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Nota de aplicación

UPLC-MS Monitoring of Water-Soluble Vitamin Bs in Cell Culture Media in Minutes

Catalin E. Doneanu, Weibin Chen, Jeffrey R. Mazzeo

Waters Corporation

Abstract

In this application note, we adapt a UPLC method coupled with multiple reaction monitoring (MRM) and UV detection method for analysis of 10 water-soluble B vitamins in fresh or spent cell culture media.

Benefits

To improve a biopharmaceutical laboratory's efficiency and productivity by demonstrating a robust, specific, and rapid UPLC/MRM-MS assay for the simultaneous monitoring of 10 water-soluble B vitamins in fresh or spent cell culture media.

Introduction

The increasing demand for protein biopharmaceuticals for therapy, diagnosis, and research has driven manufacturers to maximize protein production yield. Cell culture media is an important ingredient for increasing protein production in biomanufacturing. Therefore the biopharmaceutical industry is continually looking to optimize and efficiently monitor the composition of cell culture media.

All cell culture products contain a complex mixture of polar compounds, rich in amino acids, carbohydrates, inorganic salts and vitamins. Water-soluble vitamins are critical components because they act as catalysts or substrates to facilitate and/or control many metabolic functions during cell growth. Monitoring the concentration of B-complex vitamins during biomanufacturing allows for a better understanding of vitamin uptake by different cell lines. These measurements can be used to optimize protein production conditions in order to increase the yield of the final biopharmaceutical product.

Water-soluble vitamins from cell culture media are typically measured individually using microbiological, spectrophotometric, or titrimetric assays. A single, multi-component analysis for all B-complex water-soluble vitamins is highly desirable because this type of assay can save significant time and resources for the biopharmaceutical industry.

Simultaneous analysis of all water-soluble vitamins in cell culture media is challenging due to the complexity of the sample matrix. In addition, several vitamin B-complex components are generally very hydrophilic and are not retained well on traditional reversed-phase HPLC columns. As a result, vitamin B-complex components are usually analyzed by ion-pairing reversed-phase HPLC.

A UPLC method coupled with multiple reaction monitoring (MRM)[1] and UV detection[2] was recently introduced for rapid analysis of water-soluble vitamins in foods and beverages. The method used a high-

strength silica C_{18} column (HSS T3), a specially designed UPLC column for the retention of polar analytes, to overcome the weak retention of vitamins.

Here, we adapt this method for analysis of 10 water-soluble B vitamins in fresh or spent cell culture media.

Experimental

LC conditions

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC HSS T3 C_{18} 2.1 x 150 mm, 1.8 µm
Flow rate:	300 µL/min
Column temp.:	30 °C
Mobile phase A:	10 mM ammonium formate, 0.1% formic acid in water (pH 3.1)
Mobile phase B:	10 mM ammonium formate, 0.1% formic acid in methanol
Injection vol.:	50 µL (partial loop injection mode)

Gradient

0.0 min	0% B
2.0 min	0% B
12.0 min	35% B

13.0 min	90% B
14.0 min	90% B
15.0 min	0% B

MS conditions

MS system:	Xevo TQ MS
Ionization mode:	ESI+
Capillary voltage:	3.5 kV
Source temp.:	120 °C
Acquisition mode:	MRM
MS1/MS2 mass window:	1 Da (unit mass resolution)
Dwell time:	20 ms

Data management

Target Lynx Application Manager for MassLynx Software

Results and Discussion

Samples

Ten high-purity vitamin standards were obtained from Sigma-Aldrich (St. Louis, MO) for the following vitamins: B1 (thiamine), B2 (riboflavin), B2 phosphate (riboflavin-5'-phosphate), B3 (nicotinamide), B5 (calcium pantothenate), B6 (pyridoxal and pyridoxine), B7 (biotin), B9 (folic acid) and B12 (cyanocobalamin). Stock solutions of vitamins B1, B3, B5, B6s, B7, and B12 were prepared using deionized (Milli-Q) water. For

vitamins B2s and B9 stock solutions were prepared in 100 mM ammonium formate, pH 10, to increase their solubility in water. Sample dilutions in the range of 0.1 to 10,000 ng/mL were prepared using mobile phase A. Throughout the preparation and analysis, all solutions were protected from light exposure and stored below 5 °C.

