Simultaneous Determination of Water-Soluble Vitamins (Vitamin B₁, B₂, B₃, B₆ and C) in Dietary Supplements by High-Performance Liquid Chromatography

Hee-Jae Suh and [†]So Hee Kim^{*}

Dept. of Food Science, Sun Moon University, Asan 336-708, Korea

^{}National Institute of Food and Drug Safety Evaluation, Cheongwon 363-951, Korea*

영양보충용 식품 중 수용성비타민(Vitamin B₁, B₂, B₃, B₆ and C)의 HPLC를 이용한 동시분석법

서 희 재 · [†]김 소 희^{*}

선문대학교 식품과학과, ^{*}식품의약품안전평가원 첨가물 포장과

Abstract

시중에 유통 중인 영양보충용 제품의 수용성 비타민 B₁(thiamin), B₂(riboflavin), B₃(nicotinic acid and nicotine amide), B₆(pyridoxine), C(ascorbic acid)의 신속한 동시분석 방법을 확립하기 위하여 본 연구를 실시하였다. 영양보충용 제품은 정제, 연질캡셀, 분말, 액상의 4가지 제형에 대해 27종의 제품을 구입한 후, Ion-pair 분리기법을 사용하여 HPLC-UVD를 이용한 동시분석 방법을 검토하였다. 비타민 B₁, B₂, B₃, B₆, C의 HPLC에 의한 동시분석 조건을 검토한 결과, 이동상은 0.02% triethylamine, 17.5% 메탄올, 5 μ M sodium hexanesulfonic acid가 함유된 pH 3.5(acetic acid로 조절)의 수용액을 사용하였고, 용출시간은 다른 피크의 영향을 받지 않도록 30분으로 하였다. 수용성 비타민의 회수율은 96% 이상이었다. 본 연구에 의해 확립된 수용성 비타민의 동시분석 조건은 검량선의 직선성, 정밀성, 정확성, 기기적합성 등이 USP 및 ICH 기준에 적합하여 HPLC의 동시분석 방법으로 합당하였다. 수용성 비타민의 추출 용매는 제형에 따라 약간의 차이를 보이긴 했으나, 물이나 산성조건을 갖춘 HPLC의 이동상이 에탄올이나 메탄올보다 높은 추출 효율을 보였다. 초음파 추출기에 의한 추출 시간은 20분이 가장 적당하였다. 본 연구의 결과는 수용성 비타민의 신속한 추출 및 분석에 매우 효율적으로 이용될 것으로 기대된다.

Key words: dietary supplement, water-soluble vitamin, simultaneous determination, HPLC

INTRODUCTION

Vitamin performs a wide variety of function in human body and is essential for good health (NIH 2001). For example, vitamin B₁ (thiamin) works as a cofactor for the metabolism of carbohydrates and needs for nerve transmission. Vitamin B₂ (riboflavin) works a function as a coenzyme for a wide variety of enzymes in the intermediate metabolism (Lynch & Young 2000). Vitamin B₃ (niacin and its amide) is very helpful for

lowering high cholesterol level and elevating high-density lipoprotein cholesterol level (HDL) (NIH 2001). Vitamin B₆ (pyridoxine) is need for more than 100 enzymes involved in protein metabolism and essential for red blood cell metabolism, nervous and immune systems (NIH 2001). Vitamin C (ascorbic acid) can protect tissues and cells against oxidative damages by free radicals (Morisaki & Ozaki 1996). Individuals with a poor quality diet or an inadequate vitamin intake for an extended period may benefit from taking a vitamin supplement if they are

[†] Corresponding author: So Hee Kim, National Institute of Food and Drug Safety Evaluation, Cheongwon 363-951, Korea. Tel: +82-43-719-4351, Fax: +82-43-719-4352, E-mail: mrsksh@kfda.go.kr

unable to increase their dietary intake of vitamin (Leklem 1999; NIH 2001). Nowadays, various dietary supplements in the form of tablet, powder, capsule and liquid are available in the market.

