Asian Journal of Chemistry; Vol. 26, No. 13 (2014), 3774-3778

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.15922

Analysis of Phenolic Compounds and Determination of Loureirin B in Dragon's Blood by LC-MS and High Performance Capillary Electrophoresis

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Received: 17 June 2013; *Accepted*: 4 October 2013; *Published online*: 23 June 2014; AJC-15343

The high performance liquid chromatography-mass spectrometry (HPLC-MS) and the high performance capillary electrophoresis (HPCE) were applied to study the phenolic compounds of Dragon's blood extracts. Ten compounds were identified from Dragon's blood extracts. Moreover, under the optimized HPCE conditions, linear calibration curve of Loureirin B was obtained for the system, the accuracy of the method was validated by spiked test and the average recoveries arrive at 98.1 %. The precision and repeatability were also investigated by the relative standard deviations (RSDs) of the prepared samples in quintuplicate. The relative standard deviations of the HPCE intensity and the migration time were 3.8 % and 1.3 %, respectively. Successful separation and satisfactory quantitative results for Loureirin B in complicated Dragon's blood were obtained at pH 9.6. The developed method based on HPLC-MS and HPCE for identification of phenolic compounds and quantification of Loureirin B in complex Dragon's blood is simple and effective and the rusults can be applied to the componential analysis and quality control of herbs.

Keywords: Dragon's blood, Phenolic compounds, Loureirin B, LC-MS, HPCE.

INTRODUCTION

Dragon's blood is a deep red resin, which has been used as a famous traditional medicine since ancient times by many cultures. In China, the red resin of *Dracaena cochinchinensis* S.C. Chen, called "longxuejie"[1], was used as an ethnomedicine for the treatment of traumatic injuries, blood stasis and pain[2,3]. Chemical studies revealed that the resin contained many phenolic compounds, several steroids, terpenoids and aliphatic acids[4-9]. Moreover, the phenolic compounds were found to be the main constituents in the red resins of the plant[6,10-11]. According to structure, the phenolic compounds can be broadly divided into chalcone, dihydrochalcone, flavones, flavanoid, polymeric flavonoids, chromone, *etc*. flavonoids and other phenolic compounds[12].

Previous studies indicated that the phenolic compounds of the red resin possess a number of biological properties, such as antioxidant, antimicrobial, antiviral, antihemorrhagic, antithrombotic, antiinflammatory activities and protect myocardial cells[13-18]. Since its various pharmacological activities, it is essential to do further research about phenolic compounds of Dragon's blood. Many methods to analyze the phenolic compounds of Dragon's blood have been established. However, the new simple and convenient method is still yet to be developed. It was therefore important to try new ways to analysis the phenolic compounds of Dragon's blood. Here, we used HPLC-MS and combined with its chemical composition reported in the literature to analytical some phenolic compounds of Dragon's blood, which may provide a wider outlook on the analytical approaches of the ingredients in Dragon's blood.

It is widely known that quality control analysis of the active components is important for the safe and effective use of traditional Chinese medicines (TMC). Dragon's blood, as a name, has been applied to resins obtained from different species or different continents, there is a great need to identify them apart. There are other substitutes as well, which are available in the market for Dragon's blood[19,20]. Thus, for Dragon's blood, a novel quality evaluation standard based on the contents of active components is needed to comprehensively assess its quality. Currently, the quality standards of the domestic Dragon's blood which done by State Drug Administration (SDA) is determination of the Loureirin B by HPLC. Many literatures have used HPLC for quality control of Dragon's blood[21-23]. In addition, several reports have been published on the quality evaluation of Dragon's blood carried out by thin layer chromatography scanning (TLCS), thin layer chroma-

tography (TLC) and ultraviolet spectrometry (UV)[20,24-27]. However, TLC needs complex sample handling, TLC lacks quantitative precision, UV is difficult to eliminate interference, while HPLC requires a number of prior steps and consumes materials. By virtue of its high resolution and simple pretreatment requirement, efficient high-performance capillary electrophoresis methods (HPCE) have been used for the determination of dracorhodin in Dragon's blood[28], but there is no report for the analysis of Loureirin B. Therefore, in order to assess comprehensively the determination of the major active components in Dragon's blood, a rapid and effective HPCE method has been developed.

