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Article

Rapid Determination of Water-Soluble Vitamins in Human Serum by Ultrahigh-Performance Liquid Chromatography-Tandem Mass Spectrometry

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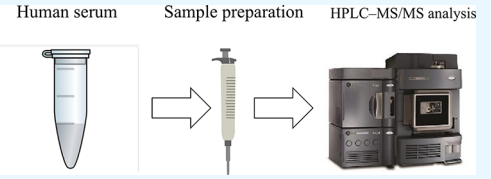
ABSTRACT: Water-soluble vitamins play essential roles in normal body functions and metabolic activities. However, few methods have simultaneously measured all nine water-soluble vitamins in biological matrices. In this study, we developed a sensitive and accurate method for the simultaneous measurement of thiamine (B1), riboflavin (B2), nicotinamide (B3), pantothenic acid (B5), 4-pyridoxic acid (B6), biotin (B7), 5-methyltetrahydrofolic acid (B9), ascorbic acid (VC), and methylmalonic acid (MMA) in human serum. Samples were analyzed using ultrahigh-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) with Waters HSS T_3 and BEH C_{18} columns, each measuring 2.1 × 50 mm with a particle size of 1.7 μm. The mass spectrometer was equipped with an electrospray ionization source, and optimized multiple reaction monitoring was employed to detect all compounds in positive ionization mode. The capillary voltage was set at 0.5 kV. Nitrogen was used as the desolvation gas, with a flow rate of 1000 L/h at 500 °C. Argon was used as the collision gas. The method's performance was validated according to the Clinical and Laboratory Standards Institute guidelines assessing linearity, limit of quantitation, precision, accuracy, carryover, matrix effects, recovery, and dilution. The results confirmed the successful validation of this method for water-soluble vitamins in serum. However, the presence of common endogenous interferences and the type of blood collection tube influenced the results for certain water-soluble vitamins. The results showed that this method was satisfactory, offering significant improvements in analytical performance, including shorter analysis time, higher sensitivity, and the requirement of a smaller sample volume.



1. INTRODUCTION

Water-soluble vitamins, including those from the B-complex group and vitamin C (VC), are crucial micronutrients involved in various metabolic and regulatory processes, primarily functioning as coenzymes or cofactors in material and energy metabolism.[1−3] The body cannot synthesize these essential micronutrients and must obtain them through the diet. Deficiencies or excesses of these vitamins can lead to specific health issues.[4,5] For example, vitamin B1 deficiency leads to neuritis and brain tissue damage,[6] whereas vitamin B2 deficiency negatively affects energy metabolism, redox balance, and lipid metabolism.[7] VC deficiency can result in scurvy, which manifests as anemia, fatigue, gum disease, and easy bruising[8]. It is essential to establish reliable reference values for vitamin concentrations including minimum and maximum thresholds. However, the accurate determination of vitamins is challenging because of their instability and complexities of the matrix.

Diverse methods are used to analyze vitamins, including chromatography, spectrophotometry, microbiology, electrochemistry, and enzymatic assays.[9] Many of these methods are cumbersome and require complex pretreatments to eliminate common interferences. They also often lack specificity for different vitamins. Multiple studies have reported simpler methods for isolating and quantifying vitamins.[10,11] Among these methods, the high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) protocol simplifies sample preparation and has high selectivity and sensitivity for vitamin identification.[12] Although numerous studies focus on determining concentrations of fat-soluble vitamins, particularly 25-(OH)vitamin D2 and 25-(OH)-vitamin D3,[13] fewer studies address the measurement of water-soluble vitamins.

HPLC-MS/MS is a novel technology that provides high sensitivity and selectivity, allowing for the analysis of complex matrices without complex sample preparation.[14] Several studies have highlighted its use for determining vitamins, including water-soluble ones.[14,15] The HPLC-MS/MS method, applied for the determination of vitamin D compounds in serum, improved sensitivity through the use of differential ion

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mobility separation techniques.[16] This method has also been used to quantify vitamins and 16 carotenoids.[17] These studies illustrate the advantages and effectiveness of HPLC-MS/MS for the simultaneous quantitative determination of serum vitamins. However, to the best of our knowledge, few studies have comprehensively investigated all water-soluble vitamins.

