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Simultaneous Analysis of Both Lactone Form and Acid Form Monacolin K in Red Yeast Rice by RP-HPLC

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역상 HPLC에 의한 홍국 중의 락톤 및 산성 모나콜린 K의 동시분석법

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Abstract

A method for the simultaneous and precise determination of lactone form and acid form monacolin K in red yeast rice by HPLC was developed in this study. The standard of acid form monacolin K was prepared by alkaline hydrolysis of its lactone form, which was purchased from Sigma company. The optimum HPLC system for the separation and quantification of acid form and lactone form monacolin K is based on the reversed-phase column, and the acidified mobile phase consisting of acetonitrile: 0.1% trifluoroacetic acid (TFA) water soln =62:38, the low limit detection amount was 5 ng (i.e.10 μl injection of 0.5 μg/ml). And the optimal extracting system for monacolins in red rice was also presented here.

Key words : RP-HPLC, lactone form monacolin K (LFMK), acid form monacolin K(AFMK).

Introduction

In the late of 1970s and the early of 1980s, Akira Endo and other Japanese researchers did much great work on monascus rubber. And they reported in succession many statins like monacolin K (also called as lovastatin, mevonolin), monacolin J[1], monacolin[2], monacolin M[3], and monacolin X[4], isolated from the liquid cultured solutions by *Monascus rubber*. And these compounds, especially monacolin K has a strong activity on lowering the cholesterol in plasma of animals and human by inhibiting 3-hydroxyl-3-methylglutaryl (HMG)-coenzyme reductase, the rate-limiting enzyme in cholesterol synthesis pathway, and are thereby effective in the therapy of hypercholesterolemia[5]. And now days monacolin K containing products like cholestin of America and Xuezhikang, Zhibituo of China, or even just the red rice powder have entered the daily life of many population, world-wide.

Recent study proved that monacolin K has two form: lactone form and acid form in most red rice

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products[6]. Since the acid form lovastatin so far has no available commercial products, and also because of the larger difference in the polarity of lactone form and acid form lovastatin, so far there has no report on the simultaneous detection method for both LFL(lactone form lovastatin) and AFL(acid form lovastatin) in red rice products by HPLC or by other means. It is very important to develop an accurate method to analyze total monacolin K in the product.

The present paper describes a very sensitive and accurate quantitative method by HPLC, providing you to simultaneously analyze this two forms of monacolin K in any red rice product.

Materials and Methods

1. Chemicals and Red Rice Samples

Lactone form monacolin K was purchased from Sigma, acid-form lovastatin was experimentally gained by alkaline hydrolysis of lovastatin in our lab. Acetic acid and HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Burdick & Johnson (U.S.A.), HPLC-grade water was prepared using a compact ultrapure water system (Compact Co. Ltd); Triethanolamine and tetrabutylammonium hydrogen sulphate, one low UV ion pair reagent, were purchased from Sigma.

The red rice samples of Dbio 1, 2, 3 were produced by the Company of Dbio Co. Ltd., Korea. Healthcare drug of Cholestin was purchased from Amercian. The healthcare drug of Xuezhikang and Zhibituo were provided by Professor Zhou in Zhejiang University of Industry, Hangzhou City, China.

2. Chromatographic Apparatus

The HPLC system was System Gold^{R} (Beckman, U.S.A.) equipped with 128 solvent module and 168 Detector and a power supplier, an 7725 auto sampler and an on-line degassing instrument. ODS column (250×4.6mm, 5 μm) from Phenomenex was used. The 168 detector was equipped with a photo-diode array (PDA) detector. Gold^{TM} Nouveau Chromatography Station was used for system control, data collection and analysis.

3. Chromatographic Conditions

The identification and quantification of monacolin K were carried out by HPLC, using a Phenomenex reverse phase column. The flow-rate was 0.8ml/min. The detection was monitored at 237nm. The injection volume was 5 μl, and the run time was 28 to 40 min. Several mobile phases as below were employed for comparison purposes, acetonitrile:water or TFA acidified water solution with the ratios ranged from 80 : 20 to 60 :40 were employed in this study.

A: acetonitrile : water (variable)
B: acetonitrile : 0.1~0.4% ethylenediamine (EDA) water soln (variable)
C: acetonitrile : 0.05~0.2% trifluoroacetic acid(TFA) water soln (variable)
D: methanol : 0.1%TFA water soln (variable)
E: acetonitrile : 0.2% tetrabutylammonium hydrogen sulphate water soln(62 : 38 v/v)

All the mobile phases were filtrated through a 0.45 μm filter membrane (Aldrich Chemical Company, Inc.), then were degassed by air pump while sonicating for 15 min.

