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A simple, sensitive and rapid centrifugation-assisted solid-phase extraction (SPE) with high-performance liquid chromatography (SPE-HPLC) method was developed for simultaneous determination of the metabolites loureirin A and loureirin B from Dragon’s blood in rat plasma and urine. The development of the extraction procedure included optimization of some important extraction phases. After evaluation, the metabolites of Dragon’s blood were extracted by centrifugation-assisted SPE and separated by using HPLC. This method showed good linearity ($R^2 > 0.99$), and in the rat plasma and urine, the recoveries were 93.1 and 95.7% for loureirin A and were 90.1 and 94.2% for loureirin B. The relative standard deviation (RSD) values of intraday and interday precision in rat plasma and urine for loureirin A were <3.84 and 2.01%, respectively. The RSD values of the intraday and interday precision in rat plasma and urine for loureirin B were below 4.25 and 5.83%, respectively. Thus, the established method is suitable for metabolism studies of loureirin A and loureirin B in rat plasma and urine.

Introduction

Dragon’s blood capsules are made from the ethanol extract of Dragon’s blood. Dragon’s blood is a deep red resin obtained from the species of Dracaena spp. (Dracaenaceae), Daemonorops spp. (Arecales) and Croton spp. (Euphorbiaceae) (1). It has been used as a medicine since ancient times by many cultures (2, 3). In traditional Chinese medicine, the resin is obtained from two species, listed in the National Herb Standards, Dracaena cochinchinensis (Lour.) S.C. Chen and Daemonorops draco Bl, which are currently the primary sources for the widely used Dragon’s blood for the purpose of facilitating the invigoration of blood circulation in the treatment of blood stasis syndrome, trauma, gynecopathy and allergic dermatitis (4–6). Recent studies reported that Dragon’s blood showed antioxidant and antimicrobial activities (7). D. cochinchinensis was first found in Yunnan Province, China, in the 1970s (8). Since then, it has been used as the main source of Dragon’s blood, substituting for the imported Dragon’s blood from Indonesia and Malaysia obtained from Daemonorops draco. There are many preparations containing Dragon’s blood (resin of D. cochinchinensis, RDC), and they are clinically used for the treatment of blood stasis syndrome, trauma, gynecopathy and allergic dermatitis. It has been proved that the pharmacological effects resulted from its flavonoid components (9).

Loureirin A and loureirin B (Figure 1) are two of the active flavonoid compounds in Dragon’s blood, used as markers to identify different sources or to control the quality of preparations containing Dragon’s blood (10). Recently, they have been used as biomarkers, identified when minimally metabolized and primarily excreted through rat urine, feces and bile in an unchanged form after dosing by using high-performance liquid chromatography (HPLC)-MS/MS (11), determination of loureirin A in rats by HPLC-UV and loureirin B in rats by HPLC-MS/MS. Those reported methods were complex and costly and not suitable for a clinical large sample study.

For further clinical application of Dragon’s blood and to obtain its pharmacokinetic information, it is necessary to develop a simple, sensitive and rapid method for the determination its metabolites. Sample pre-treatment procedures are very vital for improving the sensitivity and selectivity of analytical methods. In this study, we developed a centrifugation-assisted solid-phase extraction with high-performance liquid chromatography (SPE-HPLC) method to simultaneously measure loureirin A and loureirin B in rat plasma and urine. Compared with those reported HPLC and HPLC-MS/MS methods (11–14), our method was fast, convenient and required low cost. Furthermore, this method is suitable for clinical practice and could be applied to investigate its pharmacokinetics.

Experimental

Materials

Loureirin A (111660–200301, purity 99.2%) and loureirin B (111585–200405, purity 98.9%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Dragon’s blood capsules were purchased from Yunnan Yunhe Pharmaceutical Co., Ltd (Yunnan, China). HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Methanol was obtained from Fengchuan Chemical Co., Ltd (Tianjin, China). Acetic acid was obtained from Beijing Chemical Co., Ltd (Beijing, China). Drug-free blank plasma and urine were obtained by sacrificing male rats. HPLC-grade water from Hangzhou Wahaha Group Co., Ltd was used throughout the pre-study validation and analysis.