MRM method

MRM assays were performed on an ACQUITY UPLC System coupled to a Xevo TQ MS tandem quadrupole instrument. Table 1 shows the retention times, MRM transitions, optimized cone voltages, and collision energy values for vitamins analyzed in this study.

Vitamin compound	RT (min)	MRM transition	Cone voltage (V)	Collision energy (eV)
Thiamine (B1)	2.6	265.1 > 122.1	24	17
Riboflavin (B2)	13.8	377.1 > 243.1	42	22
Riboflavin-5'-phosphate (B2)	12.9	457.1 > 439.1	41	18
Nicotinamide (B3)	5.6	123.0 > 80.0	40	20
Calcium pantothenate (B5)	8.8	220.1 > 90.1	30	15
Pyridoxal (B6)	4.8	168.1 > 150.1	27	15
Pyridoxine (B6)	5.6	170.1 > 152.1	28	14
Biotin (B7)	13.3	245.1 > 227.1	28	13
Folic Acid (B9)	12.5	442.1 > 295.1	23	17

Vitamin compound	RT (min)	MRM transition	Cone voltage (V)	Collision energy (eV)
Cyanocobalamin (B12)	12.3	678.3 > 147.1	36	34

Table 1. Optimized experimental parameters for vitamins monitored.

UPLC separation of vitamins

Figure 1 shows that baseline separation of all 10 water-soluble B vitamins can be achieved in a single reversed-phase UPLC run without ion-pairing reagents. In addition, the use of UPLC conditions allows the separation to be completed within a short 8-min gradient.

The chromatographic conditions reported in the experimental section were obtained after systematic optimization of gradient composition and slope, flow rate, column temperature, and injection volumes. The goal of the optimization process was to achieve the best separation for all 10 vitamin standards. Special attention was paid to the separation between vitamin B9 and B12 (see Figure 1) because they are the most challenging to be resolved at the baseline.