In order to determine vitamin concentration in foodstuff, fluorometric, spectrometric, titrimetric, gas chromatographic and high-performance liquid chromatographic methods have been described, but some of these methods are currently rarely used (Albalá-Hurtado et al. 1997). High-performance liquid chromatographic methods (HPLC) to be developed for the determination of water-soluble vitamins in various foods have enabled rapid, specific and sensitive analysis. Recently, several ion-pairing chromatographic methods using reversed phase column have been reported to determine of water-soluble vitamins, such as infant milk (Viñas et al. 2003), baby foods (De Beer et al. 2003), multivitamin preparations (Lam et al. 1984) and liquid tonics (Maeda et al. 1989).

However, no references are available for the simultaneous determination of water-soluble vitamins in dietary supplements. In this study, we have developed a rapid and precise method for simultaneous determination of water-soluble vitamins in tablet, powder, capsule and liquid dietary supplements. The separation of water-soluble vitamins such as vitamin B₁ (thiamin), vitamin B₂ (riboflavin), vitamin B₃ (nicotinic acid and nicotine amide), vitamin B₆ (pyridoxine) and vitamin C was optimized using ion-pair HPLC method with a reversed phase column and an UV detection.

MATERIALS AND METHOD

1. Reagents and Solvents

Methanol, ethanol and water were of HPLC grade (Merck, Darmstadt, Germany). Acetic acid was of reagent grade. Sodium hexane-sulfonic acid and triethylamine were supplied by Sigma-Aldrich (St. Louis, MO, USA). Standards of thiamin hydrochloride, riboflavin, pyridoxine hydrochloride, nicotine amide, nicotinic acid and ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Standards solutions were prepared as follows; 1, 5, 10, 50 and 100 $\mu\text{g}/\text{mL}$ of thiamin hydrochloride, riboflavin, pyridoxine hydrochloride, nicotine amide, nicotinic acid and ascorbic acid in mobile phase. All standard solutions were filtered through a 0.45 μm membrane (Millipore, Bedford, MA, USA), stored in a dark and cool space (at 4°C in a refrigerator).

The mobile phase containing 0.02% triethylamine was prepared

as described below;

(1) Reference solution: (a) Adjust pH of the aqueous solution to 3.5, 5.0 and 6.5. (b) Add 1, 5 and 10 μM of ion-pairing reagent (sodium hexane-sulfonic acid) to the aqueous solution. (c) Add 12.5, 17.5 and 22.5 mL of LC grade methanol in 100 mL aqueous solution containing 0.02% triethylamine.

(2) Working mobile phase: The mobile phase was prepared to mix 0.02% triethylamine, 5 μM sodium hexane-sulfonic acid and 17.5% methanol in LC grade water adjusted to pH 3.5 with acetic acid. Filter the aqueous solution through a 0.45 μm membrane (Millipore, Bedford, MA, USA) before HPLC injection.

2. Apparatus

The pH of the mobile phases was measured with a pH 210 (Hanna, Italy) pH meter, equipped with a HI 1131B pH glass electrode. The ultrasonic processor power sonic 420 (Whasin, Korea) was used for extraction of vitamins from the samples.

The HPLC system was operated on a Shiseido Nanospace SI-2 separation module (Tokyo, Japan) equipped with Shiseido Nanospace SI-2 UV/VIS detector controlled by Millennium 32 chromatography manager data acquisition system. The column used for the analysis was a Capcell-Pak C₁₈ MG Column (150 $\times$ 3.0 mm, 5 μm , Shiseido, Tokyo, Japan), used for separation at 40°C. The flow rate was 500 $\mu\text{L}/\text{min}$. The detector wave-length set at 254 nm.

3. Sample Preparation

The dietary supplements were presented in the form of powder, liquid, tablet and capsule. In order to improve the extraction efficiency of vitamins, different types of dietary supplements has been prepared as following method. (a) Powder and liquid form samples: Powder and liquid samples were weighed as amount 10 to 50 $\mu\text{g}/\text{mL}$ as water-soluble vitamins (based on the Nutrition Facts of the product) into four 50 mL volumetric flask. Then, 10 mL of four types of solution (mobile phase, water, ethanol and methanol) were added into the flask, respectively. The four flasks were protected from light by covered with aluminum foil and the mixtures were thoroughly extracted for 1, 10, 20, and 30 min at ultra sonic bath. The extracts were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) before HPLC analysis. A sample blank and control sample were included in each analytical schedule. (b) Other types of samples: The Korean Food Code stipulates that the experimental method for net contents must be performed with collected twenty tablets

