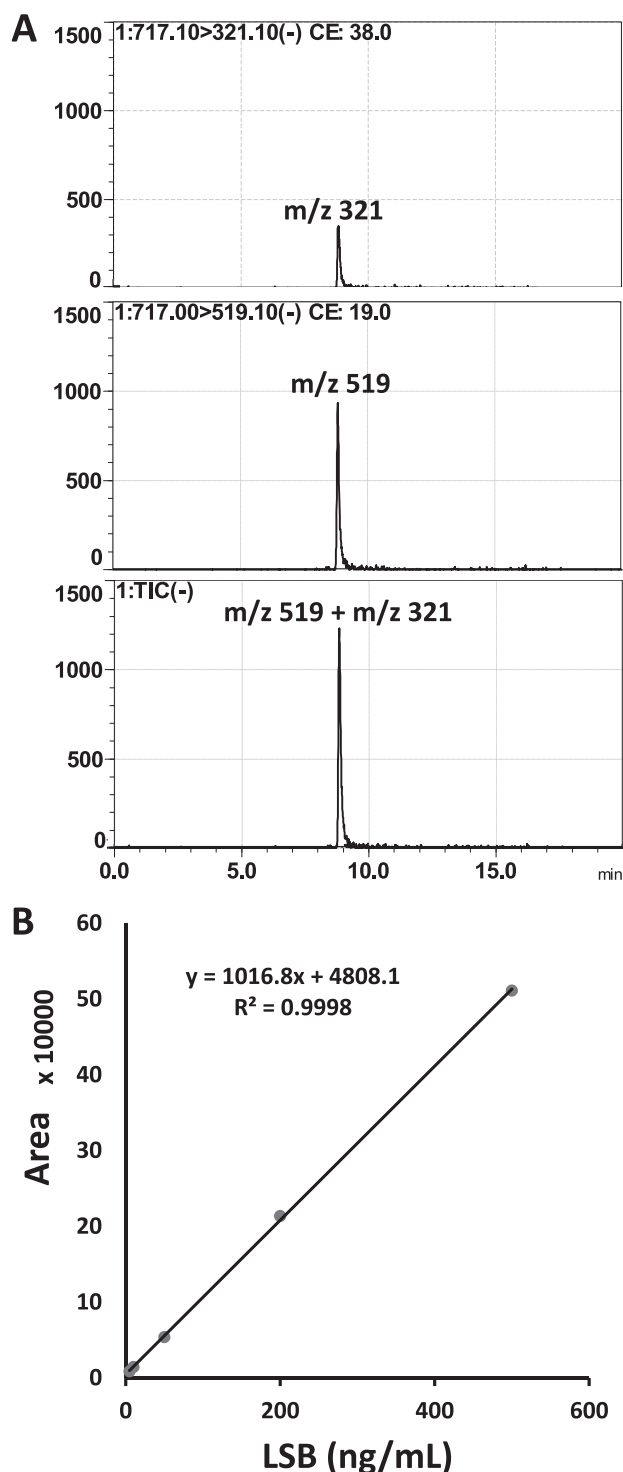


Fig. 2 – (A) Detection of LSB of 5 ng/mL in MRM mode. The upper figure was filtered with *m/z* value of 321, the middle one was filtered with *m/z* value of 519, and the lower one represented the total ion current chromatogram (TIC). **(B)** Calibration curve of LSB in the range of 5–500 ng/mL.

salt and presumably impede ionization of LSB in LC/MS analysis. In an alternative strategy, caffeic acid was added in the extraction solvent to outcompete LSB for binding to plasma proteins. It was assumed that excess supplement of