To achieve comprehensive and simultaneous determination of nine water-soluble vitamins, B1, B2, B3, B5, B6, B7, B9, MMA, and VC, HPLC-MS/MS was used for the direct quantitative analysis of these vitamins in serum. The main purpose of this study was to establish and validate a method for the reliable and highly sensitive measurement of water-soluble vitamins in serum. This approach allows the simultaneous quantification of all nine vitamins without requiring separate analyses.

2. MATERIALS AND METHODS

2.1. Chemicals and Instruments. Methanol was obtained from J&K Scientific (Beijing, China). Metaphosphoric acid was sourced from Merck (Darmstadt, Germany). Acetonitrile was purchased from Macklin (Shanghai, China). A vacuum centrifugal concentrator (CV100-DNA-H) was acquired from Jiaimu (Beijing, China). The Waters Acquity TQS mass spectrometer, which includes an Acquity UPLC system, was obtained from Waters (Milford, USA).

2.2. Standard and Internal Standard Solutions. Thiamine (B1) and biotin (B7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Riboflavin (B2) was obtained from IsoSciences (Trevose, PA, USA). Nicotinamide (B3) and pantothenic acid (B5) were received from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). 4-Pyridoxic acid (B6), methylmalonic acid (MMA), and ascorbic acid (VC) were acquired from Bepure (Beijing, China). 5-Methyl-tetrahydrofolate (B9) was obtained from Cayman (Michigan, USA). All isotope standards were obtained from Bepure (Beijing, China). The structural formulas for the water-soluble vitamins are listed in Figure 1. VC standard stock solution and internal standard stock solutions were prepared with 0.1% formic acid, and the other vitamins were prepared with 10 mM ammonium acetate. These solutions were stored at −70 °C.

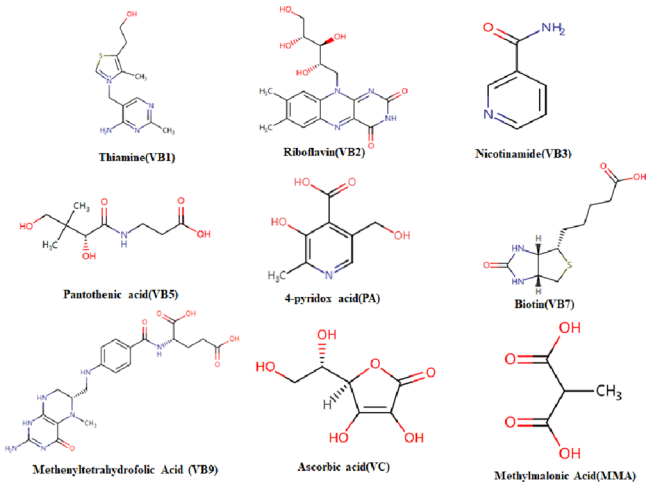

Figure 1. Structural formulas of vitamins B1, B2, B3, B5, B6, B7, B9, MMA, and VC.

2.3. Sample Collection and Preparation. For the preparation of B1, B2, B3, B5, B6, B7, B9, and VC, the following pretreatment method was used under dark conditions. One hundred μL of serum was transferred to a centrifuge tube; 20 μL of an isotope-labeled internal standard was added, and the mixture was vortexed for 30 s followed by the addition of 400 μL of protein precipitation solution (1% formic acid acetonitrile solution). The mixture was vortexed for 3 min and then centrifuged at 12,000 rpm for 5 min at 4 °C. Then, 200 μL of the organic layer was transferred to a new centrifuge tube and evaporated to dryness at room temperature. The sample was reconstituted in 120 μL of a formic acid solvent (0.5% formic acid aqueous solution). After vortexing and mixing for 3 min, the sample was centrifuged at 12,000 rpm for 5 min at 4 °C. Subsequently, 100 μL of the supernatant was transferred to a 96-well sample collection plate, and 5 μL was used for injection and separation.

