HPLC–MS Analysis of the Riboflavin Crude Product of Semisynthesis

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Abstract

An accurate and simple analysis method by high-performance liquid chromatography–mass spectrometry for the riboflavin crude product of semisynthesis is established in this study. In this method, a C_{18} column is used, and the mobile phase is methanol–water. Under the optimal analysis conditions established in this study, all components in the riboflavin crude product are well separated and identified, respectively. The external standard method is used for the quantitative analysis, and the results are proven to be accurate.

Introduction

Riboflavin [7,8-dimethyl-10-(D-1-ribitol)-isoalloxazin, Vitamin B_2] acts as an essential coenzyme in many oxidation-reduction reactions involved with carbohydrate metabolism and is important for the growth and development of living things (1–4). Currently, riboflavin consumption is much higher than before because a mass of riboflavin products are also used as food and feed additives as oppose to for medical use only (5–7). In recent years, in order to enhance output, the semisynthesis method was developed in industrial production (8,9), by which the reaction of 1-deoxy-1-[4, 5-dimethyl-2-(phenylazo) phenylamino]-D-ribitol (PAR) with barbituric acid gives a crude riboflavin product. The crude product still needs to be further purified to obtain the pure riboflavin. As expected, the quality of the crude product will directly affect the yield of the pure production. Therefore, it is obvious that establishing an analysis method for riboflavin crude product that is simple and accurate is of great importance to strengthening quality control and enhancing the industrial yield, accordingly.

For medicinal purpose, the quality standard of riboflavin, as stated in the Chinese Pharmacopeia (10), Chinese National Standards (11), US Pharmacopeia (12), and Europen Pharmacopeia (13), already has strict regulations. In both the Chinise Pharmacopeia and the Europen Pharmacopeia, the method used for riboflavin content analysis is by UV–vis spectrophotometry, and the method used by the US Pharmacopeia is the fluorimetric method. However, the described methods often result in higher-than-expected results because of the fact that the impurities in riboflavin crude product have not been well separated before the riboflavin was quantitatively determined. In recently years, other analysis methods for riboflavin have been reported, which mostly focused on pure riboflavin (14–18). In this study, a new, accurate, and simple analysis method for riboflavin crude product from semisynthesis was established. In this method, all impurities in the crude product were well separated and identified, respectively, before quantitative determination.

Yan Lu et al. (19) previously reported an analysis method for riboflavin crude product. However, in that method, an uncommon –CN column was used, and the impurities in crude riboflavin were not identified. For the purpose of convenience and accuracy, in this study, a new method by high-performance liquid chromatography (HPLC)–mass spectrometry (MS) is reported for riboflavin crude product analysis, which uses the common C_{18} column and methanol–water as the mobile phase. By this method, all the impurities in riboflavin crude product were successfully separated and identified by the MS data. Under the optimal chromatographic conditions established in this study, the main components in the crude product were also quantitatively determined by the external standard method, with a diode-array detector (DAD).

Experimental

Samples, chemicals, and reagents

Methanol (HPLC-grade), bidistilled water (filtered by 0.45-µm filter membrane), riboflavin crude product (self-synthesized), riboflavin standard sample [recrystallized 5 times, melting point (MP): 279.5–280.5°C], and PAR standard sample (recrystallized 5 times, MP: 175.5–176.5°C) were purchased from Merck

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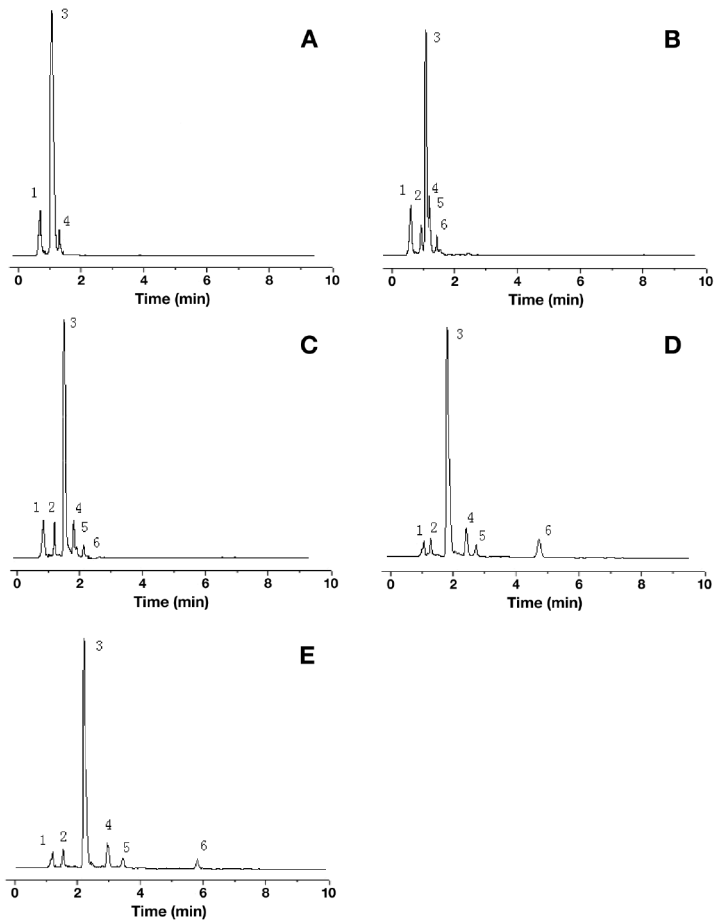