In this study, high performance liquid chromatography (HPLC) with electrospray ionization mass spectrometry (ESI-MS) and the high performance capillary electrophoresis (HPCE) were developed for the characterization and determination of the phenolic compounds in Dragon's blood extracts. The validation results revealed that the developed method was highly efficient and reliable and hence suitable for qualitative and quantitative analysis of Dragon's blood samples.

EXPERIMENTAL

Loureirin B standard compound was purchased from Guangxi Institute of Traditional Chinese Medicine and used as received. Dragon's blood was mango brand and purchased from Guangxi College of Traditional Chinese Medicine Pharmaceutical Factory. Analytical grade SDS was obtained from Shanghai Test a Chemical Reagent Co., Ltd. Analytical grade borax, sodium dihydrogen phosphate and polyamide resin were obtained from Sinopharm Chemical Reagent Co., Ltd. Analytical grade ethanol (95 %), chloroform, petroleum ether and HPLC grade methanol were obtained from Kermel, Tianjin Chemical Reagent Co., Ltd. All other reagents were of analytical grade and the experiment water was ultrapure water.

An Agilent LC-1200 series system equipped with a quaternary pump, a vacuum degasser, an auto-sampler and a column heater-cooler was used for the chromatographic separation. Samples were separated on a Narrow-Bore C18 column (150 mm × 2.1 mm, 5 μm, Agilent Corp.,, USA) with the column temperature at 25 °C. The mobile phase was consisted of methanol (A) and 10 mM ammonium acetate (B). The following linear elution gradient was used: 0-30 min, from 20 % to 95 % A, maintained 10min, 40-50min, from 95 % to 20 % A. The injection volume was 5 μL and the elution time is 50 min.

The above HPLC system was connected by an electrospray ionization interface to an Aglient 6520 Q-TOF tandem mass spectrometer equipped with Mass Hunter software (Aglient Corp.,, USA). The same chromatographic conditions were used during the HPLC-MS analysis. The optimized mass spectrometry operating conditions were as follows: scan spectra from *m/z* 100 to 800, negative ionization mode , whilst the conditions of the ESI source were: drying gas (N_2) flow rate, 10 L min^{-1}; drying gas temperature, 325 °C; nebulizer pressure, 30 psig; capillary voltage, 4 000 V.

The quantitative determination of Loureirin B was carried out by HPCE and experiments were performed with a P/ACE MDQ capillary electrophoresis system equipped with an on-column diode-array detector (DAD) system, operated under gold system software for control, data acquisition and analysis (Beckman Co., USA). The column used for the determination of Loureirin B was an uncoated fused-silica capillary of 60 cm in length and 75 μm in internal diameter (Hebei YongNian optical fiber factory, China). The buffers were prepared from 20 mmol L^{-1} SDS, 80 mmol L^{-1} borax and 10 % methanol (pH = 9.6). Then operated at a condition of voltage at 15 kV, pressure injection at 0.5 psis, the separation temperature at 25 °C and the wavelength at 280 nm. Prior to analysis, fused-silica capillary was purged for 2 min with 0.1 mol L^{-1} NaOH, 5 min with water and 15 min with buffer, respectively. After the end of each day's analysis, the fused-silica capillary must be purged for 5 min with 0.1 mol L^{-1} NaOH and water.

Preparation of test solution for LC-MS: The experiment use ultrasonic-microwave-assisted-extraction method to get the sample. Steps are as follows: 36 g of dried Dragon's blood powder was weighed accurately in a round bottom flask and then extracted twice with 200 mL chloroform respectively. Microwave power 100 w heating up to 40 °C and the extraction was performed at 50 °C for a period of 30 min. Filtration, the extract was extracted with petroleum ether for three times, concentrated through flash concentrator, purified with polyamide adsorption resin and then the eluent was concentrated to get the determinand. Before measurement, the preparation solution was diluted with ethanol and filtered with a 0.22 μm membrane.

Preparation of reference solution for HPCE: To prepare reference solution, accurately weighed amount of the Loureirin B dissolved in 95 % ethanol to give concentration of 0.3164 mg mL^{-1} to provide stock solutions.

Preparation of test solution for HPCE: Amount of dried Dragon's blood was porphyrized and then 1 g of its powder was weighed accurately in to a 25 mL colorimetric cylinder. Next ultrasonic extracted with appropriate 95 % ethanol for 15 min. After cooling, filled volume with 95 % ethanol and filtered with 0.22 μm millipore filter. Afterwards, 300 μL filtrate was diluted to 2 mL with buffer (filtered with 0.22 μm filter membrane).