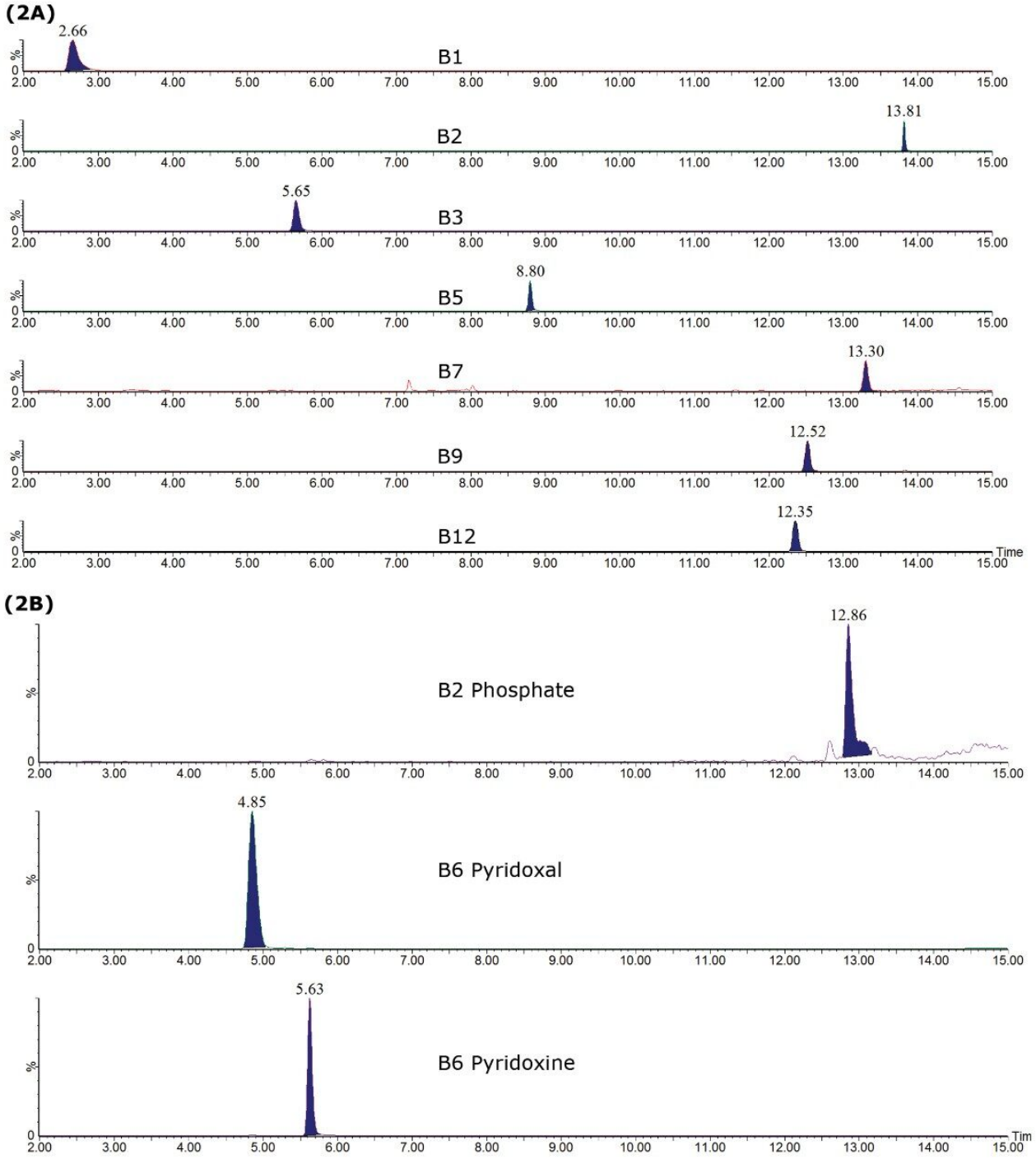

Figure 1. MRM chromatograms of 10 water-soluble B vitamins. (A) Seven B-complex vitamins detected in spent cell culture media; (B) three vitamin standards not detected in the spent media sample: riboflavin-5'-phosphate (13 ng/mL), pyridoxal (200 ng/mL), and pyridoxine (100 ng/mL).

Detection of vitamins by the MRM method

Detection of all vitamin analytes by UV absorbance was initially attempted in a spent cell culture media sample, at three different wavelengths (232, 260, and 278 nm). Figure 2 shows a UV chromatogram recorded at 260 nm, and clearly demonstrates that samples are highly complex. The presence of other interfering species in the spent cell culture media greatly hinders the utility of a UV-only detector method for monitoring vitamins in this chemical background. A more selective and sensitive detection method is clearly needed.

