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응용 자료

UPLC/PDA/MS Analysis of Riboflavin and Related Compounds

Andrew J. Aubin, Katherine Hynes

Waters Corporation

Abstract

This application note will show the UltraPerformance LC (UPLC) separation of these compounds utilizing Photodiode Array detection and Single Quadropole Mass Spectrometry (UPLC/PDA/MS) for compound confirmation.

Benefits

Separation of riboflavin and its degradation products is easily achieved using UltraPerformance LC in less than four minutes

Introduction

Riboflavin (vitamin B_2) is a water soluble B-complex vitamin important in human and animal nutrition. Riboflavin metabolizes into two coenzymes, Flavin Adenine Dinucleotide (FAD) and Flavin Mononucleotide (FMN), and also undergoes photodegradation primarily to 7,8-Dimethylalloxazine (Lumichrome). Previously, HPLC separations of these related compounds have been done in approximately twenty five minutes on 150 mm columns with minimal resolution and poor peak shapes.[1] Separations in less than eight minutes have been achieved with poor resolution between FAD and FMN and indications of co-eluting compounds based on the peak shape.[2] This application note will show the Ultra Performance LC (UPLC) separation of these compounds utilizing photodiode array detection and single quadrupole mass spectrometry (UPLC/PDA/MS) for compound confirmation.

Experimental

UPLC/PDA/MS Conditions

The Waters ACQUITY UPLC System consisted of a Binary Solvent Manager (BSM) and a Sample Manager (SM) equipped with a 5 µL loop. Dual detection was employed using an ACQUITY UPLC Photodiode Array Detector in series with a Waters ZQ 2000 Mass Detector. For system control, data collection, and processing, Waters Empower 2 chromatography data Software was employed.

Linear gradient separation (Table 1) of the four related compounds (and other unknown impurities) was achieved on a 2.1 x 100 mm ACQUITY BEH C_{18} Column containing 1.7 µm particles at a flow rate of 650 µ

L/min at 40 °C. Injections of 3 µL were done using the 'Partial Loop Needle Overfill' mode with the ACQUITY UPLC System. To eliminate carryover, 200 µL of strong wash (1:1 water/acetonitrile) followed by 600 µL weak wash (95:5 water/ acetonitrile) was used. ZQ 2000 mass detector conditions are described in Table 2.

Time	*Profile**	
(min)	*%A*	*%B*
0.0	80	20
2.0	60	40
3.0	1	99
3.5	1	99
4.0	80	20

*Where Solvent A = 3.85 g/L Ammonium Acetate in Water and Solvent B = 3.85 g/L Ammonium Acetate in Water/Acetonitrile 7:3.

Table 1. UPLC linear gradient separation conditions.

ES capillary (kV): 3.60	Source Temp (°C): 350
Cone (V): 30	Cone Temp (°C): 120
Extractor (V): 3	Cone Gas Flow (L/Hr): 50
RF Lens (V): 0	Desolvation Flow (L/Hr): 550
LM Resolution: 14	Scan Time: 0.15 s
HM Resolution: 15	Inter-scan Delay: 0.05 s
Multiplier: 650	Scan Range: 150–900 m/z
Probe: ES^+	

Table 2. ZQ instrument parameters and tune page parameters.

Sample Preparation

Riboflavin, flavin adenine dinucleotide, flavin mononucleotide, lumichrome, and ammonium acetate were obtained from Sigma-Aldrich Co. Acetonitrile (Optima grade) was obtained from Fisher Scientific. Stock solutions (0.5 mg/mL) of each of the standards were prepared in hot 1M sodium bicarbonate. After cooling, mixed working solutions (0.05 mg/mL of each compound) were prepared in 1M sodium bicarbonate at room temperature. All stock and working solutions were then stored in the dark at 4 °C.

Results and Discussion

As can be seen in Figures 1 and 3, the four compounds of interest are well separated in under four minutes with UPLC. Examination of the mass spectral data provided positive identification for the four compounds of interest (Figure 4), with the base peak masses matching the molecular weights of the four compounds. In addition to the four identified compounds, another relatively large unknown compound was also resolved from the mixture. This unknown compound (RT=1.98 minutes) had a UV spectrum that was similar to both FMN and riboflavin as can been seen in Figure 5, indicating that it was likely a related compound. The

unknown compound at 1.98 minutes showed a mass of M+H=439.1 (Figure 6).