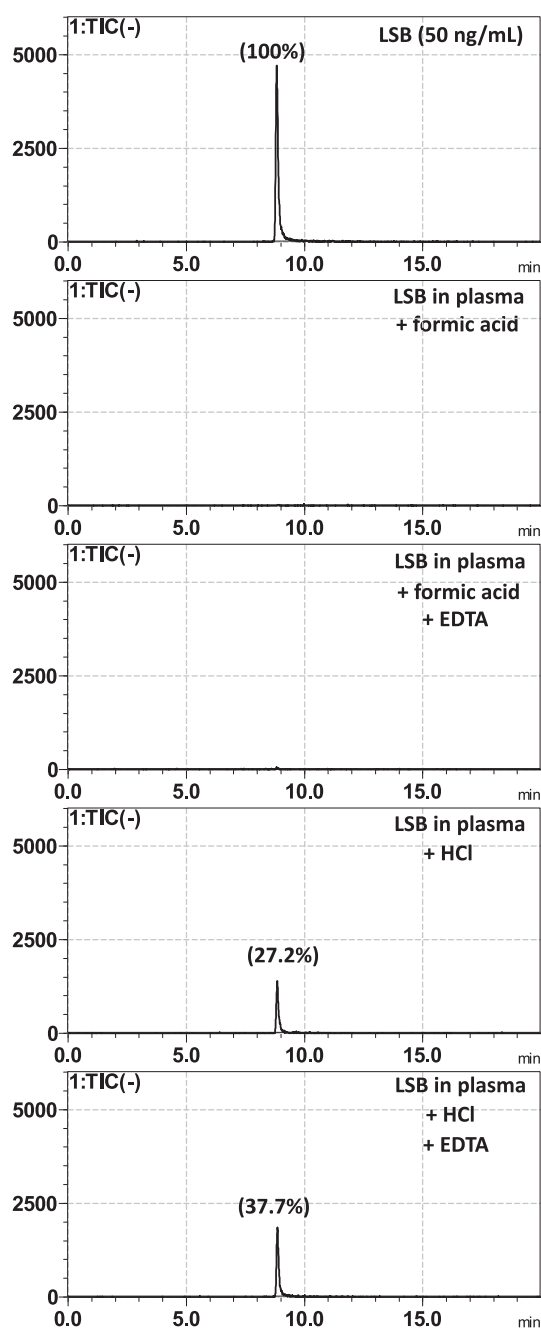


Fig. 3 – Detection of LSB in rat plasma in MRM mode. LSB of 50 ng/mL in rat plasma was added with formic acid or HCl in the presence or absence of EDTA. The recovery yield of LSB in each treatment was labeled on top of the peak detected in MRM mode.

caffaic acid might be able to saturate and block the capacity of plasma proteins for binding to LSB (a tetramer of caffeic acid), such that LSB captured by plasma proteins could be largely released to increase the recovery yield of LSB extracted from rat plasma. As expected, the recovery yield of LSB (50 ng/mL) extracted from rat plasma was significantly elevated after adding caffeic acid of 40 or 400 $\mu\text{g/mL}$ (Fig. 5). The highest recovery yield (90.7%) was achieved when LSB mixed in rat plasma was acidified by HCl in the presence of EDTA and