Apparatus

Chromatographic separation and evaluation were performed on an HPLC system consisting of a vacuum degasser, an autosampler,
a quaternary pump and a diode-array detector (Agilent 1200 Series; Agilent Technologies, CA, USA). Vortex machine from Hanuo (Shanghai, China) was used for vortex-mixing. An ultrasonic bath from Kunshan ultra-sonic instrument plant (Jiangsu, China) was used for extraction of samples. A HC-3018R centrifuge (Anhui, China) was used for complete phase separation.

Preparation of standard solutions
The stock solutions of loureirin A and loureirin B were prepared by dissolving 0.622 mg of loureirin A and 0.843 mg of loureirin B in 20.0 mL of methanol and then diluted with methanol to give the final concentration of 31.1 and 42.2 ng mL\(^{-1}\). Working solutions were prepared by appropriate dilution of the stock solution with methanol. All the solutions were stored at 0 °C before use.

Calibration standards and quality-control samples
The calibration standards of loureirin A and loureirin B were prepared by diluting the standard working solution (10.0–1,000 μL) with drug-free rat plasma (100.0 μL) and urine (100.0 μL) to span a calibration standard range of 0.310–31.1 ng mL\(^{-1}\) (0.310, 0.620, 2.45, 4.98, 14.93 and 31.1 ng mL\(^{-1}\)) and 0.210–42.3 ng mL\(^{-1}\) (0.210, 0.420, 1.69, 3.38, 10.13 and 42.3 ng mL\(^{-1}\)).

All quality-control (QC) samples used for validation and metabolites study were prepared immediately before analysis, following the same procedure for the calibration standards. The three concentrations of loureirin A and loureirin B in rat plasma and urine of QC samples were 0.310, 2.45 and 14.93 ng mL\(^{-1}\) and 0.420, 3.38 and 10.13 ng mL\(^{-1}\), respectively.

Preparation of the sample
The content of the Dragon’s blood capsule was accurately weighed, equated to about 85.5 mg of loureirin A and 45.3 mg of loureirin B, extracted with 25.0 mL water–methanol (75:25, v/v) and promoted by an ultrasonic bath under the frequency of 40 kHz for 20 min.

SPE method
The blank plasma and urine sample (0.100 mL) were transferred into a 2.00-mL plastic test tube with 10.0 μL of standard solution. After vortex-mixing for 10 s, a mixture of plasma or urine sample was transferred into an activated SPE column (1.10167.1000; C-18 was obtained from Merck, Germany); the average particle size of C-18 was 40–63 μm and the aperture was 60 Å. The surface area of C-18 was 500 m\(^2\)/g, and the % carbon loading of the C-18 particles was 17%; 3.00 g of C-18 (the length of C-18 was about 4 cm) was loaded in 10.0 mL centrifuge tubes, then the compounds were eluted with 1 mL of methanol–water (90:10, v/v) and centrifuged at 10,000 rpm for 5 min (Figure 2). The obtained eluate was evaporated to dryness under nitrogen at 40 °C and redissolved in 100 μL methanol, then vortex-mixed and transferred to an autosampler vial. An aliquot of 10.0 μL of mixture was injected into the HPLC system. The solid-phase extraction (SPE) method was performed by using HLB (186000115; Oasis\textsuperscript{®} HLB, Waters Corporation, Milford, MA, USA; HLB SPE sorbent [poly(divinylbenzene-co-N-vinylpyrrolidone]) and physical characteristics: the average particle size of HLB was 60 μm and the aperture was 100 Å in the experiment and 500 mg), which was the same with C-18 column.

It should be mentioned that the C-18 column was preconditioned with 1 mL methanol and 1.00 mL ultra-pure water before use, and then the C-18 column was dried. This method was also applied to other samples.