For preprocessing of MMA, the details are as follows: 100 μL of serum was transferred to a centrifuge tube, 20 μL of an isotope-labeled internal standard was added, and the mixture was vortexed for 30 s followed by the addition of 500 μL of an antiextractant (0.5% formic acid and 99.5% methyl *tert*-butyl ether). The mixture was vortexed for 3 min and then centrifuged at 12,000 rpm for 5 min at 4 °C. Subsequently, 400 μL of the organic layer was transferred to a new centrifuge tube and evaporated to dryness at room temperature under a uniform nitrogen gas flow. One hundred μL of a derivatization reagent (10% acetyl chloride and 90% *n*-butanol) was added, and the mixture was shaken at 65 °C for 20 min and then evaporated to dryness at room temperature under a uniform nitrogen gas flow. The sample was reconstituted in 120 μL of the termination solvent (50% methanol aqueous solution); after vortexing and mixing for 3 min, the sample was centrifuged at 12,000 rpm for 5 min at 4 °C. A 100 μL portion of the supernatant was transferred to a 96-well sample collection plate, and a 10 μL injection volume was used for injection and separation. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki.

2.4. HPLC-MS/MS Analysis. The analytes, including vitamins B1, B2, B3, B5, B6, B7, B9, and VC, were extracted using a Waters HSS T_3 column (1.7 μm, 2.1 × 50 mm; Waters, Milford, USA). Chromatographic separation was performed at 35 °C. Mobile phase A was a mixture of 10 mM ammonium acetate with 0.1% formic acid, whereas mobile phase B was a mixture of 10 mM ammonium acetate in methanol with 0.1% formic acid. The flow rate was 0.40 mL/min, and the sample injection volume was 5 μL. The HPLC-MS/MS analysis was conducted in positive electrospray ionization mode. Mass spectrometry conditions were as follows: a capillary voltage of 0.5 kV, a temperature of 500 °C, a desolvation gas flow rate of 1000 L/h, and a cone gas flow rate of 150 L/h.

For HPLC-MS/MS analysis of MMA, chromatographic separation was conducted at 40 °C. The chromatographic column used was a Waters BEH C_{18} column (1.7 μm, 2.1 × 50 mm; Waters, Milford, MA, USA). Mobile phase A consisted of 10 mM ammonium formate, and mobile phase B consisted of methanol (100%). The flow rate was 0.40 mL/min, and the sample injection volume was 10 μL. The analysis was performed in positive electrospray ionization mode with the following mass spectrometric parameters: a capillary voltage of 3.0 kV, a temperature of 500 °C, a desolvation gas flow rate of 1000 L/h, and a cone gas flow rate of 150 L/h.

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Water-soluble vitamin levels in human serum were measured using the MRM mode. The mobile phase and mass spectrometry parameters mentioned above yielded the highest sensitivity for the respective product ions. The MRM conditions for the nine water-soluble vitamins are summarized in Table 1.

Table 1. MRM Parameters for Nine Water-Soluble Vitamins and Their Deuterated Forms

compounds	parent (m/z)	daughter (m/z)	dwell (s)	cone (v)	collision (v)
B1	265.3	122.1	0.010	30	22
B1-d4	269.3	121.9	0.010	30	22
B2	377.3	243.1	0.020	35	20
B2-d6	383.3	249.2	0.020	35	20
B3	123.0	80.2	0.010	25	22
B3-d4	127.0	84.1	0.010	25	22
B5	220.1	90.1	0.020	30	12
B5-d4	224.2	93.9	0.020	30	12
B6	184.2	148.1	0.010	25	25
B6-d3	187.1	150.0	0.010	25	25
VB7	245.1	227.1	0.020	10	12
VB7-d4	249.1	231.1	0.020	10	12
B9	460.4	313.3	0.020	20	17
B9-d5	465.3	313.3	0.020	20	17
MMA	231.2	119.2	0.05	20	10
MMA-d3	234.2	122.2	0.05	20	10
VC	177.0	95.0	0.010	16	25
VC-d6	183.0	100.0	0.010	16	25

