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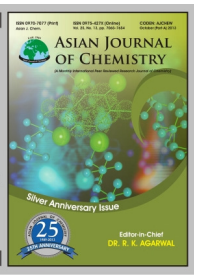

Biopartitioning Micellar Chromatography Determination of Nucleosides and Bases in Bailing Capsule

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In this work, we reported the determination of contents of three nucleosides (uridine, guanosine and adenosine) and two bases (uracil and adenine) in bailing capsule by biopartitioning micellar chromatography. The analytes were carried out on a Sepax-C_{18} (4.6 mm × 250 mm, 5 µm) and mobile phase consisted of 0.02 mol L^{-1} polyoxyethylene (23) lauryl ether (Brij35), 0.02 mol L^{-1} sodium dihydrogen phosphate-disodium hydrogen phosphate (pH = 7.4) and 9.2 g L^{-1} NaCl. The flow rate was 0.6 mL min^{-1}, the detection wavelength and column temperature were set up at 260 nm and 37 ºC, respectively. Uridine, guanosine, adenosine, uracil and adenine could be completely separated from each other and had good linerarity. The method is simple, sensitive,accurate, stable with a better reproducibility. So, it can assess and control the quality of preparations of fermental cordyeps-bailing capsule comprehensively.

Key Words: Biopartitioning micellar chromatography, Bailing capsule, Nucleosides and bases, Contents determination.

INTRODUCTION

Biopartitioning micellar chromatography (BMC)[1] is a mode of micellar liquid chromatography[2] which uses polyoxyethylene (23) lauryl ether (Brij35) solution above the critical micellar concentration (CMC) as a mobile phase under adequate experimental conditions having the same pH and ion intensity with physiological environment. The success of BMC in describing drug's biological behaviour could be attributed to the fact that the characteristics of the BMC systems are similar to biological barriers and extracellular fluids[2].

Biopartitioning micellar chromatography can't only perform the basic function of chromatographic separation, but also can simulate the biopartioning process of many kinds of drugs. The chromatographic retention parameters getting from the BMC system could be applied to predict oral absorption of drugs[3], drugs penetration across the bloodbrain barrier[4], skin permeability of drugs[5] and described their bioactivities[1].

Bailing capsule is included in China pharmacopoeia. It is derived from the spawn of a sexual generation synnematium sinense of a well-known traditional Chinese medicine named *Cordyceps sinensis* which is one kinds of funguses belonging to clavicipitaceae and made out of mycelia dry powder after deep fermentation[6]. Bailing capsule with functions of regulating and nourishing the lung and kidney can treat with the prescription of cough, asthma and haemoptysis associated with lung-kidney vacuity, meanwhile, aches in limbs and waist, chronic renal insufficiency can also be given adjuvant therapy by bailing capsule.

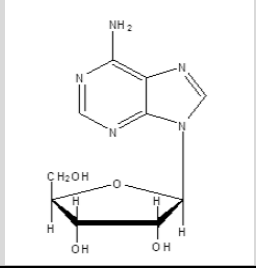
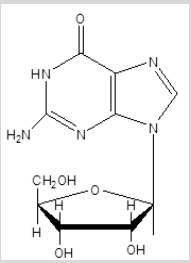
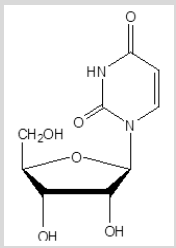
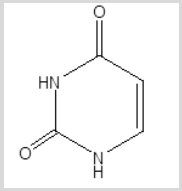
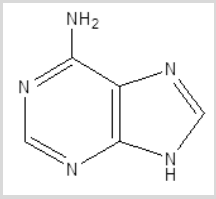
Nucleoside is the most important active component, but China pharmacopoeias pecification[6] and many researches just control the content of adenosine in Bailing capsule[7,8]. Only one article researched about the determination of five constituents (uridine, guanosine, adenosine, uracil and adenine) in 28 preparations of fermental cordyceps using RP-HPLC method[9]. However, determining the content of five constituents by biopartitioning micellar chromatography has never been reported.

In this study, a simple, accurate and reliable analytical method for the simultaneous determination of five active components (Table-1) for their structures, including three nucleosides (uridine, guanosine and adenosine) and two bases (uracil and adenine) in bailing capsule was firstly established by biopartitioning micellar chromatography method coupled with diode array detection. From these results, the proposed method in this paper is particularly suitable for the routine analysis of Bailing capsule and its quality control.