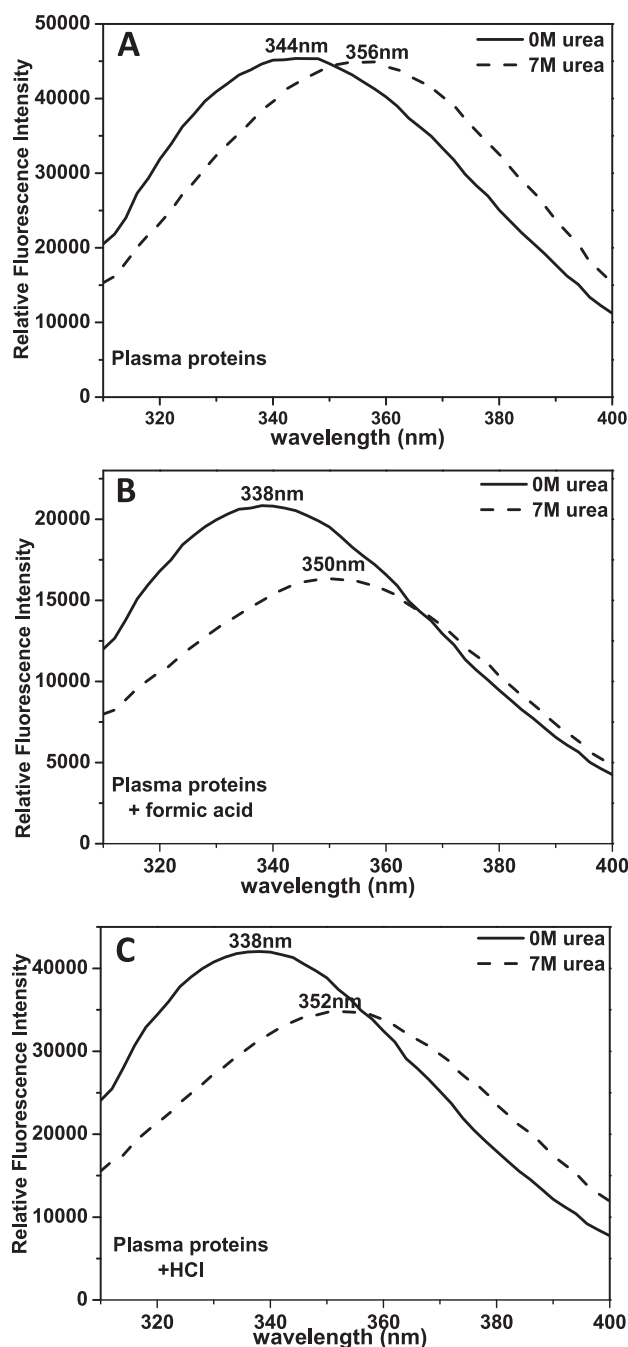


Fig. 4 – Fluorescence spectra of Trp and Tyr in plasma proteins acidified by (A) none, (B) formic acid and (C) HCl. In each condition, spectra of the samples treated with or without 7M urea were shown in dash and solid lines, respectively.

caffaic acid of 400 $\mu\text{g/mL}$. Meanwhile, the saturation of binding capacity plasma proteins by caffeic acid (400 $\mu\text{g/mL}$) was also demonstrated as high recovery yield (93.1 \pm 1.7%) of caffeic acid extracted from rat plasma was detected.

This optimized extraction protocol was used to further examine the extraction recovery yields of LSB of three different concentrations in rat plasma. The results showed that the recovery yields of LSB of 10, 50 and 500 ng/mL were

94.8 ± 3.7%, 90.4 ± 6.0% and 88.9 ± 3.3%, respectively. The results showed that the protocol developed in this study seemed to be an adequate method to quantitatively detect the recovery yield of LSB extracted from rat plasma.

3.5. Calibration curve and method validation

To establish the calibration curve of LSB extracted from rat plasma, recovery yields of six different concentrations of LSB

in the range of 5–500 ng/mL were determined. The areas of peaks versus the concentration of LSB were plotted, and the linear responses were found in two regions; one between 5 and 20 ng/mL (Fig. 6A), and the other between 20 and 500 ng/mL (Fig. 6B). LOD and LOQ evaluated by the linear curve between 5 and 20 ng/mL of LSB were 1.8 and 5.4 ng/mL, respectively. The accuracy and precision were estimated by the peak areas of the inter- and intra-day (frozen at –80 °C for one day) QC samples with LSB of 5, 50 and 500 ng/mL in rat