or capsules in order to secure the representativeness of the sample (KFDA 2000). Thus this study conducted the experiment with twenty samples collected, including soft and hard capsules. Twenty sample tablets were weighed, ground to fine powder, then, sample preparations were as described for powder and liquid type samples. Each twenty soft and hard capsules were collected, removed the capsules, and homogenized the contents. The samples homogenized were weighed the amount of 10 to 50 $\mu\text{g}/\text{ml}$ as water-soluble vitamins. Then, sample preparations were as described for powder and liquid type samples.

RESULTS AND DISCUSSION

1. Analytical Characteristics of HPLC Performance

The pH, the concentration levels of ion-pairing reagent and the proportion of methanol in mobile phase were adjusted to check the optimum conditions for water-soluble vitamin analysis.

The pH of the mobile phase was a highly important factor for the separation of water-soluble vitamins, as previous reports by Maeda et al. (1989) and Lam et al. (1984). The studies of ion-pairing reagent in mobile phase were necessary to improve resolution of B group vitamins (De Beer et al. 2003).

Because reversed phase column was unstable under basic condition, mobile phase was adjusted pH 3.5, 5.0 and 6.5 with acetic acid, respectively. The results were obtained as shown in

Fig. 1. The separation of vitamin B₁ (thiamin), vitamin B₂ (riboflavin), vitamin B₃ (nicotinic acid and nicotine amide) and vitamin C was accomplished successfully in all pH condition. However, the retention time for vitamin B₆ (pyridoxine) in both pH 5.0 and 6.5 was too short and the retention time for vitamin B₂ (riboflavin) was too long compare to pH 3.5 condition. Therefore, optimum pH in mobile phase appeared pH 3.5 for analyzing of water soluble vitamins.

Sodium hexane-sulfonic acid was used as an ion-pairing reagent. The concentration of sodium hexane-sulfonic acid were varied as 1, 5 and 10 μM and the resolution capacity of each peak was assessed as shown in Fig. 2. In case of 1 μM concentration of sodium hexane-sulfonic acid, the separation of vitamins except vitamin B₁ (thiamin) was completed. But, because the retention time was great short, it might be overlapped with other substances such as organic acid. All vitamins were successfully separated in 5 μM and 10 μM concentration and the retentions time were within 20 minutes and 25 minutes, respectively. Therefore, taking both time-saving and sensitivity into consideration, 5 μM concentration of sodium hexane-sulfonic acid in mobile phase was most appropriate.

The proportion of methanol in mobile phase was also an essential factor to improve resolution in water-soluble vitamin analysis (Lam et al. 1984; Maeda et al. 1989). This study was obtained the results shown in Fig. 3, as analyzing all types of

Fig. 1. Chromatogram of mobile phase adjusted by pH.

Fig. 2. Effect of concentration of sodium hexane-sulfonic acid in mobile phase on separation of water soluble vitamins.

Fig. 3. Chromatogram of mobile phase adjusted by methanol concentration.

Table 1. Retention time of mobile phase as affected by pH, ion-pairing reagent and solvent

Conditions	Vitamin B ₁	Vitamin B ₂	Nicotinic acid	Nicotinamide	Vitamin B ₆	Vitamin C
pH						
3.5	15.10	17.99	1.95	2.66	5.48	1.40
5.0	10.88	22.35	1.99	2.47	4.83	1.19
6.5	8.93	21.22	1.00	2.43	3.91	1.09
Ion-pairing reagent (μ M)						
1	4.64	17.66	1.87	2.33	3.24	1.44
5	14.71	17.91	1.95	2.64	5.34	1.41
10	17.31	24.48	2.02	2.84	6.06	1.40
Methanol (%)						
12.5	29.40	41.58	2.41	3.68	9.14	43.28
17.5	14.85	17.14	2.10	2.54	5.38	1.46
22.5	8.43	9.28	1.97	2.24	3.89	1.25

vitamins adjusted the methanol concentration 12.5%, 17.5% and 22.5% respectively. Each vitamin was separated in all methanol concentration. The retention times of vitamin B₁ (thiamin) and vitamin B₂ (riboflavin) in 12.5% methanol concentration were 30 minutes and 45 minutes, respectively, which were much longer time. The retention time of vitamins in 22.5% of methanol concentration was too fast within 10 minutes, which were inappropriate as a simultaneous analysis condition for 5 types of water-soluble vitamins. Thus, it concluded that 17.5% methanol concentration was the most feasible condition for the separation.