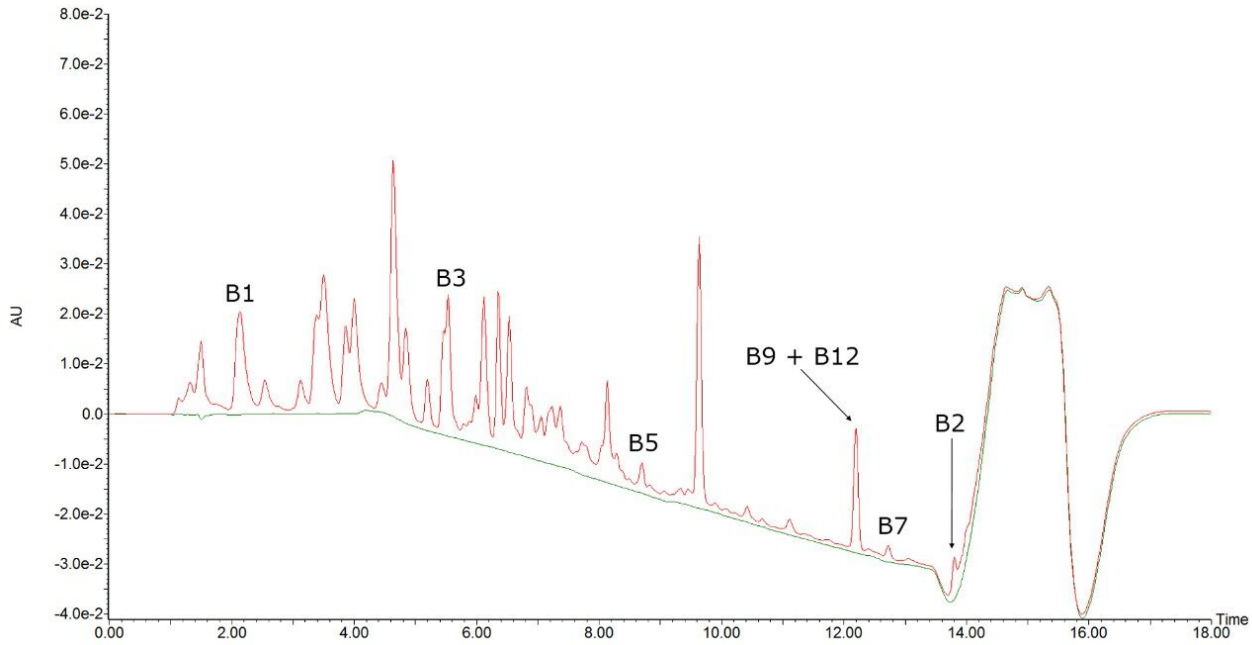

Figure 2. UPLC chromatogram (260 nm detection) acquired for a spent cell culture media sample after 1:50 sample dilution with eluent A. The green trace corresponds to a blank injection analyzed under the same conditions.

A triple quadrupole instrument, operated in MRM mode, is known to provide selectivity and sensitivity that cannot be readily matched by other detectors (e.g., UV, fluorescence), especially in the case of a complex sample matrix as shown here. With the developed MRM method, each of the 10 B-complex vitamins can be confidently identified (Figure 1).

In addition, the MRM method shows very good reproducibility and linearity as illustrated by the calibration curve of vitamin B2 presented in Figure 3. Even in the absence of an internal standard, the MRM response was linear over two orders of magnitude. For concentrations above 10 ng/mL, the peak area RSD (%) were better than 10% for all analytes, indicating the utility of this MRM assay for monitoring the vitamin uptake during protein biomanufacturing.

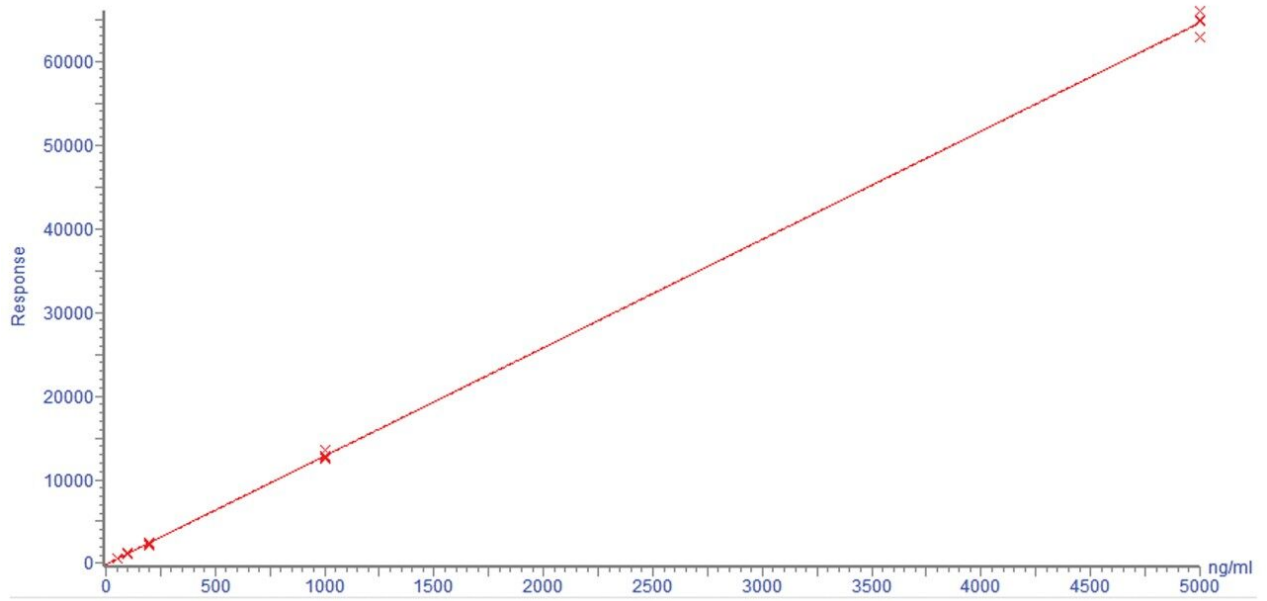

Figure 3. TargetLynx calibration curve obtained for the MRM assay of vitamin B2 in the concentration range of 50 to 5000 ng/mL. The average peak area RSD (%) for this concentration range was 5.6% (n=5).

The limits of quantification (LOQs) determined for all 10 vitamins are displayed in Table 2. These are the lowest analyte concentrations showing a residual plot deviation of 50% or lower.