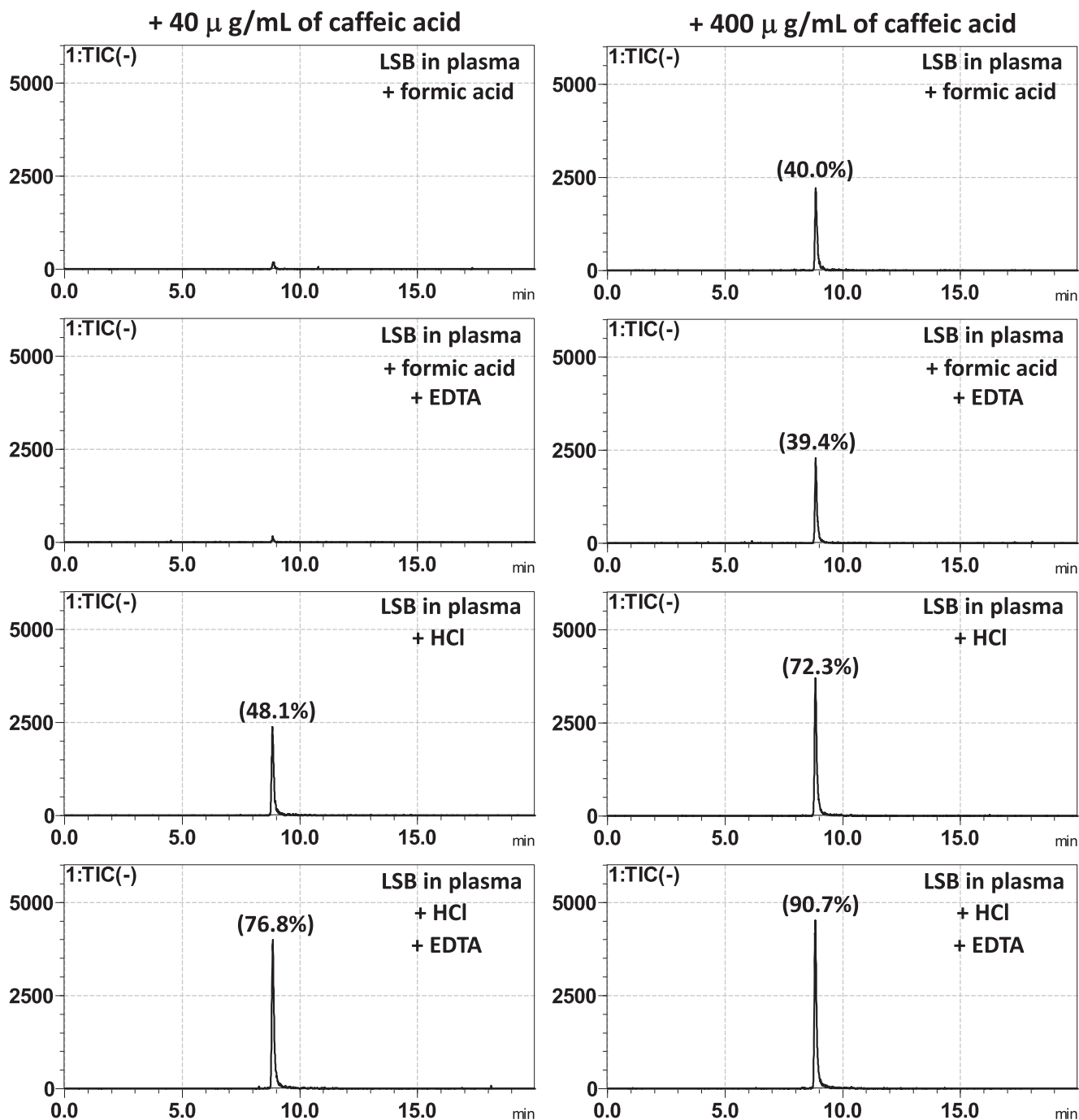


Fig. 5 – MRM peaks of LSB extracted from acidified rat plasma in the presence of EDTA and caffeic acid. LSB of 50 ng/mL in rat plasma acidified by formic acid or HCl in the presence or absence of EDTA was added with caffeic acid of 40 or 400 μg/mL. The recovery yield of LSB in each treatment was labeled on top of the peak detected in MRM mode.

plasma. The accuracy of intra- and inter-day QC samples ranged from 93.3 to 108.5%, and the precision ranged from 3.8 to 8.1% (Table 1). The accuracy was within $\pm 10\%$, and the precision of each intra- and inter-day QC sample was less than 10%. No obvious matrix effect was found as high recovery yields of LSB of 10, 50 and 500 ng/mL were detected in the optimized method. Moreover, the data showed that LSB in plasma samples could be maintained in good stability at $-80\text{ }^{\circ}\text{C}$ for one day.

3.6. Bioavailability of LSB after oral administration in rats

Furthermore, the optimized extraction method was used to detect the oral bioavailability in rat. The mean plasma concentration of LSB versus time profile was shown in Fig. 7. Pharmacokinetic parameters of LSB (oral dose of 100 mg/kg) were calculated and listed in Table 2. After feeding rats with LSB of 100 mg/kg, the maximum plasma concentration (C_{\max}) was found to be $1034.3 \pm 510.5\text{ }\mu\text{g/L}$ at t_{\max} around 10 min, and the area under the plasma concentration-time curve (AUC) was $1414.1 \pm 851.2\text{ }\mu\text{g}\cdot\text{h/L}$. According to this observation, the optimized method was demonstrated to be successfully employed to monitor the oral bioavailability of LSB in rats.