RESULTS AND DISCUSSION

Under the optimized conditions, the established LC-MS method (negative ionization mode) was applied to evaluate the LC/Q-TOF total ion current chromatogram of products extracted from Dragon's blood as shown in Fig.1. The total ion chromatogram and the corresponding mass spectrum were analyzed, the known chemical compositions were identified by its molecular ion peak and chemical compounds reported in the literature. Here, 10 phenolic compounds of Dragon's blood's extracts which were mostly flavones and flavanoids were identified. The results were shown in Table-1.

Optimization of HPCE conditions

Effect of buffer concentration: In capillary electrophoresis separations, if the concentration of buffer was too low, the separation is not satisfactory. However, if the concentration of buffer was too high, the density of negative charge of test

TABLE-1
CHARACTERIZATION OF 10 COMPOUNDS IN DRAGON'S BLOOD EXTRACTS

t_R (min)	*m/z*	m.w.	Adscription	Identification	Reference
6.254	253	254.26996	$[M-H]^-$	6-Hydroxy-7-methoxy-3-(4′-hydroxybenzyl)chromane	7
8.064	301	302.36435	$[M-H]^-$	4,4′-Dihydroxy-2,6-dimethoxy two hydrogen chalcone	29
9.246	299	300.34729	$[M-H]^-$	4′,5,7-Trihydroxy high isoflavone	30
10.610	241	242.29972	$[M-H]^-$	2,4′,4-Trihydroxy chalcon	6
13.610	285	286.35928	$[M-H]^-$	4′-Hydroxy-2,6-dimethoxy dihydrochalcone (cochinchinenin A)	7
13.889	315	316.39185	$[M-H]^-$	4′-Hydroxy-2,4,6-trimethoxy dihydrochalcone (loureirin B)	7
16.626	255	256.32403	$[M-H]^-$	4′-Hydroxy-3,5-dimethoxy styrene	31
17.536	497	498.59201	$[M-H]^-$	Cochinchinenin	32
30.130	269	270.47840	$[M-H]^-$	4′,7-Dihydroxy high isoflavone	30
31.337	283	284.50448	$[M-H]^-$	4′,7-Dihydroxy-8-methyl high isoflavone	30