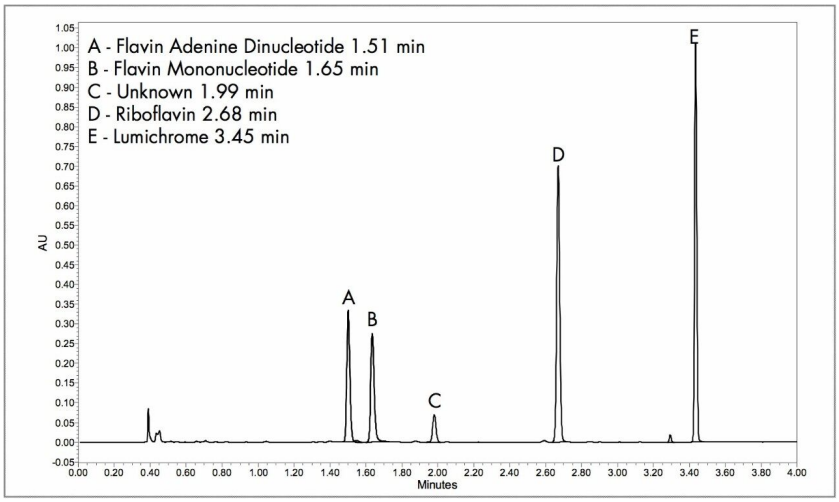

Figure 1. UPLC Separation of Riboflavin, FAD, FMN, and Lumichrome (266 nm).

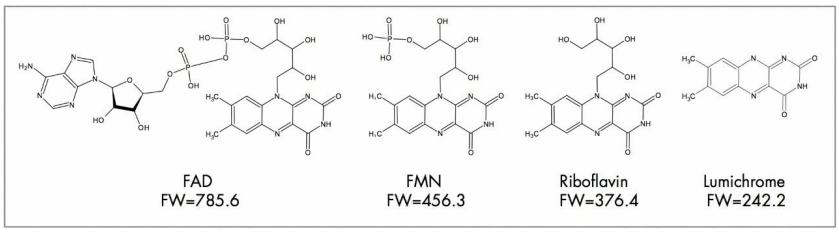

Figure 2. Structures of riboflavin and related compounds.

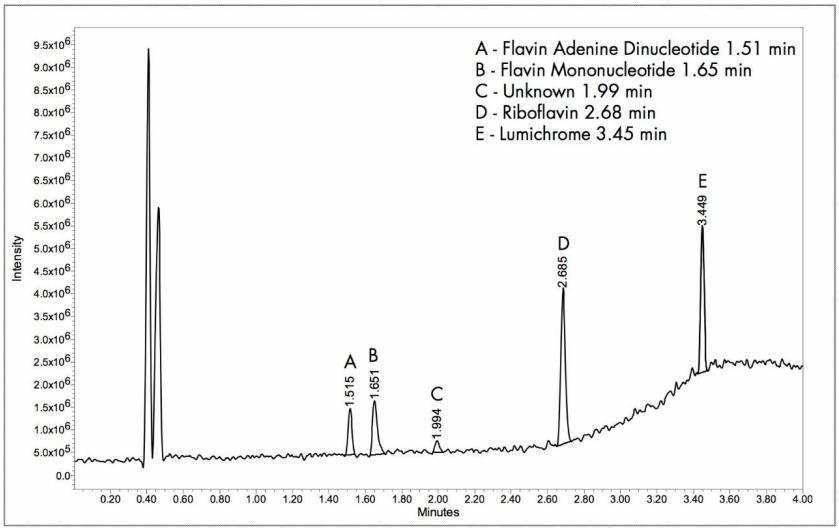

Figure 3. Total ion chromatogram UPLC separation of riboflavin, FAD, FMN and lumichrome.