Figure 1. The effects of changing the mobile phase ratio on the separation of the riboflavin crude product: methanol–water: 90:10, A; 85:15, B; 80:20, C; 75:25, D; 70:30, E.

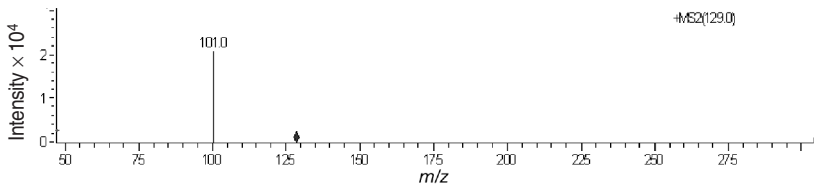

Figure 2. MS spectrum of peak 1

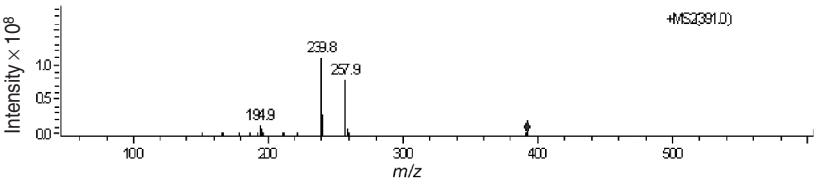

Figure 3. MS spectrum of peak 2

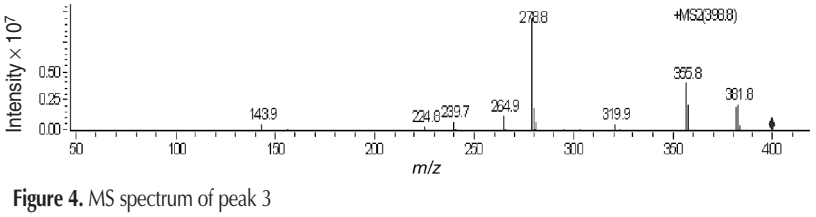

Figure 4. MS spectrum of peak 3

(Darmstadt, Germany), and barbituric acid (AR) was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Apparatus and chromatographic conditions

An Agilent 1100 HPLC with a DAD, HP1100 liquid chromatograph–mass selective detector, C_{18} column (ZORBAX SB-C18, 5 µm, 150 × 4.6 mm) were also used (Agilent, Palo Alto, CA).

Other chromatographic conditions were: column temperature, 30°C; mobile phase, methanol–water (75:25); flow-rate, 1.0 mL/min; λ = 224 nm; sample injected, 20 µL. Quantitative analysis was carried out by external standard method. Other conditions were: ion source, electrospray ionization; N_2 temperature, 350°C; flow-rate, 11 L/min; *m/z*, 50–2000.

Results and Discussion

Selection of the HPLC conditions

Selection of the detection wavelength

In this study, the UV detection wavelength was set at 224 nm. UV spectrum of the riboflavin standard sample showed that there were very strong absorptions at both 224 and 267 nm. However, the wavelengths of reactant residues in crude product were usually below 250 nm. For the sensitivities of both riboflavin and the reactant residues, the UV detection wavelength was set at 224 nm.

Effect of the mobile phase composition

The mobile phase was methanol–water. To study the effect of the mixture ratio (methanol–water) on the separation, some HPLC conditions were set as follows: column temperature, 30°C; λ = 224 nm; flow-rate, 1.0 mL/min. The ratio of methanol–water was changed in turn to 70:30, 75:25, 80:20, 85:15, or 90:10. The corresponding effects on the crude product separation are shown in Figure 1. It can be seen from Figure 1 that the ratio of the mobile phase plays an important role in the separation. When the ratio of methanol–water was 80:20, 85:15, or 90:10, the components in the crude product were not separated completely, and some peaks were overlapped. When the ratio of methanol–water was 70:30 or 75:25, all components in the crude product were separated completely. However, when the mobile phase ratio was set at 75:25, less time was needed to obtain the complete chromatogram, and the peak forms (Figure 1D) were also better than those obtained at the ratio of 70:30 (Figure 1E). Therefore, 75:25 was the optimum ratio of the mobile phase.