2.5. Method Validation. The method was validated in accordance with guidelines from the Clinical and Laboratory Standards Institute (C62-A and C50-A) and the Ministry of Health of the People's Republic of China (WS/T 492-2016 and WS/T 480-2012). Additionally, it followed relevant documentation from the Clinical Mass Spectrometry Committee of the Chinese Medical Doctors Association of Laboratory Medicine. The validation process included the following parameters: the calibration curve, limit of quantitation (LOQ), precision, recovery, carryover, matrix effect, stability, and dilution in serum.

2.5.1. Calibration. Calibration curves were constructed by calculating the peak area ratio against its concentration. The LOQ was determined by performing repeated injections of low-concentration serum samples near the LOQ and by calculating the standard deviation. The LOQ is defined as the mean value of the determination of samples with the coefficient of variation (CV) ≤20% and bias <15% by repeating the determination of samples close to the detection limit.[18]

2.5.2. Precision and Accuracy. The assay was performed on quality control samples at both high and low concentrations. Each sample was analyzed three times to evaluate intrabatch precision and over a period of 5 days to assess interbatch precision. Accuracy was determined based on intra- and interbatch precision and was expressed as bias (%). For all levels of quality control, both intra- and interbatch CVs should not exceed the allowable laboratory imprecision. That is, the total allowable error (TEA) within intrabatch is CVs ≤ 20%, and TEA within interbatch is CVs ≤ 6.66%.[19]