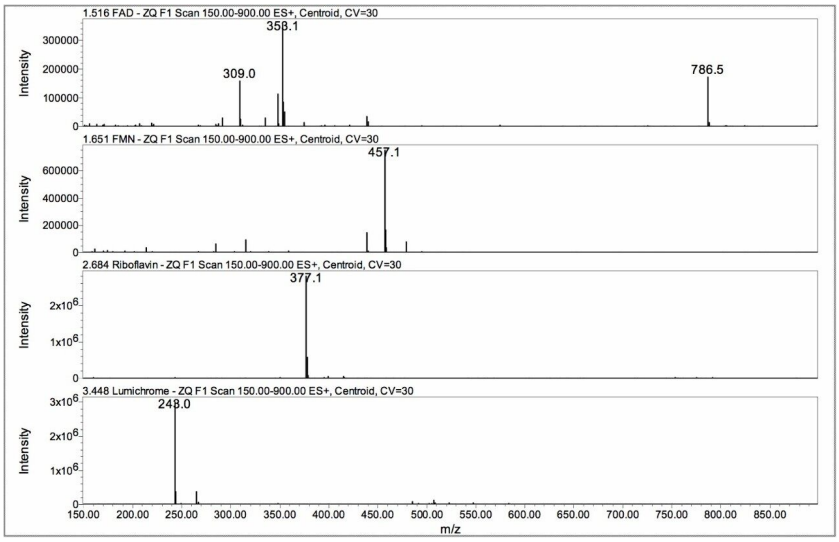

Figure 4. Mass spectra obtained from the ZQ of FAD, FMN, riboflavin, and lumichrome.

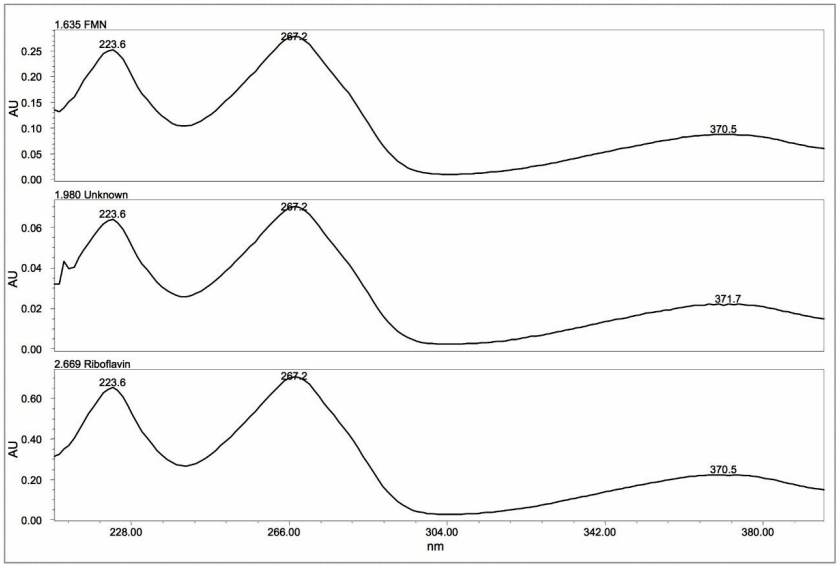

Figure 5. UV spectra of FMN (top), the unknown at 1.98 minutes (middle), and riboflavin (bottom).

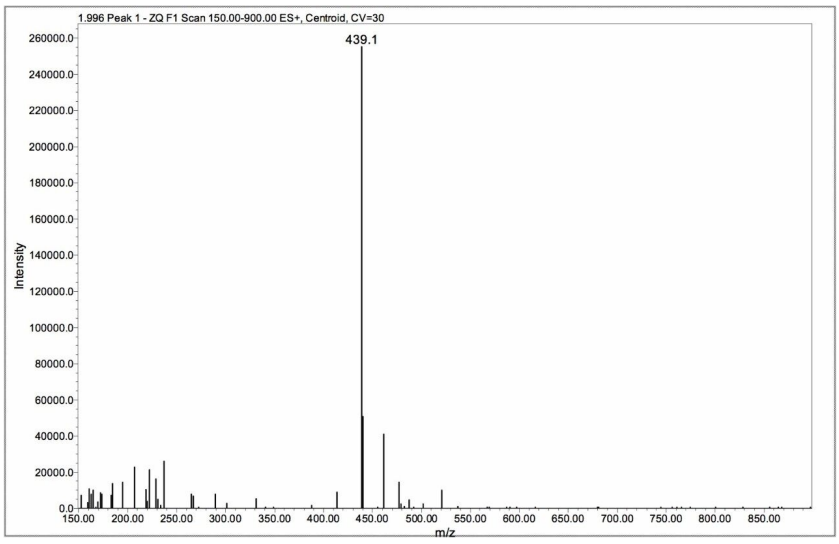

Figure 6. Mass spectrum obtained from the ZQ of the unknown at 1.98 minutes.