4. Preparation of Standards

1) For Lactone Form Monacolin K

A stock solution of LFMK(lactone form monacolin K) was made by dissolving 0.100 g monacolin K powder in 80% acetonitrile aqueous solution and made to volume in 100 ml volumetric flasks. Then transferred the solution to a brown reagent bottle and stored at -20℃ if not using it. This stock solution was used to prepare the working standard solutions of different concentrations by gradient dilution with 80% acetonitrile aqueous solution: 50, 100, 300, 600, 900, 1,000 μg/ml. From these working solutions, with 5 μl injection, running on the developed optimum chromatographic condition, the calibration line of peak area (X) versus analyte concentration (Y) were plotted. And the linear regression equation was:

$$Y = 3.582140 \times 10^{-5} X + 0.018, \quad r = 0.9999$$

The stock solution of 500μg/ml AFMK(acid form monacolin K) was prepared in 80% acetonitrile aqueous solution, alkalized by adding 10 μl 0.1 N NaOH

per 1 ml. Then the work standard solutions of 20, 50, 100, 300, 400, 500 μg/ml were made by dilution with the alkalized acetonitrile aqueous solution. With the same method for LFMK, the linear regression equation for AFMK was plotted:

$$Y = 4.782880 \times 10^{-5} X + 0.023, \quad r = 0.9987$$

5. Analytic Limit and Recovery of the Screened HPLC System

The accuracy and the precision of the screened optimum HPLC system (i.e with the mobile phase of ACN : 0.1% TFA water soln = 62: 38) were estimated by the recovery rate and the relative standard deviation (R.S.D.) respectively, calculated from the running of 300 μg/ml standard LFMK and 300 μg/ml AFMK 8 times. The concentration of 300 μg /ml was considered as true value to calculate the recovery rate.

6. Sample Extraction and Clean Up

All the extraction done in this study was processed as following: 0.200g red rice powder or its product was extracted with 1 ml extracting solvent in 1.5 ml Enpendorf tube by sonicating for 20 mins at 25℃[7]. Then centrifuge it at 12,000rpm for 5 mins, then transferred the supernatant to a 5ml vial, and repeated extracting the residue with 1ml extracting solvent for another 4 times. Then combined these 5 times extraction solutions and make it up to 5ml with the responding extracting solvent. Then took 1 ml the extracted solution and subjected to 0.2 μm PTFE sample filter or 0.45 μm PVDF sample filter (Whatman, England). The filtrated solutions were used for HPLC analysis. Stored in refrigerator at 4℃ if not used it.

7. Screening of the Optimum Extracting Solvent

Among ACN, EtOH, MeOH, EtOAC and their water solutions, red rice of Dbio 2 was chosen for screening the optimum extracting solvent for total monacolin K extraction job. For each treatment, the extracting steps just followed the procedures described above. The filtered extracted solution was subjected to the screened optimum HPLC condition, i.e with the mobile phase of ACN : 0.1% TFA water soln = 62 : 38. The extracting efficiency of 80% MeCN was relatively considered as 100%, and the relative percentages of other solvents were calculated and used to estimate their extracting efficiency. Based on the primary screening, the secondary screening work was done among the solvents of 80%, 70%, 65%, 60%, 57%, 53%, 50%ACN, each treatment was repeated up to 3 times.

8. Analytic Recovery

To determine the reliability of the screened optimum extraction method, 2 ml of 1mg/ml standard LFMK solution was mixed thoroughly with 2.000g Cholestin powder then dried completely at room temperature, then mixed again. Then for 5 times, 0.200 g mixture was taken and subjected to the extraction method as described above, extracted with 60% ACN water solvent. The filtered extracted solution was applied to the screened optimum HPLC condition, i.e with the mobile phase of ACN : 0.1% TFA water soln = 62: 38. Cholestin was used as control. The average recovery rate and relative standard deviation (R.S.D.) were calculated.

Results and Discussion

1. Chromatographic Conditions

For the testing column, the optimum composing of the mobile phase was acetonitrile : 0.1% TFA water soln = 62 : 38 (v/v). Refer to Fig. 1.(D). The addition of TFA to mobile phase had a greater influence on the retention time of ALMK than that of LFMK. (Refer to Fig. 1.). With the ratio of acetonitrile : TFA water soln = 65: 35, the rentention time of AFMK increased from around 2.20 mins to 15.00 mins with the increase of the final concentration of TFA water solution from 0% to 0.2%. We also found the ratio (from 80:20 to 55:45) of acetonitrile to TFA acidified water solution of a given concentration, greatly but almost evenly effected the retention times of LFMK and AFMK. On the whole, the higher ratio the more delayed retention time of both AFMK and LFMK. Refer to Fig. 1. C, D for this point.