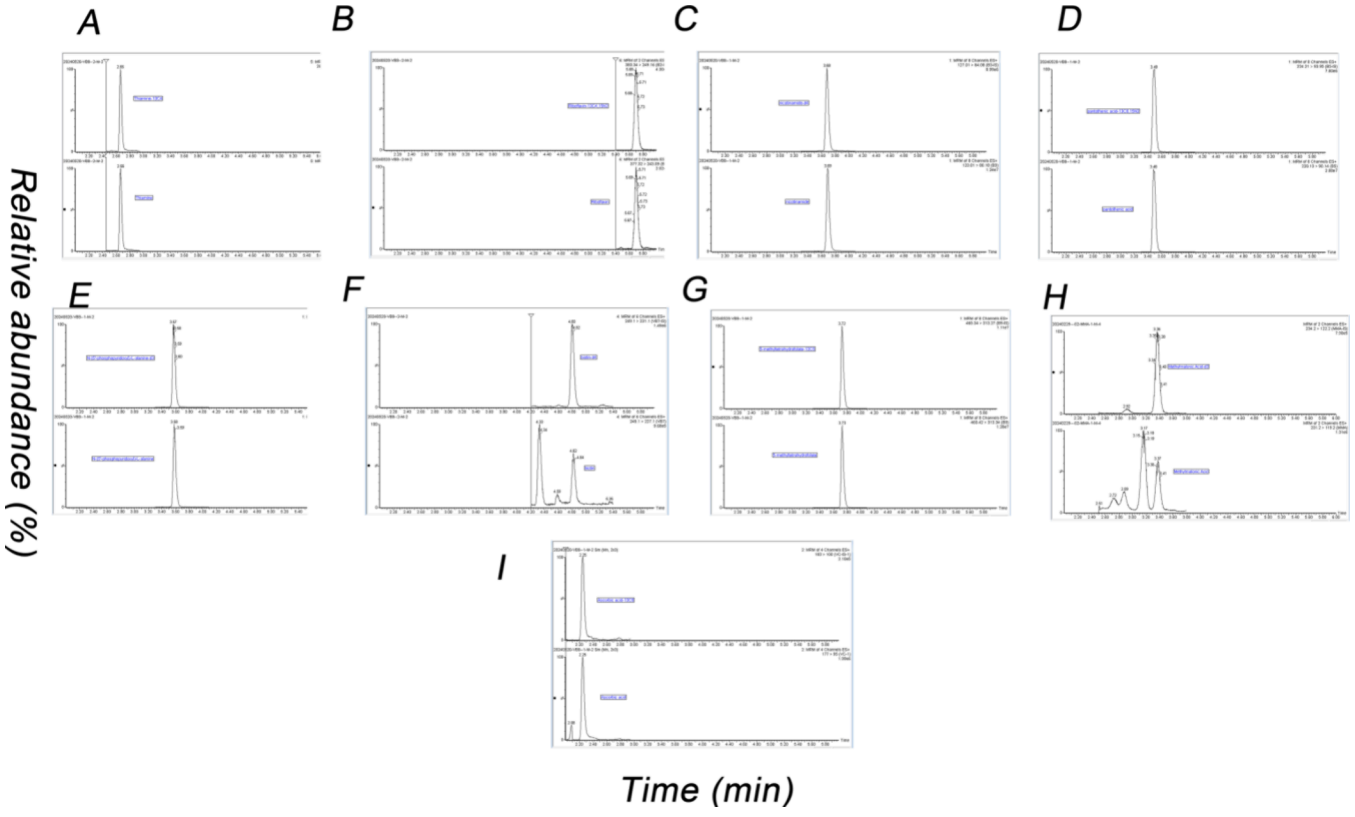

Figure 2. Chromatograms of nine water-soluble vitamins and their corresponding internal standards detected simultaneously using the HPLC-MS/MS method. (A–I) Chromatograms of B1, B2, B3, B5, B6, B7, B9, MMA, and VC.

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Table 2. LOQs and Linear Ranges of Water-Soluble Vitamins

compounds	LOQ (ng/mL)	linear range (ng/mL)	regression equation	R^2
B1	1.00	2.00−30.00	$y = 1.008x - 0.1222$	0.9997
B2	1.25	2.50−35.00	$y = 0.9987x + 0.1959$	0.9999
B3	4.00	8.00−120.00	$y = 0.997x + 0.7538$	0.9998
B5	1.875	15.00−225.00	$y = 1.0127x + 0.5014$	0.9999
B6	1.00	4.00−60.00	$y = 0.9886x + 0.3286$	0.9999
B7	0.04	0.08−1.20	$y = 0.9936x + 0.0155$	0.9979
B9	2.50	5.00−75.00	$y = 0.989x + 0.4323$	0.9993
MMA	2.50	10.00−400.00	$y = 0.9818x + 4.6792$	0.9989
VC	0.25 μg/mL	2.00−30.00 μg/mL	$y = 1.006x - 0.0112$	0.9999

Table 3. Validation of Precision and Recovery

	intrabatch precision (CV%)		interbatch precision (CV%)		
vitamins	high concentration	low concentration	high concentration	low concentration	recovery (%)
B1, thiamine	1.58	2.20	2.20	2.23	104.13
B2, riboflavin	1.95	3.00	1.46	4.20	110.51
B3, nicotinamide	3.89	2.96	2.61	1.36	87.24
B5, pantothenic acid	1.35	1.10	0.91	1.02	100.39
B6, pyridoxamine	1.45	2.45	1.68	1.59	110.29
B7, biotin	4.47	5.78	4.60	7.50	91.39
B9, folic acid	2.00	3.03	1.98	3.42	107.29
MMA, methyl methacrylate	4.21	5.29	3.39	5.09	95.78
C, ascorbic acid	1.16	1.38	2.03	1.63	100.25

2.5.3. Interference. Interference studies were conducted by adding potential interfering substances at five different concentrations to the serum pools. The substances tested included hemoglobin, bilirubin, and lipids. Significant interference was defined as a change >10% deviation from the threshold value.[20] The relative bias for each sample was calculated based on the observed and baseline values. Figures and tables were used to illustrate the effects of these common interferences.

2.5.4. Carryover. Carryover reflects the level of contamination between the samples. To evaluate carryover, measurements were repeated for a low-concentration sample, alternating between high- and low-concentration samples. The carryover was assessed by comparing the results of the first measurement of the low-concentration sample to subsequent measurements. If the carryover was less than the predetermined standard (20%), then it was deemed acceptable for determining samples at high concentration and subsequent concentrations.