The generation of ultraviolet (UV) and mass spectra of unknown peaks provides information that can aid the process of identification. In the case of this assay, it was observed that the unknown UV spectrum was very similar to FMN and riboflavin. The unknown mass was consistent with the loss of water from FMN. Its longer retention time is also indicative of this loss. Further identification of this molecule would require the use of

more advanced techniques such as time-of-flight mass spectrometry (TOF MS) for exact mass information, and MS/MS for structural confirmation.

Further examination of the PDA data also shows many other well resolved unknown related compounds (Figure 7). No attempt was made to identify these compounds, although the small peak (RT=3.29 min) immediately preceding lumichrome had a UV spectra that was very similar to lumichrome (Figure 8), indicating that it is likely related to lumichrome or is possibly another photodegradation product of riboflavin.

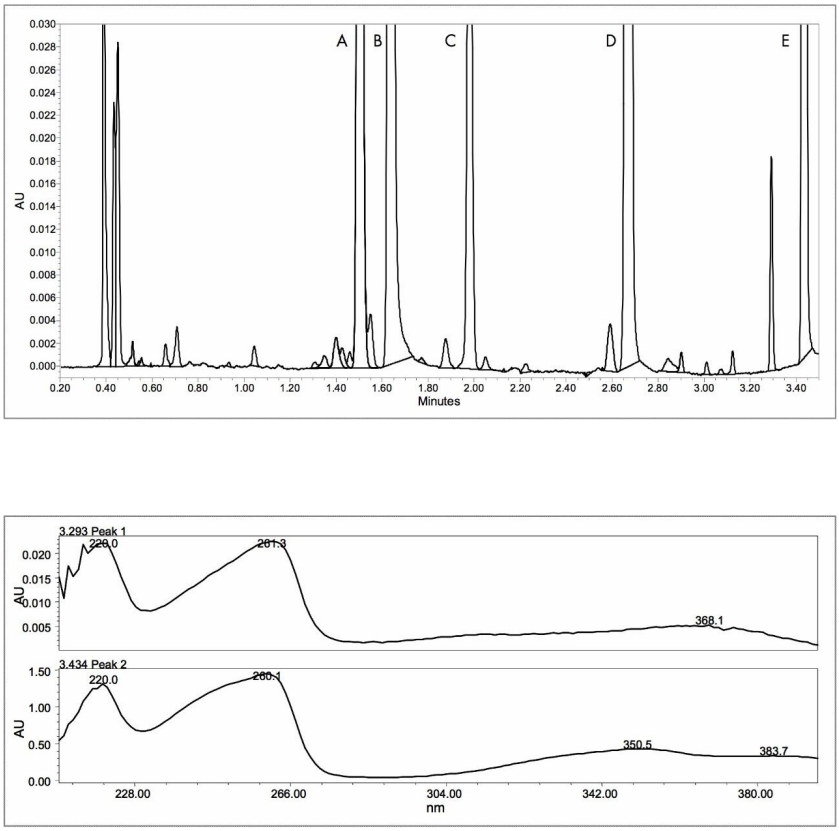

Figure 8. UV spectra of the unknown at 3.29 minutes (top), and lumichrome (bottom).

Conclusion

The separation of riboflavin and its degradation products is easily achieved using UltraPerformance LC in less than four minutes, a vast improvement in speed and quality of data as compared to what could be

previously achieved with traditional HPLC. Additionally, the use of ACQUITY UPLC photodiode array detection in series with single quadrupole mass spectrometry provides a powerful system platform for positive compound identification of known compounds. The use of UPLC/PDA/MS also aided in the initial identification of other related compounds in the mixture.

References

1. *J Chromatogr A*. 2000 Jun 9;881(1-2):285–97.
2. *J Chromatogr B Biomed Sci Appl*. 2000 Feb 28;739(1):219–24.

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ACQUITY UPLC System <https://www.waters.com/1000396>

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