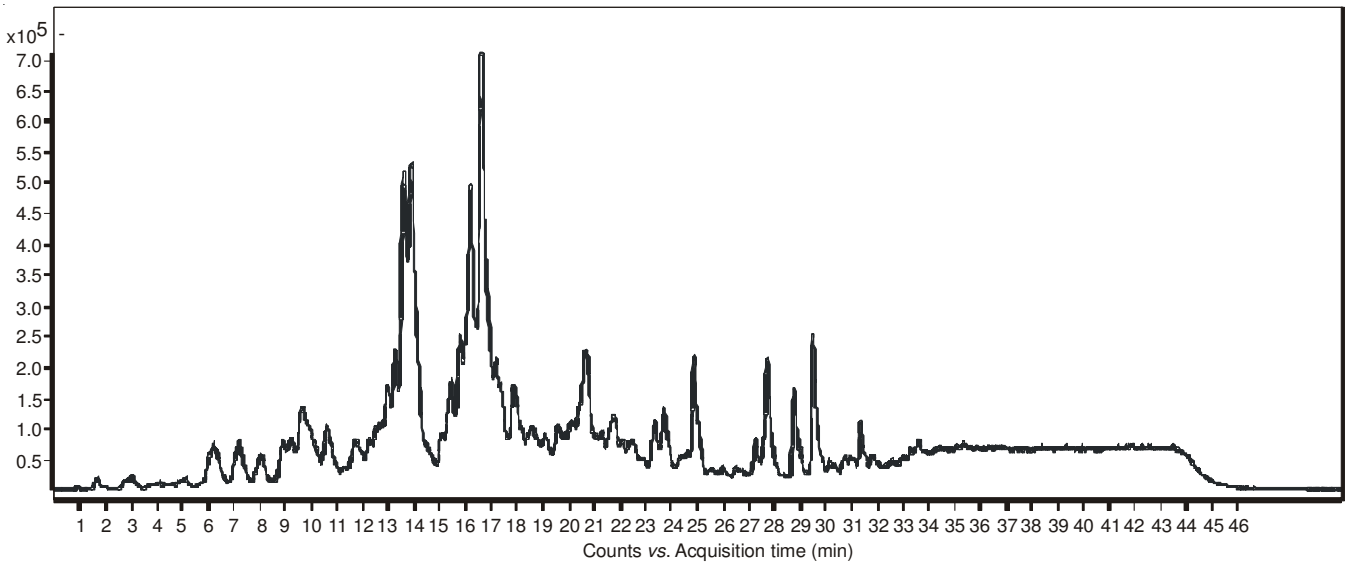

Fig. 1. LC/Q-TOF total ion current chromatogram of products extracted from Dragon's blood

solution's acidic component may be increased and caused the migration time became longer. The effect of the concentration of borax-SDS run buffer at 9.6 on these separations were examined at concentrations of 40-100 mmol/L (borax),with 10-40 mmol/L SDS. As a result of this study, the 80 mmol/L borax-20 mmol/L SDS buffer was taken to be optimal.

Effect of the pH: The pH of running buffer was the most important parameter for changing the selectivity of capillary electrophoresis. The effect of pH value of running buffer on the separation of Loureirin B samples was examined by varying the pH value from 8.5 to 9.8. It was found that with the increase of pH value, the migration time prolonged, but the resolution was improved. Under the pH value (pH 9.6) of running buffer, satisfactory separation efficiency was obtained.

Effect of organic modifier: Organic modifiers added to the running buffer altered the polarity and the viscosity of electrolyte, thus affecting both electroosmotic flow and the electrophoretic mobility of the analytes. By the means of testing, we found that methanol took important role to improve the separation efficiency of Loureirin B samples. Corresponding to the rising of the concentration of methanol, the resolution would increase and the migration time would be prolonged. The effect of concentration of methanol on the migration time was investigated over a concentration range from 5 to 20 % (v/v) and a methanol concentration of 10 % (v/v) was taken to be optimal.

Effect of applied voltage: The effect of the applied voltage on the separation of Dragon's blood samples was examined by varying the applied voltage from 10 to 25 kV. We found that the migration time of the analytes was significantly shortened and their corresponding electrophoretic peaks were sharpened when the applied voltage was increased. However, if the applied voltage was too large, more joule heat was produced by the higher current inside of the capillary and separation efficiency was reduced. In these studies, 15 kV was chosen as the optimum applied voltage.

Determination of Loureirin B in Dragon's blood extracts: Under the optimum conditions described above (80 mmol/L borax-20 mmol/L SDS buffer, pH 9.6, 10 % methanol, 15 kV voltage, separation temperature at 25 °C and the wavelength at 280 nm), the electropherograms of Loureirin B reference solution and Dragon's blood sample were showed in Figs. 2 and 3. Then the prepared Dragon's blood samples were analyzed in triplicate and the average concentration of the Loureirin B in Dragon's blood using our methods was determined as 4.116 mg g^{-1}.

Linearity: Standard stock solutions of Loureirin B were diluted with buffer solution into a series of mass concentration of the reference solution then calculated the peak area integral value. The linearity of the standard curve was confirmed by plotting the peak area value of the Loureirin B standard as