2.5.5. Matrix Effects and Recovery. Matrix effects and recovery were studied following the method described by Matuszewski et al.[21] Three sets of six samples each were prepared at three different concentration levels. Sets A and B were prepared with blank matrices spiked before and after extraction, respectively. Set C was prepared using neat mobile phase A. The matrix effect was measured as the ratio of the peak area in set B to the peak area in set C. The recovery rate was determined by averaging the response of analytes and internal standards across all three sets. No significant matrix effects were considered to exist when the response values of the mixture samples showed deviations <20% at low concentrations and <15% at medium and high concentrations.

2.5.6. Dilution. Dilution was assessed by adding concentrations near the upper limit of the analytical measurement range to all of the compounds. The samples were diluted at various folds with the corresponding blank matrices and then analyzed for accuracy. Each sample was analyzed three times. The assay values for each dilution of the samples were expressed as mean values. Dilution integrity was validated when the accuracies ranged from 80 to 120%.

2.5.7. Comparison of Blood Collection Tubes. Two types of blood collection tubes were used in this study: white-capped tubes (additive-free tubes, Lingen Precision Medical Products (Shanghai, China) Co., Ltd.) and powdery-capped tubes (procoagulant-added tubes, Lingen Precision Medical Products (Shanghai, China) Co., Ltd.). Eleven healthy volunteers were enrolled to compare the effects of these different collection tubes on water-soluble vitamins. Serum samples were prepared using validated standard operating procedures. To assess the effect of the tube type, the concentrations of water-soluble vitamins in the serum samples from the different types of collection tubes were compared. The results are presented as ratios in the diagram.

3. RESULTS AND DISCUSSION

3.1. HPLC-MS/MS Analysis. Vitamins are endogenous substances, and therefore, we used an equivalent human matrix for labeling. Based on existing methods, the serum was extracted using formic acid and acetonitrile. All vitamin analytes were then separated by using high-performance liquid chromatography.

Simultaneous and rapid quantitative analysis of blood samples for water-soluble vitamins was performed by using the XEVO TQS system. Figure 2A−I displays the chromatograms of the nine water-soluble vitamins in serum detected by using the HPLC-MS/MS method. The elution time for these vitamins was reduced to 5 min, which is shorter than that reported in a previous study.[15] The method offers the advantages of requiring a small sample volume (i.e., 100 μL) and simplified sample preparation. The method used formic acid acetonitrile solution for sample extraction of eight water-

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Table 4. Measurements of Water-Soluble Vitamins Using HPLC-MS/MS in the Presence of Interfering Substances

(a) Hemolysis									
concentration of interferents (mg/dL)	B1	B2	B3	B5	B6	B7	B9	MMA	C
10	−0.67	1.06	−4.87	−1.39	−1.71	25.00	6.12	12.74	−2.21
50	−1.11	0.14	−4.90	−1.61	−2.77	50.00	0.95	15.18	−5.15
100	−2.90	−1.88	−7.93	−2.36	−4.90	75.00	15.07	5.29	−2.21
250	−0.45	1.79	−7.19	1.37	−4.05	91.67	−96.13	−0.68	55.51
500	−2.23	4.06	−5.53	8.22	−2.56	170.83	−95.92	1.14	−88.24
(b) Bilirubin									
concentration of interferents (mg/dL)	B1	B2	B3	B5	B6	B7	B9	MMA	C
2	−3.94	−2.60	−3.86	−4.29	−4.02	−4.70	−4.51	−6.25	−2.75
5	−4.61	−3.21	−5.32	−4.86	−4.35	−4.94	−4.29	−7.91	−4.07
10	−4.20	−4.16	5.77	−5.64	−3.93	−10.80	−3.56	−12.21	−7.01
20	−4.04	−2.67	−6.41	−6.26	−4.87	−9.39	−3.91	−53.66	−4.49
30	−3.62	−6.06	−6.67	−7.19	−5.13	−9.32	−3.72	−4.94	−4.98
(c) Lipids									
concentration of interferents (mg/dL)	B1	B2	B3	B5	B6	B7	B9	MMA	C
150	1.91	4.40	−0.05	−0.07	1.76	9.32	2.16	−12.02	1.91
750	−0.29	−2.04	−1.71	−2.68	0.06	11.94	0.92	−21.34	−1.33
1500	0.84	2.04	0.28	−1.82	0.79	11.93	1.58	−32.78	−2.27
2250	−0.37	2.57	−1.28	−2.48	0.52	10.99	0.98	−35.82	−1.01
3000	−2.85	1.13	−3.15	−4.03	−1.56	27.56	−1.23	−23.70	−5.43