HPLC–MS qualitative analysis

Figures 2–7 are the mass spectra of the six components in riboflavin crude product using HPLC–MS analysis. The mass spectrometric data are shown in Table I. By the HPLC standard addition method, peaks 1, 3, and 6 found in Figure 1 can be assigned to barbituric acid, riboflavin, and PAR, respectively. The results of MS analysis were in accordance with those by the HPLC standard addition method (see Table I), which indicate that there were still some reactant residues in the crude product.

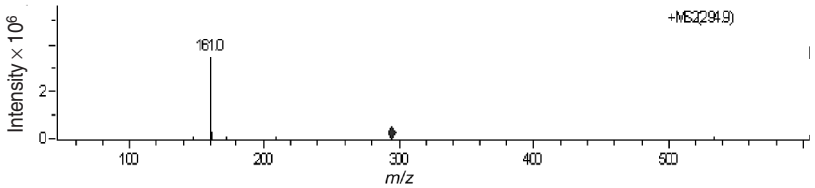

Figure 5. MS spectrum of peak 4

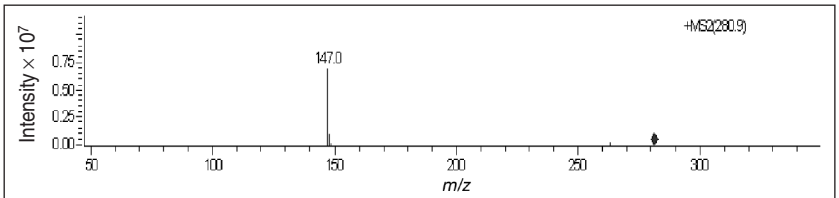

Figure 6. MS spectrum of peak 5

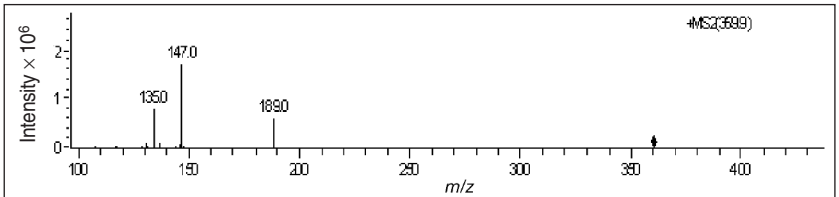

Figure 7. MS spectrum of peak 6

From the MS data, peak 2 can be concluded to be the decomposition product of riboflavin, and the fragment ion of *m/z* 391 can be interpreted as the quasi-molecular ion (M+K) of the decomposition product of riboflavin, primarily because riboflavin decomposed easily with the following reaction (Figure 8) (20–22).

Peaks 4 and 5 (found in the chromatogram Figure 1) can be assigned to lumiflavin and 7,8-dimethylisoalloxazine. When exposed to light, riboflavin decomposed to 7,8-dimethylisoalloxazine, whereas in alkaline conditions, it decomposed to lumiflavin (Figure 9) (23).

Figures 5 and 6 show the MS spectrum of peaks 4 and 5, respectively. The fragment ions of *m/z* 295 (Figure 5) and *m/z* 281 (Figure 6) differ by 14, which is equal to the difference between the molecular weights (MW) of lumiflavin (MW 256) and 7,8-dimethylisoalloxazine (MW 242). Therefore, the fragment ion of *m/z* 295 in Figure 5 can be assigned to the quasi-molecular ion (M+K) of lumiflavin (MW 256), and similarly, the fragment ion of *m/z* 281 in Figure 6 can be attributed to the quasi-molecular ion (M+K) of 7,8-dimethylisoalloxazine (MW 242).

Table I. MS Data of the Components in the Riboflavin Crude Product

Peak number	Components	Ion fragment *(m/z)*
1	Barbituric acid	129.0 (M+H), 101.0
2	Decomposition product of riboflavin	391 (M+K), 257.9, 239.8, 194.9
3	Riboflavin	398.8 (M+Na), 381.8, 355.8, 319.9, 278.8, 264.9, 239.7, 224.8, 143.9
4	Lumiflavin	294.9 (M+K), 161.0
5	7,8-Dimethylisoalloxazine	280.9 (M+K), 147.0
6	PAR	359.9 (M+H), 189.0, 147.0, 135.0

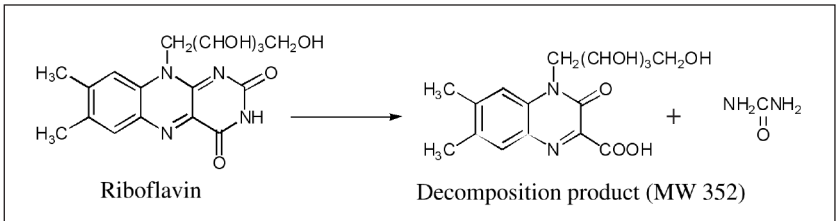

Figure 8. Scheme of riboflavin decomposition reaction.