Acetonitrile protein precipitation
The plasma and urine samples (0.100 mL) were transferred to a 2.00-mL plastic test tube with 10.0 μL of standard solution (100 μg mL\(^{-1}\), vortex-mixing for 10 s, and then acetonitrile (300 μL) was added with vortex-mixing for 1 min and centrifuged at 10,000 rpm for 10 min to separate the aqueous and immiscible organic layers. The clear organic layer was transferred into a 1.50-mL microcentrifuge tube. Then, ethyl acetate
(200 μL) was added to the microcentrifuge tube, and the upper layer was quantitatively transferred to a 1.50-mL glass tube and evaporated to dryness at 40 °C. The residue was redissolve with 100 μL methanol, then vortex-mixed and transferred to an autosampler vial. An aliquot of 10.0 μL of the mixture was injected into the HPLC system.

Contents of loureirin A and loureirin B in Dragon’s blood capsules

To calculate the administrated dose, contents of loureirin A and loureirin B in Dragon’s blood capsules were quantitatively determined by using HPLC. The content of loureirin A was 8.55 mg g⁻¹, and of loureirin B was 4.53 mg g⁻¹.

Chromatography

Chromatography was performed with a HPLC system consisting of quaternary pumps, a diode-array detector, an auto injector (Waldbronn, Germany) and a chromatography workstation. Chromatographic separations were achieved using a Kromasil 100-5-C18 reversed-phase column (150 mm × 4.6 mm, 5 μm). The mobile phase consisted of 0.1% acetic acid–acetonitrile (67:33, v/v), and the flow rate was 1.0 mL min⁻¹. The 0.1% acetic acid was prepared fresh daily with HPLC-grade purified water. All solutions were filtered through a 0.45-μm membrane (Ø 50 mm, 0.22 μm; Shanghai Xingya Purifying Material Factory, China) and degassed prior to use.

The column was kept at 30 °C. The diode-array detector was configured to acquire data at 280 nm, and 10.0 μL of the samples were injected into the HPLC for each chromatographic analysis. The total run time of each chromatographic analysis was 15 min.

Method application

Animals, drug administration, plasma and urine collection

Male rats (n = 20, weight = 160–180 g) were obtained from Laboratory Animal Center of Kunming Medical University. All rats were kept in a controlled environment at 23 ± 2 °C and 50 ± 10% relative humidity of a 12 h dark–light cycle. Food and water were allowed ad libitum. The rats received free access to water during the experiment, after a 12 h fast before the experiment.

The rats were randomly divided into two groups (n = 6) and administrated an oral dose of 10.0 g kg⁻¹ Dragon’s blood capsules. Plasma samples were collected in a sodium citrate tube via the heart of the rat at 0.5, 1.0 and 2.0 h after dosing (merge to collected plasma) for analysis. Urine samples were collected in a citrate tube within 24 h of dosing (merge to collected urine) for analysis.

Results

Comparison of data of three studied systems

To obtain the best analytical performance, we compared the different sample pre-treatment methods by using C-18, HLB and acetonitrile for protein precipitation. The chromatographic peak area and the recoveries were used to evaluate the extraction efficiency under different experimental conditions (Figure 3 and Table 1).

Optimization of the separation conditions

During method development, several combinations of buffer and organic phase were tested. The mixture with the mobile phase that consisted of 0.1% acetic acid–acetonitrile (67:33, v/v) showed a good peak for loureirin A and loureirin B. The UV detector was operated at 280 nm.

In this study, all the experiments were performed five times and the average of the results was used for optimization. Potential interference from endogenous compounds was investigated by analyzing plasma and urine from six different rats.

Characteristic performance data

Effect of the concentration of elution

The recovery factor depends on the weight of different elutions. The effect of different concentrations of the elution solvent on recoveries was examined, and the results are presented in Figure 4. On the basis of these experimental results, 90% of methanol was adopted as the optimum amount to achieve best analytical signals and extraction efficiency. The other parameters, including weight of C-18 and centrifugation time, were optimized in subsequent experiments.

Effect of volume of C-18

The SPE method was significantly affected by the amount of C-18. The recoveries continued to rise, but it had a little change after 3.00 g. The appropriate concentration was at 90% methanol, which was adopted as the optimum amount to achieve best analytical signals and recovery. An amount of 3.00 g of C-18 was chosen in the process (Figure 5).