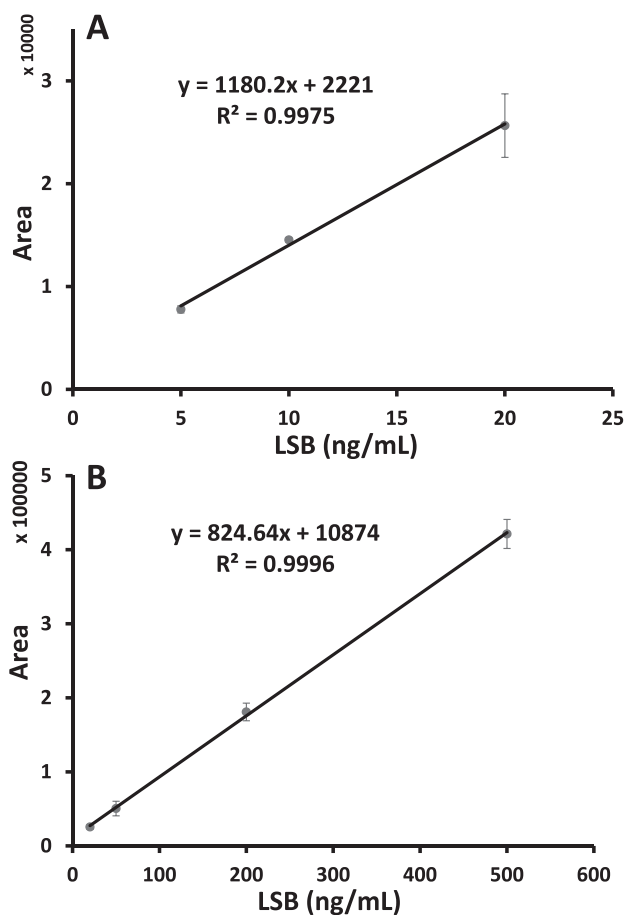


Fig. 6 – Calibration curves of LSB extracted from plasma in the ranges of (A) 5–20 and (B) 20–500 ng/mL. The areas of peaks in MRM model against concentrations were plotted ($n = 3$).

Table 1 – Accuracy and precision for the detection of LSB in rat plasma.

Theoretical concentration (ng/mL)	Assayed concentration ^a (ng/mL)	Accuracy (%)	Precision (%)
Intra-day ($n = 3$)			
10	9.3 ± 0.40	93.3	4.2
50	47.5 ± 3.87	95.0	8.1
500	533.9 ± 20.29	106.8	3.8
Inter-day ($n = 3$)			
10	9.2 ± 0.53	91.7	5.8
50	46.8 ± 2.73	93.5	5.8
500	542.6 ± 23.95	108.5	3.8
Mean		98.1	5.3

^a Values of assayed concentrations are mean \pm S.D., $n = 3$.

4. Discussion

Similar to the detection of many drug molecules at nanogram level, it is difficult to quantitatively detect LSB in plasma by HPLC coupled with a UV–Visible detector due to its low bioavailability and high binding affinity to plasma proteins [20]. In a previous study, LSB of 300–90,000 pg/mL in dog plasma was effectively detected via removing the plasma interference by using LC/MS spectrometry in MRM mode [26]. In this study, LSB at nanogram level in rat plasma was successfully monitored via avoiding plasma interference through the selection of parent and corresponding product ions of LSB (m/z 717 \rightarrow 519, 321) under the same technical approach. Mass spectrometry seemed to be an adequate tool to detect drug molecules in plasma by eliminating non-target signals or noise via filtration of m/z values.

Plasma, the liquid part of blood, is approximately composed of 90% water and 7% protein, and three major groups of plasma proteins are found to be serum albumins (55%), globulins (38%) and fibrinogens (7%) [27,28]. In blood fluid, serum albumins are able to regulate blood volume via maintaining the oncotic pressure, and to serve as carriers for