Vitamin compound	LOQ (ng/mL)
Thiamine (B1)	20
Riboflavin (B2)	50
Riboflavin-5'-phosphate (B2)	13
Nicotinamide (B3)	20
Calcium pantothenate (B5)	20
Pyridoxal (B6)	40
Pyridoxine (B6)	20

Vitamin compound	LOQ (ng/mL)
Biotin (B7)	20
Folic Acid (B9)	50
Cyanocobalamin (B12)	20

Table 2. Limits of quantification (LOQ) found for MIX-10 water-soluble B vitamins assuming a residual plot value below 50%.

Vitamin analysis in spent cell culture media

Table 3 displays the calculated concentrations and peak area RSD (%) obtained for a sample of spent cell culture media when using the UPLC/MRM-MS method described here. These concentrations were measured using the standard addition method, after spiking 100 to 200 ng/mL of each vitamin standard into a spent cell culture media sample diluted 1:50 with mobile phase A.

Vitamin compound	Conc. (µg/mL)	Peak area RSD (%) (n=5)
Thiamine (B1)	7.6	2.5
Riboflavin (B2)	1.5	5.3
Riboflavin-5'-phosphate (B2)	ND	–
Nicotinamide (B3)	9.2	1.1
Calcium pantothenate (B5)	10.7	0.7
Pyridoxal (B6)	ND	–

Vitamin compound	Conc. (µg/mL)	Peak area RSD (%) (n=5)
Pyridoxine (B6)	ND	–
Biotin (B7)	0.2	2.6
Folic Acid (B9)	9.1	3.9
Cyanocobalamin (B12)	5.7	3.1

Table 3. Calculated concentrations and peak area RSD (%) values for 7 water soluble B vitamins measured in spent cell culture media

According to Table 3, the MRM assay generates very good RSD (%) values, better than 5% for all vitamins. It was found that dilution of spent media by 50–100 fold was one of the critical factors in maintaining the robustness of the separation column over hundreds of injections.

Table 4 shows the variance of peak area (RSD, %) for selected vitamins from a spent cell culture media. Each value was calculated based on the integrated peak areas that were continuously acquired over 24 hours for the same spent cell culture media sample (72 injections). The entire experiment was repeated in triplicate using freshly thawed/diluted samples each time. The RSD values observed in this study were less than 10%, demonstrating the robustness of the UPLC/MRM system solution for this application.

Vitamin compound	Experiment 1	Experiment 2	Experiment 3
Thiamine (B1)	4	7.2	5.1
Riboflavin (B2)	10	5.8	4.4

Vitamin compound	Experiment 1	Experiment 2	Experiment 3
Nicotinamide (B3)	4	5	3.4
Calcium pantothenate (B5)	2.6	2.2	1.6
Biotin (B7)	3.9	3.2	3.1
Folic Acid (B9)	2.6	4.3	2.8
Cyanocobalamin (B12)	4.4	2.1	3.2
Biotin (B7)	0.2	2.6	
Folic Acid (B9)	9.1	3.9	
Cyanocobalamin (B12)	5.7	3.1	

Table 4. Peak area RSD (%) for seven water-soluble B vitamins monitored in spent cell culture media over 24 hours (72 injections) during three independent experiments.

Conclusion

A single MRM-based assay for simultaneously measuring 10 water-soluble B vitamins was developed. The assay can be applied for monitoring these analytes in cell culture media.

The assay meets a number of key performance criteria:

- The linearity is at least two orders of magnitude in concentration, with the LOQ lower than the vitamin concentration levels commonly found in cell culture media
- Minimal sample preparation/handling is required and the assay is very appealing for fast, continuous monitoring of vitamin uptake during biomanufacturing
- The UPLC/MRM-MS assay is robust, with peak areas with less than 10% RSD during three replicate experiments, eachconsisting of 72 injections performed over a 24-hour interval.

Biopharmaceutical organizations that adopts UPLC/MRM-MS methodology stands to benefit from gains in efficiency and productivity. Being able to rapidly react to cell culture changes or to prevent cell culture failures can save time and improve product yields, with a direct contribution to the profitability of the organization.

References

1. The Rapid, Simultaneous Analysis of 12 Water-Soluble Vitamin Compounds. Waters Application Note. 2009; 720003052en.
2. Rapid Analysis of 10 Water-Soluble Vitamins, Caffeine, and Six Common Food Dyes using ACQUITY UPLC with UV Detection. Waters Application Note. 2009;720003188en.

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