Effect of centrifugation time

The effect of centrifugation time on recovery test was examined under 10,000 rpm. The results showed that the recovery increased from 1 to 5 min and decreased thereafter (Figure 6).

Method validation

Specificity

The analytical methods had been validated for parameters such as linearity, repeatability, precision, accuracy and stability. The relative standard deviation (RSD %) was taken as a measure of precision, repeatability and stability. The representative chromatograms are shown in Figure 3. The retention times of loureirin A and loureirin B were ~8.2 and 11.5 min, respectively. The peak corresponded to Dragon’s blood capsule and its standards were well resolved from instrumental noise and background interference. No significant interference peaks were observed at the retention times of the analyte and their standards in rat plasma and urine samples during the analysis.

Linearity

The calibration curves were constructed by analyzing at least six different concentrations of standard solutions. As a result, good
The linearity ($r^2 > 0.99$) of the investigated concentration ranges was observed.

The injection concentration, which could be detected at the signal-to-noise ratio of 3, was considered to be the limit of detection (LOD). The limit of quantification (LOQ) was the injection concentration corresponding to the peak heights with a signal-to-noise of 10. The LOD was 0.100 ng mL$^{-1}$ for lourierin A, and 0.150 and 0.0700 ng mL$^{-1}$ for lourierin B. The LOQ was 0.310 ng mL$^{-1}$ for lourierin A, and 0.420 and 0.210 ng mL$^{-1}$ for lourierin B. The details of the calibration curves are listed in Table II.

**Figure 3.** Chromatograms of (A) blank plasma and urine; (B) a plasma sample spiked with 2.49 ng mL$^{-1}$ lourierin A (Peak 1) and 1.69 ng mL$^{-1}$ lourierin B (Peak 2); (C) a urine sample spiked with 2.49 ng mL$^{-1}$ lourierin A and 1.69 ng mL$^{-1}$ lourierin B; (D) after oral administration of 10 g kg$^{-1}$ Dragon’s blood capsules detection of lourierin A and lourierin B in rat plasma (at 0.5, 1.0 and 2.0 h) and urine (within 24 h), respectively. The highest peak refers to using C-18, the middle peak refers to using HLB and the lowest peak refers to for protein precipitation; (E) the recoveries were used to show the extraction efficiency under different experimental conditions.

**Precision**

Different QC samples were used to evaluate the accuracy and precision of the assay. The QC samples for lourierin A in plasma and urine were 0.310, 2.490 and 15.93 ng mL$^{-1}$, and for lourierin B in plasma and urine were 0.420, 3.38 and 10.13 ng mL$^{-1}$. The accuracy for the assay of lourierin A in plasma and urine were less than $\pm 8.9\%$. The accuracy of lourierin B in all samples ranged from $-7.6$ to 10.4%. For assessment of the intraday precision, the RSD values of two analytes were below 3.84 and 4.25% for the QC samples in plasma and urine, respectively. For assessment
of the interday precision, the RSD values were below 5.90% at three QC levels for loureirin A and loureirin B in all matrices. Both accuracy and precision results were satisfactory.

Recovery
In the recovery test, the proposed method was applied to the samples spiked with the mixed standard solution at low, middle and high concentration levels. Each level was performed three times. The recovery was calculated by comparing observed concentrations with nominal concentrations. This method showed good recovery, and in the rat plasma and urine the recoveries were 93.1 and 95.7% and 90.1 and 94.2% for loureirin A and loureirin B, respectively. The RSD values of each concentration level were <8.09%.

Repeatability and stability
Injection repeatability was examined by the injection of six samples that are prepared with the same sample preparation procedure.

The stability of loureirin A and loureirin B in plasma and urine was evaluated. After the three freeze–thaw cycles, loureirin A and loureirin B in all samples were found to be rather stable at the three observed concentrations. The RSD values were between 4.18 and 6.36% for loureirin A and loureirin B. The results from storage stability at −20°C for 7 days and at room temperature showed that loureirin A and loureirin B were stable (RSD of ±9.84% or less) under the above two storage conditions.