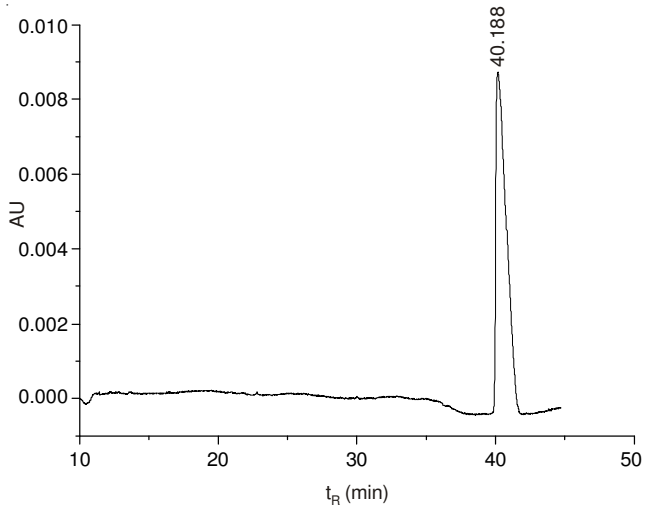

Fig. 2. Electropherogram of Loureirin B reference solution

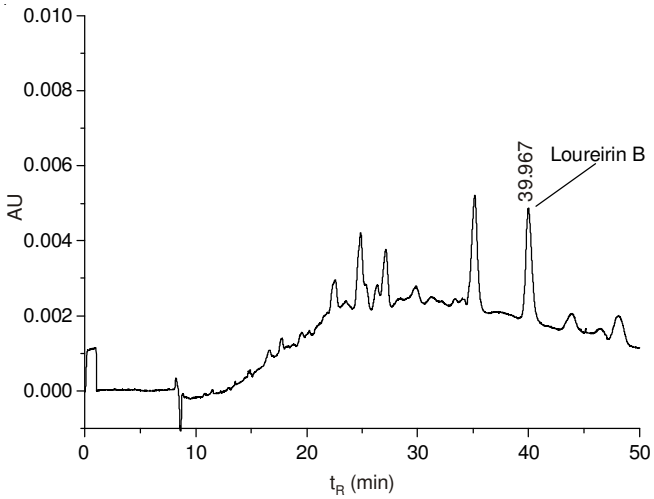

Fig. 3. Electropherogram of Dragon's blood sample

ordinate (Y) *vs*. the mass concentration of the corresponding Loureirin B standard as abscissa (X) and found that the linear concentration of Loureirin B ranged from 0.0053 to 0.1054 mg/mL (r = 0.9963) under the chosen condition. Meanwhile the regression equation was Y = 4700090X—13217.19 1116. The linear calibration curve of Loureirin B as shown in Fig. 4.

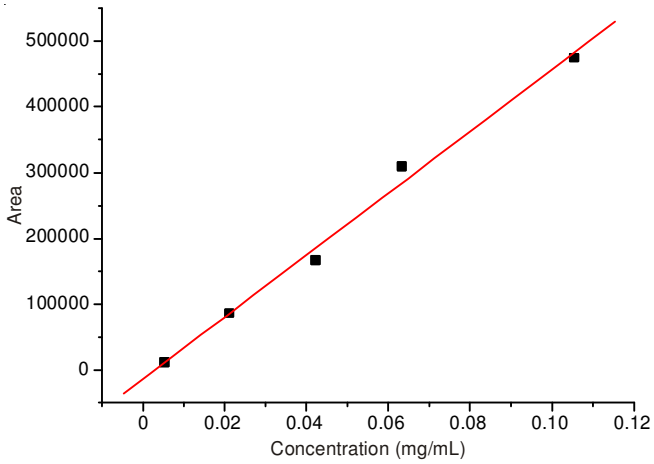

Fig. 4. Linear calibration curve of Loureirin B reference solution

Precision: The intra-day precision was determined by calculating the RSD (%) for five determinations within one day as well as calculated the migration time and peak area precisions (RSD), respectively. The overall RSD precision value (intra-day) for the migration times and peak-areas were found to be 1.3 % and 3.8 % on average.

Recovery: Six different concentrations of Loureirin B reference solutions were added to the corresponding prepared samples, respectively. The mean recoveries of the Loureirin B were calculated at six concentration levels. Consequencely, the average recovery was 98.1 % and the relative standard deviation (RSD) was 1.6 %.

Conclusion

In this work, the HPLC-MS method was established to analysis the phenolic compounds of Dragon's blood and ten phenolic compounds were accurately identified form Dragon's blood. At the same time, a simple, rapid, specific and repeatable HPCE method was proposed and its related influencing factors of the quantification method were investigated and optimized. Analytical application of the developed method was firstly suggested for quantitative determination of Loureirin B in complicated Dragon's blood. These results could be helpful to study Dragon's blood further. The method can be expected to achieve rapidly more componential analysis and quality control of herbs.

ACKNOWLEDGEMENTS

This project was supported by The National Natural Science Foundation of China (No. 21205145, 21276006, 21036009), The Open Funds of State Key Laboratory of Chemo/Biosensing and Chemometrics of Hunan University (No. 201111), the Special Fund for Basic Scientific Research of Central Colleges, South-Central University for Nationalities (No. CZZ10005, CZQ11012, YZZ12002, XTZ13002, XTZ10001) and the 'Five-twelfth' National Science and Technology Support Program (2012BAI27B00).

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