soluble vitamins. The sample extraction of MMA was performed using an antiextractant.

The pretreatment method is simple and low cost and has higher recovery rates.[22] Additionally, after 200 consecutive injections of different serum samples, no change was observed in the peak patterns of the compounds. Moreover, this method causes minimal damage to the instruments during testing. It provides significant advantages over previously reported methods in terms of the blood processing volume, overall analysis time, and sensitivity and can be accurately quantify vitamins at physiological and clinical deficiency levels.[22,23]

3.2. Method Validation. *3.2.1. Linearity and LOQ.* The LOQs were determined according to the sample pretreatment protocol, with the LOQs set at the level of the lowest calibrator. Table 2 presents the calibration curves established by plotting the peak area ratios of the analytes against their corresponding internal standard peak areas. The method demonstrated an excellent linear range, with correlation coefficients (R^2) ranging from 0.997 to 0.999 for the vitamins. The LOQs for B1, B2, B3, B5, B6, B7, B9, VC, and MMA were 1.00, 1.25, 4.00, 1.875, 1.00, 0.04, 2.50, and 0.25 μg/mL and 2.50 ng/mL, respectively. These values are consistent with those of other published techniques, indicating that our assay data are reliable.[22]

3.2.2. Precision and Accuracy. Table 3 summarizes the intra- and interbatch precision and accuracy results for all water-soluble vitamins. The high and low concentrations for each vitamin are summarized in Table S1. The intra- and interbatch CVs ranged from 0.91 to 7.50% for all analytes, and the recovery ranged from 87.24 to 110.51%. These results meet the acceptance standards for precision and accuracy, indicating that accuracy is sufficient for subsequent assays.

3.2.3. Assay Interferences. In the assay for water-soluble vitamins, potential interfering substances, such as hemoglobin (up to 500 mg/mL), bilirubin (up to 30 ng/mL), and lipids (up to 3000 ng/mL), were tested, as shown in Table 4. Significant interference was observed in the measurements of B7, B9, and VC in hemolyzed samples (Figure 3A).

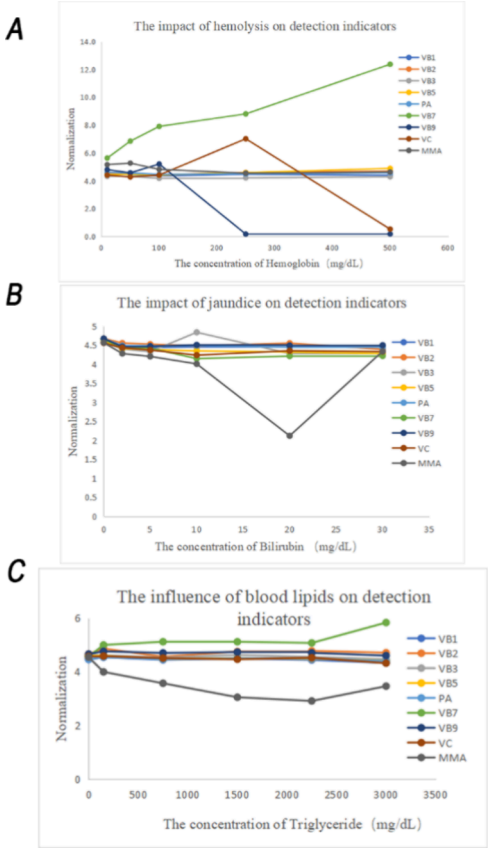

Figure 3. Assessment of interference caused by common endogenous substances: (a) hemolysis, (b) bilirubin, and (c) lipids.