EXPERIMENTAL

High purity grade polyoxyethylene (23) lauryl ether (Brij35) and five reference substances (adenosine, guanosine, uridine, uracil, adenine) were purchased from Aladdin reagent (Shanghai,China). Bailing capsule was supplied by the east China pharmaceutical Co., Ltd. (Hangzhou, China). HPLC grade methanol procured from TEDIA (USA) was used. Sodium

TABLE-1
CHEMICAL STRUCTURE OF FIVE ACTIVE COMPONENTS

Active components	Adenosine	Guanosine	Uridine	Uracil	Adenine
Chemical structure					

dihydrogen phosphate, disodium hydrogen phosphate, diethyl ether, sodium chloride, phosphoric acid, sodium hydroxide and other reagents were all of analytical reagent grade. HPLC grade water obtained from Millipore system (Millipore Inc., USA) was used throughout the analysis.

The HPLC (Dalian elite analytical instruments Co. Ltd.) equipped with a P230 high-pressure pump, a 7725i manual injector, diode array detector and QM QT-330 column oven was used in this study. All separations were carried out on a Sepax-C_{18} column (250 mm × 4.6 mm with 5.0 μm particle size) from Sepax Technologies (USA). Other instruments included an electronic balance (Model: FA2104), a ultrasonic cleaner (Model: KQ 5200 DE), a pH 211 Microprocessor pH meter and a Milli-Q Biocel ultra-pure water machine (Millipore Inc.,USA).

Preparation of standard solution: Five stock solutions (uridine 1.07 mol L^{-1}, uracil 1.01 mol L^{-1}, guanosine 1.03 mol L^{-1}, adenosine 1.06 mol L^{-1}, adenine 1.03 mol L^{-1}) were prepared in water and stored at 4 ºC when not in use. Working solutions of the lower concentration to study accuracy, precision, linearity, limits of detection (LOD) and quantitation (LOQ) were prepared by appropriate dilution of the stock solution.

Preparation of sample solution: Ten bailing capsules contents powder was mixed and 0.5 g powder precisely weighed into a flask with bottle stopper. 20 mL diethyl ether was added,then soaked of 0.5 h. After filtration, the residue was put into another flask with bottle stopper, then dissolved in 50 mL of 0.5 % phosphoric acid solution for 0.5 h in an ultrasonic bath. After static cooling down,the total solution was filtered through a syringe filter (0.45 μm) before injected into the HPLC system for analysis.

RESULTS AND DISCUSSION

Determination of five active components

Chromatographic conditions: The HPLC separation was done on a Sepax-C_{18} column (250 mm × 4.6 mm, 5.0 μm), with the mobile phase of 0.02 mol L^{-1} Brij35 aqueous solution comprised of 0.02 mol L^{-1} phosphate buffer (pH = 7.4) and 9.2 g L^{-1} sodium chloride delivered at a flow rate of 0.6 mL/min. The chromatograms were recorded at 260 nm using diode array detector. The injection volume was 20 μL,column temperature was maintained at 37 ºC. Under the chromatographic conditions, the number of theoretical plates of all peaks is over 4000, separating degrees are all greater than 1.5. It was indicated that a good separation was obtained under the described condition and no interfering peaks were found at the retention time of analytes. Five active components had a symmetrical peak shape with acceptable retention time as shown in Fig. 1.

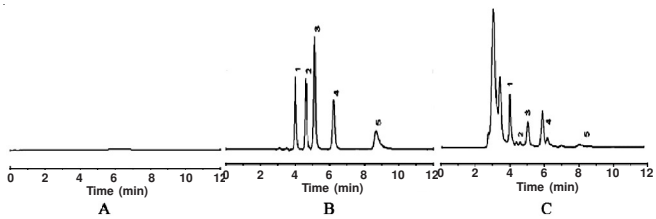


Fig. 1. HPLC chromatograms of bailing capsule (A blank solution, B tandard solution, C sample solution). Peak 1. uridine Peak 2. uracil Peak 3. guanosine Peak 4. adenosine Peak 5. adenine

Linearity: The linearities of uridine, uracil, guanosine, adenosine, adenine were studied by preparing standard solutions at five different levels. The data were subjected to statistical analysis using a linear-regression model. The standard deviation of slope and intercept were calculated and shown in Table-2. The results indicated a good linearity of uridine, uracil, guanosine, adenosine, adenine with a linear range of 5.35-85.6, 0.505-8.08, 5.15-82.4, 1.06-33.9, 0.206-3.30 μg mL^{-1}, respectively.