Discussion
The characteristics of cumulative excretion from the urine and plasma were determined. After oral administration of 10.0 g kg⁻¹ Dragon’s blood capsules, both loureirin A and loureirin B were
found in the plasma and urine of the rats. The contents of loureirin A and loureirin B were 0.855 and 0.453% in Dragon’s blood capsules, respectively. In other words, 10.0 g of Dragon’s blood capsules corresponds to 85.5 mg of loureirin A and 45.3 mg of loureirin B. In our previous study, the recovery of the SPE method by using C-18 was >90%. This method showed a good linearity ($r^2 > 0.99$) over the concentration range of 0.310–31.1 ng mL$^{-1}$, with the low LOQ of 0.310 ng mL$^{-1}$ and the low LOD of 0.100 ng mL$^{-1}$ for loureirin A in rat plasma and urine, respectively. The linearity of calibration curves of loureirin B in rat plasma and urine was $r^2 > 0.99$ over the concentration range of 0.420–42.3 and 0.210–42.3 ng mL$^{-1}$, with the LOQ of 0.420 and 0.210 ng mL$^{-1}$ and the LOD of 0.150 and 0.0700 ng mL$^{-1}$, respectively. This method showed good linearity ($r^2 > 0.99$), and in the rat plasma and urine, the recoveries were 93.1 and 95.7% for loureirin A, and 90.1 and 94.2% loureirin B, respectively. In rat plasma and urine, the intraday and interday precision were less than 3.84 and 2.01%, respectively, for loureirin A, and were below 4.25% and 5.83%, respectively, for loureirin B. After oral administration of Dragon’s blood capsules, a cumulative supersession study of loureirin A and loureirin B in rat plasma and urine was performed. Thus, the established method is suitable for metabolism studies of loureirin A and loureirin B in rat plasma and urine. The centrifugation-assisted SPE method by using C-18 improved the accuracy of the metabolite study. In this study, only free loureirin A and loureirin B were determined in rat plasma and urine samples. Metabolites of Dragon’s blood capsules in plasma still need further study.

**Conclusion**

Loureirin A and loureirin B in rat plasma and urine using centrifugation-assisted SPE have been proved to be an effective preconcentration method. It is specific and sensitive for the analysis of Dragon’s blood capsules after oral administration by HPLC. The following points are worth noting: (i) loureirin A and loureirin B have higher recovery, good linearity and repeatability and (ii) the process of the SPE also reveals that the C-18 as a SPE has influence on enrichment. The effect of the concentration of elution and the weight of C-18 may be related to space structure and polarity of loureirin A and loureirin B. In addition, three systems (C-18, HLB and acetonitrile) have been compared by the recoveries. The recoveries of acetonitrile for protein precipitation extraction in plasma and urine of the rats were about 70%. Nevertheless, the recoveries of the method by using HLB were about 95% and by using the C-18 SPE method, the recoveries were >90%. The method by using C-18 was as good as HLB, and the C-18 SPE method was cheaper and more useful. This validated method was shown to be suitable for metabolites study of loureirin A and loureirin B after oral administration of Dragon’s blood capsules. These results might be helpful for further study and clinical application of this rare traditional herb.

**Acknowledgments**

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**Table II**

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>Linear range (ng mL$^{-1}$)</th>
<th>Calibration equation</th>
<th>Correlation coefficient ($r^2$)</th>
<th>LOD (ng mL$^{-1}$)</th>
<th>LOQ (ng mL$^{-1}$)</th>
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<tbody>
<tr>
<td>Plasma</td>
<td>Loureirin A</td>
<td>0.31–31.1</td>
<td>$y = 252.3x - 109.97$</td>
<td>0.9956</td>
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<td>Loureirin B</td>
<td>0.42–42.3</td>
<td>$y = 146.18x + 106.34$</td>
<td>0.9946</td>
<td>0.15</td>
<td>0.42</td>
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<tr>
<td>Urine</td>
<td>Loureirin A</td>
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<td>$y = 389.91x - 159.10$</td>
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<tr>
<td></td>
<td>Loureirin B</td>
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<td>$y = 208.13x + 197.56$</td>
<td>0.9953</td>
<td>0.07</td>
<td>0.21</td>
</tr>
</tbody>
</table>

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