HPLC quantitative analysis

Calibration curve of riboflavin analysis

A series of concentrations of riboflavin standard solutions (0.4, 0.3, 0.2, 0.1, 0.05, 0.02, 0.001, and 0.0005 mg/mL) were prepared (5) and were sampled under the chromatographic conditions established in this study. The calibration curve was Y = –46.8 + 3.24e4X (Y was defined as peak area, and X was defined as mg/mL), and the related coefficient was 0.9999. The limit of quantitation (LOQ) was 0.5 µg/mL under the signal-to-noise ratio (s/n) of 10.

Precision and recovery of riboflavin

The spiked recovery method was used in the precision and recovery tests, and the results are reported in Table II, which shows that the recoveries ranged between 100.2% and 98.8%, and the repeatability [relative standard deviation (RSD%)] was between 0.5% and 1.9%. The results were satisfactory.

Calibration curve of the major impurities in riboflavin crude product

Calibration curve of PAR analysis. A series of concentrations of PAR standard solutions (0.002–0.8 mg/mL) were prepared and were ana-

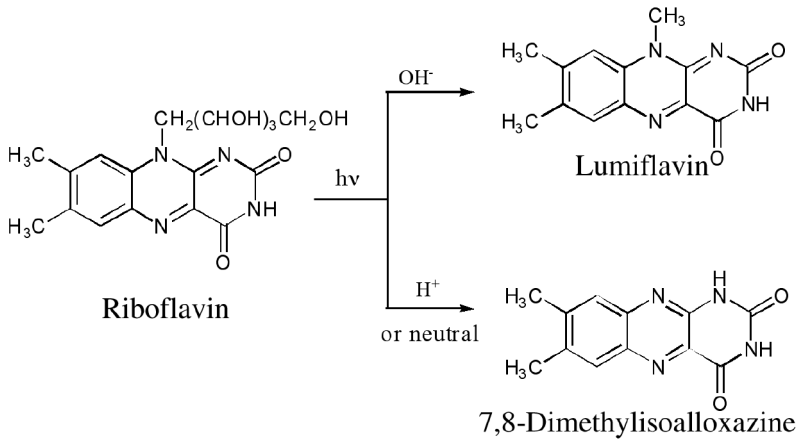

Figure 9. Scheme of riboflavin decomposing to lumiflavin when exposed to light and to 7,8-dimethylisoalloxazine when under alkaline conditions.

Table II. The Precision and Recovery of Riboflavin

Added (mg)	Found (mg) 1	2	3	RSD (%)	Mean found (mg)	Recovery (%)
11.20	11.12	11.08	11.01	0.50	11.07	98.8
8.25	8.33	8.12	8.35	1.5	8.27	100.2
5.79	5.61	5.72	5.83	1.9	5.72	98.8

Table III. Quantitative Analysis of Riboflavin Crude Product

Batch number	Components Riboflavin (%)	1-Deoxy-1-[4, 5-dimethyl-2-(phenylazo) phenylamino]-D-ribitol(%)	Barbituric acid (%)
1	85.64	2.15	4.21
2	72.39	3.76	8.75
3	78.62	2.62	7.59
4	66.83	4.17	5.37
5	68.96	3.93	9.21

lyzed under the chromatographic conditions established in this study. The calibration curve was Y = –169 + 3.66e4X (Y was defined as peak area, and X was defined as mg/mL), and the related coefficient was 0.9997. The RSD was 1.9%, and the LOQ was 1 µg/mL (s/n = 10).

Calibration curve of barbituric acid analysis. Under the chromatographic conditions established in this study, the RSD of barbituric acid analysis was 0.6% at the linear range of 0.001–0.1 mg/mL. The linear related coefficient was 0.9991, and the LOQ was 0.6 µg/mL (s/n = 10).

Quantitative analysis of riboflavin crude product

Five batches of riboflavin crude product (synthesized by our group) were analyzed by the analysis method established in this study. The results are shown in Table III.

Conclusion

In this study, the HPLC–MS analysis method for riboflavin crude product was established. By this method, the components in riboflavin crude product were well separated and identified, respectively. The results of quantitative analysis are accurate, and the method proved to be simple, rapid, and accurate.

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