TABLE-2
REGRESSION EQUATIONS AND RELATED COEFFICIENTS OF FIVE COMPONENTS

Component	Regression equation	Related coefficient
Uridine	A = 129.91C + 245.43	0.9996
Uracil	A = 118.07C + 7.3713	0.9999
Guanosine	A = 65.035C + 70.831	0.9999
Adenosine	A = 89.681C – 14.544	0.9998
Adenine	A = 122.40C – 15.353	0.9993

Limits of detection and quantitation: Limits of detection and quantitation represent the concentration of the analyte that would yield signal-to-noise ratio of 3 for limit of detection (LOD) and 10 for limit of quantitation (LOQ), respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by injecting blank samples and calculating the signal-to-noise ratio by injecting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. The results were given as following: LODs of uridine, uracil, guanosine, adenosine,adenine were 0.0321, 0.0084, 0.0172, 0.0151 and 0.0515 μg mL^{-1}, respectively. The quantitation limit was subsequently validated by the analysis of a suitable number of samples near at quantitation limit. The results were given as following: LOQs of uridine, uracil, guanosine, adenosine,

adenine were 0.0107, 0.0253, 0.0515, 0.0454 and 0.1030 μg mL^{-1}.

Precision, repeatability and stability: The precision of the method was assessed by determining the intra-day and inter-day variations. The intra-day variation was investigated by analyzing the same mixed standard solution for five times within one day and the inter-day variation was determined in consecutive five days. Variations were expressed by relative standard deviations (RSD). The precision result of the solution at medium concentration was presented as following in Table-3.

TABLE-3
PRECISION, REPEATABILITY AND STABILITY OF THE METHOD (n = 5)

Component	Precision		Repeatability	Stability
	Intra-day RSD (%)	Inter-day RSD (%)		
Uridine	0.06	0.27	1.50	0.30
Uracil	1.00	1.78	1.72	1.59
Guanosine	0.11	0.26	1.45	0.69
Adenosine	0.66	1.33	1.28	0.88
Adenine	1.20	1.20	1.64	1.31

The repeatability was determined by the injection of five different samples which were obtained through the same sample preparation. The stability was tested with the same sample solution at room temperature and analyzed at 0, 2, 4, 8, 12, 24 and 48 h. The results of precision, repeatability and stability were shown in Table-3.

The results from these studies indicated, the sample solution was stable and there was no impurity peak observed.

Recovery: Recovery test was used to evaluate the accuracy of this method. 0.2164 mg uridine, 0.0273 mg uracil, 0.3096 mg guanosine, 0.1614 mg adenosine, 0.0128 mg adenine were added to the sample of each in quintuplicate and then extracted and analyzed using the proposed procedure to calculate recoveries. The results listed in Table-4.

TABLE-4
RECOVERY OF THE METHOD (n = 5)

Component	Average recovery (%)	RSD (%)
Uridine	98.35	1.85
Uracil	95.19	1.41
Guanosine	103.4	1.54
Denosine	102.7	1.77
Adenine	90.56	1.98

Sample analysis: The different batches of samples were prepared by the same prescription and the same manufacturing process. Determination of the content of five components in the three batches was consistent in general. The datas were shown in Table-5.

Due to the complicate compositions of traditional Chinese medicinal preparations, it is difficult to obtained good separation. It was necessary to investigate various factors in BMC, such as mobile phases and flow rate.The following was the optimization of the chromatographic conditions we have done.

Different concentrations of phosphate buffer in mobile phase systems were investigated in attempts to obtain the best separation and resolution of five components. By comprehensive comparison of the chromatograms, the mobile phase consisting of 0.02 M phosphate buffer was found to give the best separation of these compounds. The effect of flow rate was also investigated and the results showed that the separation were suitable at 0.6 mL/min.

TABLE-5
RESULTS OF CONTENT DETERMINATION OF FIVE COMPONENTS(n=5)

Component	(Lot No.) 100646	(Lot No.) 100749	(Lot No.) 101009
Uridine	0.8343 (1.37)	0.8811 (1.28)	0.8658 (1.35)
Uracil	0.1087 (1.50)	0.1099 (1.10)	0.1094 (1.60)
Guanosine	1.2022 (1.56)	1.2410 (1.49)	1.2392 (1.78)
Adenosine	0.8189 (0.30)	0.8234 (0.60)	0.8214 (0.58)
Adenine	0.0480 (1.88)	0.0592 (1.76)	0.0560 (1.85)

Note: RSD (in the parentheses, %).

Conclusion

To out best of knowledge, this is the first report of an accurate and reliable analytical method for the simultaneous determination of five major bioactive constituents (uridine, uracil, guanosine, adenosine, adenine) in Bailing capsule by using biopartitioning micellar chromatography method coupled with diode array detection. High linearity, precision, repeatability, stability and accuracy were presented in the method validation procedure. The proposed method is promising to improve the quality control of Bailing capsule and other related nucleoside drugs.

The chromatographic retention parameters obtained from the BMC system, such as capacity factor, could be applied to establish the models of quantitative retention membrane permeability relationship (QRPR) and quantitative retention-activity relationship (QRAR)[10] and then to predict oral absorption of drugs and describe their bioactivities.

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