To find out whether this TFA acidulation is sui-

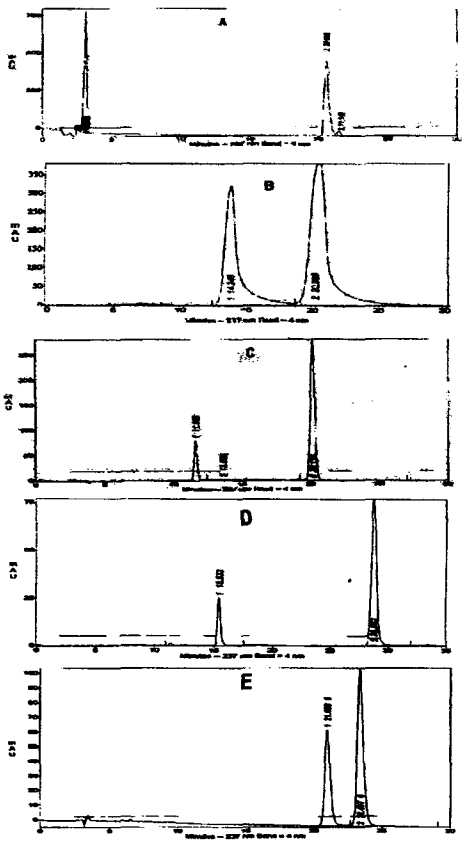

Fig. 1. The chromatographic characteristics of AFMK and LFMK under different mobile phases. The mixture of LFMK and AFMK was used for the case of comparing the separation efficiency of various mobile phases. ODS column from Phenomenex (250×4.6mm, 5 μm) was used and detection wavelength was 237 nm, and the flow rate was 0.8 ml/min. A : the chromatogram run with the mobile phase of acetonitrile/water soln. =65/35, B: the chromatogram run with the mobile phase of acetonitrile / 0.1%EDA water soln. = 65/35, C : the chromatogram run with the mobile phase of acetonitrile / 0.1%TFA water soln. = 65/35, D : the chromatogram run with the mobile phase of acetonitrile / 0.1%TFA water soln. = 62/38, E : the chromatogram run with the mobile phase of methanol / 0.1%TFA water soln. = 80/20.

table either to MeOH-H_2O system. We also tried the mobile phase of D in section 2.3. And we found if not adding TFA in mobile phase, AFMK was eluted with in 3 mins just as with the mobile phase of Acetonitrile-water system. If the ratio of methanol : 0.1%TFA water soln was less than 70:30, the peak of LFMK appeared only after 40 mins run and with a quite big width of 50% height. So we raised the ratio to 80:20 and the retention times of AFMK and LFMK were 21.033 mins, 23.467 mins respectively. (for its chromatogram refer to Fig. 1. E.). And later run of the red rice produced in our lab with this mobile phase found that at least two peaks were missing compared with the mobile phase of acetonitrile : 0.1% TFA water soln = 62 : 38(v/v).

Further experiments were carried out by adding tetrabutylammonium hydrogen sulphate, one Ion-pair-reagent PIC-A, to the mobile phase of ACN-H_2O system. The results suggested that no improvement was gained in the resolution, as reported in the literature[8], on the contrary, suggested that reduced both retention time and column sensitivity when comparing with our screened optimum mobile phase of acetonitrile : 0.1 TFA water soln. = 62 : 38 (v/v).

2. Assay Evaluation

1) Estimation of this HPLC System

With 300 μg/ml LFMK and 300 μg/ml AFMK, the accuracy and precision of this screened optimum HPLC system was estimated by the recovery rate and R.S.D. calculated from 8 repetitions. The recovery rates of LFMK and AFMK were 99.6% and 99.7%, and the R.S.D. were 1.21% and 1.27% (n= 8), respectively, which indicated that this HPLC system had a very high accuracy and precision. The result was shown in Table 1.

2) The Optimum Extraction Solvent

The relative extracting efficiency of 80% MeCN for AFMK and LFMK was considered as 100%. And 60% ACN was screened as the optimum extraction solvent for AFMK and LFMK with the relative extracting efficiency of 117.7% and 113.6% respectively, after primary and secondary screening with red rice sample of Dbio 2. The results were shown in Fig. 2. and Table 2.

3) Analytical Recovery

Table 1. Recovery rate and R.S.D. of the diluted standards of both LFMK and AFMK

Operation times (n)	300 μg/ml standard LFMK				300 μg/ml standard AFMK			
	Conc.	mean ± S.D.	Rcvy* (%)	R.S.D (%)	Conc.	mean ± S.D.	Rcvy* (%)	R.S.D (%)
1	297.421				294.330			
2	295.380				306.653			
3	301.765				298.602			
4	297.428	298.856	99.62	1.21	297.890	299.24	99.7	1.27
5	305.070	± 3.624			299.988	±3.814		
6	300.373				302.046			
7	293.780				295.820			
8	299.628				298.617			

* : recovery rate= (mean / 300) × 100%.