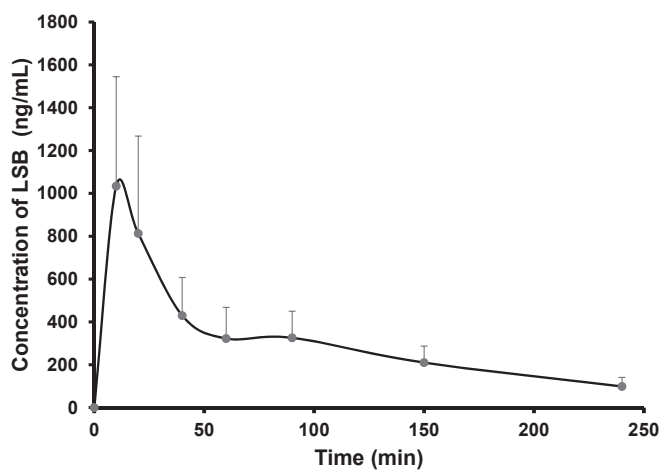


Fig. 7 – Mean plasma concentration of LSB versus time profile in rats following oral administration of LSB (100 mg/kg) ($n = 5$).

Table 2 – Pharmacokinetic parameters of LSB after oral administration (Mean \pm SEM, n = 5).^a

	t_{\max} (min)	C_{\max} ($\mu\text{g/L}$)	AUC ($\mu\text{g}\cdot\text{h/L}$)	MRT (h)	CL/F (L/h/kg)
LSB (100 mg/kg)	10.0 \pm 0	1034.3 \pm 510.5	1414.1 \pm 851.2	4.4	70.7

^a t_{\max} , time to C_{\max} ; C_{\max} : maximum concentration in plasma; AUC: area under the curve; MRT: mean residence time; CL/F: apparent total body clearance from plasma.

transporting cations and hydrophobic molecules, free fatty acids, hormones, bilirubin, and many drugs, such as warfarin, phenobutazone, clofibrate, and phenytoin [29,30]. As expected, most of LSB (83.78 \pm 10.5%) in plasma was captured by serum proteins as analyzed by ultrafiltration method and fluorescence quenching experiment [31,32]. It was reported that addition of acid in the extraction solution could increase the recovery yield of LSB captured by plasma proteins [2,18]. Accordingly, the extraction recovery of LSB in plasma acidified by HCl was found to be increased to 27.2% in this study (Fig. 3). However, the recovery yield (<30%) of LSB extracted from rat plasma was unsatisfied for quantitative detection. Further modification of the extraction solution showed that high recovery yield (~90%) could be achieved when LSB in rat plasma acidified by HCl was added with EDTA and excess caffeic acid (Fig. 5). The resultant effect of HCl, EDTA and caffeic acid in the extraction solution seemed to be synergistic as the individual effects of HCl, EDTA and caffeic acid on the extraction efficiency of LSB trapped in the rat plasma were 27.2%, 0% and 40%, respectively. Conclusively, an optimized extraction method was successfully developed to effectively release LSB captured by plasma proteins.

Strong interactions between proteins and phenolic compounds are often formed by intermolecular H-bonding, hydrophobic and van der Waals interactions [33]. These strong interactions, as observed in the tight binding between rat plasma proteins and LSB, are usually hard to be dissociated, and thus severely impede the extraction of phenolic compounds from their binding proteins. The success of effective extraction of LSB in plasma in this study should be primarily attributed to the addition of excess caffeic acid in the optimized extraction solution. Similar binding interactions with plasma proteins via carboxylic acid and aromatic ring are predictable between caffeic acid and LSB (generally regarded as a covalently linked tetramer of caffeic acid). Structurally, the interaction between plasma proteins and caffeic acid is apparently weaker than that between plasma proteins and LSB. Nevertheless, utilization of excess caffeic acid was demonstrated to effectively substitute LSB for binding to plasma proteins. Although LSB released from plasma was accompanied with excess of caffeic acid in the extraction, the quantitative detection of LSB was not influenced since caffeic acid had no signals via filtration of m/z 519 and 321 in the MS2 spectrum.

In the animal study, the maximum concentration of LSB in plasma was observed at round 10 min, and thus LSB was obviously absorbed very quickly in vivo. Values of C_{\max} and AUC, 1034.3 \pm 510.5 $\mu\text{g/L}$ and 1414.1 \pm 851.2 $\mu\text{g}\cdot\text{h/L}$, observed in this study were apparently higher than those reported previously (C_{\max} ~ 40 $\mu\text{g/L}$) [20]. This drastic difference might be mainly resulted from the massive capture of LSB by plasma proteins. Conclusively, the usefulness of this protocol was

verified via the detection of oral bioavailability of lithospermate B in the rat model.

Conflicts of interest

All authors declare no conflicts of interest.

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