Specifically, the presence of hemoglobin led to an increased response for B7 at elevated concentrations. When hemoglobin concentration exceeded 250 mg/dL, the assay result for B9 approached zero and the VC measurement first increased and then decreased to near zero with a rising of hemoglobin levels. Additionally, significant interference with MMA detection was

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observed in serum samples, particularly with bilirubin and lipids (Figure 3B,C). In summary, certain endogenous substances can significantly interfere with the detection of some water-soluble vitamins, highlighting the need for clinical vigilance and timely detection.

3.2.4. Carryover. Carryover for the nine water-soluble vitamins was assessed in serum (Table S2). VB3 had the highest carryover rate at 14.93%. The carryovers for all analytes met the acceptance criteria.

3.2.5. Matrix Effects and Recovery. The spiking levels used in the recovery experiment for each vitamin are presented in Table S3. The matrix effect data for all analytes are summarized in Table S4. No significant matrix effects were observed at low, medium, or high concentrations. The average extraction recoveries at different concentrations and the mean absolute recoveries for all analytes ranged from 86.79 to 112.10%. The validation results confirmed that there were no significant matrix effects in the serum samples.

3.2.6. Dilution. The recoveries of vitamins B1, B2, B3, B5, B6, B9, MMA, and VC for 8-fold diluted serum samples ranged from 80 to 120%, meeting the criterion (Table S5). The recovery of B7 was 113.9% for 4-fold diluted serum and 129.6% for 8-fold diluted serum samples, respectively. The maximum dilution for B1, B2, B3, B5, B6, B9, MMA, and VC within the clinically reportable range was 8-fold, and the maximum dilution for B7 was 4-fold. If the sample concentrations exceed the limit of the analytical measurement range, then they can be reanalyzed after appropriate dilution.

3.2.7. Influence of Different Blood Collection Tubes on Water-Soluble Vitamins. The impact of the tube type on the measurement of water-soluble vitamins was evaluated. A significant difference in vitamin levels was observed between the two types of collection tubes (Figure 4). This suggests that

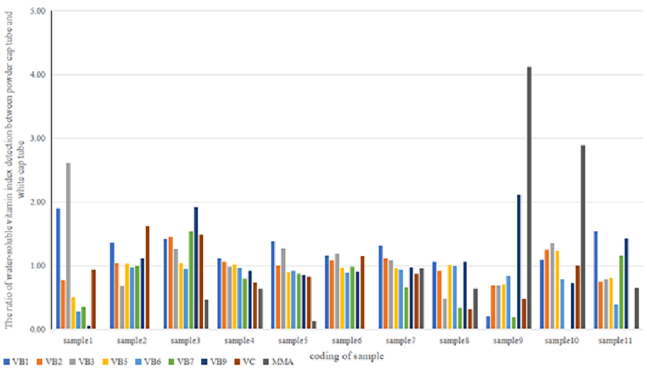

Figure 4. Ratio of water-soluble vitamins measured using white-capped versus powdery-capped tubes.

the presence of procoagulants can affect the detection of water-soluble vitamins in serum. Therefore, using additive-free tubes is recommended for accurate water-soluble vitamin testing in clinical practice.

4. CONCLUSIONS

In conclusion, we have developed an efficient and sensitive HPLC-MS/MS method for the determination of nine water-soluble vitamins in serum. This method features a simple sample pretreatment protocol and is suitable for large-scale clinical sample analysis.

■ ASSOCIATED CONTENT

Data Availability Statement

Data in the study are available from corresponding authors on request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c07968.

(Table S1) Concentration, (Table S2) carryover, (Table S3) spiking levels, (Table S4) matrix effects (ME) and recovery, and (Table S5) dilution (PDF)

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Notes

The authors declare no competing financial interest.

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