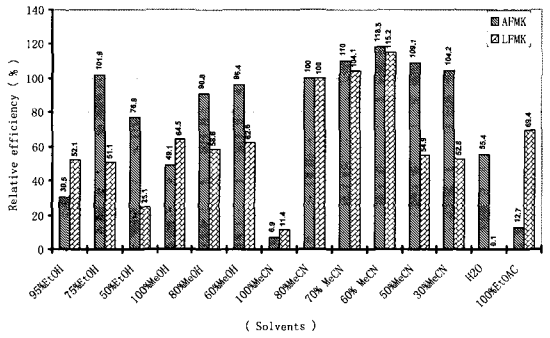

Fig. 2. The primary screening for optimum solvent for monacolin K extraction.

The recovery rate of the lactone form monacolin K added to the healthcare drug of cholestin was shown in Table 3. The recovery reached to 98.397 ± 2.81% (R.S.D. : 2.854 %, n=5). This indicated that around 98% of total monacolin K can be extracted out from red rice or its product with 60% ACN, 5 times extraction.

Conclusions

We have developed an HPLC system for the separation and quantification of monacolins in red yeast rice. The system is based on a reversed-phase column, and an acidificational mobile phase consisting of acetonitrile- water-trifluroacetic acid.

This method was shown here to provide producible, accurate and sensitive results, allowing the acid-form monacolin K and lactone-form monacolin K in the red yeast rice to be simultaneously determined using a simple extraction procedure.

Table 2. The secondary screening for the optimum extracting solvent

Solvents	AFMK			LFMK		
	Avr.conc.* (μg/ml)	R.S.D.(%)	R.E.E**(%)	Avr.Conc.*(μg/ml)	R.S.D.(%)	R.E.E** (%)
80%CAN	216.562 ± 2.858	1.32	100.0	43.442 ± 0.582	1.34	100
70%CAN	239.950 ± 4.007	1.67	110.8	45.353 ± 0.571	1.26	104.4
65%CAN	250.778 ± 3.235	1.29	115.8	47.830 ± 0.660	1.38	110.1
60%CAN	254.893 ± 3.645	1.43	117.7	49.350 ± 0.730	1.48	113.6
57%CAN	248.829 ± 3.456	1.39	114.9	47.178 ± 0.612	1.30	108.6
53%CAN	241.899 ± 3.628	1.50	111.7	37.881 ± 0.564	1.49	87.2
50%CAN	236.485 ± 3.476	1.47	109.2	26.195 ± 0.414	1.36	60.3

Avr.conc.* : The concentrations of 25 times diluted extracted soln of Dbio2, mean ± S.D.(n=3)

R.E.E.*: Relative extracting efficiency, comparing to the extracting efficiency of 80% ACN.

Table 3. Recovery of 2 mg LFMK added to 2 g Cholestin powder

		Conc. of mixture & control (μg/ml)*	
		AFMK	LFMK
Repetition (n)	1	56.670	118.080
	2	54.985	116.423
	3	56.780	119.396
	4	56.082	117.064
	5	57.183	117.720
Avr. Conc.		56.340±0.854	117.736±1.124
Control[a]		56.320±0.845	78.378±1.00
Recovery[b] (%)		98.397	
R.S.D[c]. (%)		0.320	

*: The concentrations of 25 times diluted extracted soln of LFMK added Xuezhikang powder by 60% CAN Water soln.

a : The control Cholestin was repeated 3 times. means ± S.D., n=3.

b : Recovery (%) of added LFMK = [(Avr. Conc. Control[a]) / 40 $\mu g \cdot ml^{-1}$] X 100% n=5.

c : R.S.D. = (S.D. / Avr. Conc.) × 100% X (Avr. Conc. Control[a].)/ Avr. Conc., n=5.

요 약

홍버섯쌀은 현재 혈압과 혈중 고지혈 강하제로서 의약품 뿐만 아니라 기능성 식품으로서 많은 관심을 불러 일으키고 있다. 그러나 이 홍버섯에 함유된 유효성분인 monacolin K의 정성법과 정량적인 분석 방법이 제시되지 못하였다.

본 연구에서는 monacolin K의 lactone과 acid 형태에 대한 각각의 정확한 정성법과 정량가능한 분석법을 제안하였다. 이 방법은 HPLC의 역상컬럼을 사용하여 acetonitrile과 0.1%TFA를 62:38의 비율로 혼합한 용매를 사용하는 것이다. 이 제시된 방법을 활용했을 때 홍국중의 monacolin K의 최소 검출농도는 5ng이었다.

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