Table 1 is shown the retention time of mobile phase as affected by pH, ion-pairing reagent and solvent in standard water-soluble vitamins.

By checking concentration of ion-pairing reagent, methanol and pH in mobile phase, suitable mobile phase was determined to mix 0.02% triethylamine, 17.5% methanol and 5 μ M sodium hexane-sulfonic acid in water adjusted to pH 3.5 with acetic acid.

2. Validation of HPLC Method

1) Linearity

Linearity was verified by a regression of calibration for standard solution of vitamins (Fig. 4). Coefficients of determination (R^2) were obtained above 0.9977 for all standard curves of vitamins ($p < 0.001$).

2) Precision

Ten times measurement of each vitamin standard solution were carried out using the same mobile phase and equipments to evaluate the method precision. The relative standard deviations for retention time and vitamin content in all standard solution were obtained as Table 2. The relative standard deviation for retention time ranged from 0.09 to 0.21% and the relative standard deviations for vitamin content ranged from 0.212 to 0.257%. These results were satisfied according to USP and ICH standard (less than 1.0%) (ICH 1994).

3) Recovery

Recovery ranged from 96.4% to 99.0% for vitamin B₁ (thiamin),

Fig. 4. Standard curves for vitamin B₁, B₂, B₃ (nicotinic acid and nicotine amide), B₆ and vitamin C

Table 2. System precision, recovery and system suitability for standard solution of vitamins

Vitamins	System precision				Recovery		System suitability		
	Retention time (min)		Vitamin content ($\mu\text{g}/\text{mL}$)		Recovery (%)	LOD ²⁾ ($\mu\text{g}/\text{mL}$)	Area RSD (%)	Tailing factor	Resolution
	Mean	RSD ¹⁾ (%)	Mean	RSD(%)					
Vitamin B ₁	14.71	0.21	10.074	0.213	97.6 $\pm$ 2.0	0.2	0.54	1.86	19.68
Vitamin B ₂	17.91	0.21	10.070	0.257	98.9 $\pm$ 2.4	0.2	0.54	0.96	3.48
Nicotinic acid	1.95	0.09	10.097	0.212	96.4 $\pm$ 1.7	0.1	0.45	1.08	3.22
Nicotine amide	2.64	0.09	10.053	0.221	96.7 $\pm$ 1.2	0.1	0.48	0.97	4.03
Vitamin B ₆	5.34	0.12	10.032	0.241	97.2 $\pm$ 1.3	0.2	0.51	0.91	12.09
Vitamin C	1.44	0.09	10.044	0.231	99.0 $\pm$ 1.4	0.1	0.32	1.14	-

¹⁾ Relative standard deviation, ²⁾ Limit of detection.

vitamin B₂ (riboflavin), vitamin B₃ (nicotinic acid and nicotine amide), vitamin B₆ (pyridoxine) and vitamin C (ascorbic acid). The detection limits for vitamin B₃ (nicotinic acid and nicotine amide) and vitamin C (ascorbic acid) were 0.1 $\mu\text{g}/\text{mL}$ and the detection limits for vitamin B₁ (thiamin), vitamin B₂ (riboflavin) and vitamin B₆ (pyridoxine) were 0.2 $\mu\text{g}/\text{mL}$ (Table 2).

Moreno & Salvado (2000) used the solid-phase extract (SPE) column to analyze vitamins in multi-vitamin pharmaceutical formulation by HPLC. They reported that the recovery rates of vitamin B₁, vitamin B₂ and vitamin B₆ were 100, 116, and 98 percent, respectively (Moreno & Salvado 2000). These results for vitamin B₁ and vitamin B₆ were higher than recoveries in the

present study (97.6% and 97.2%). On the other hand, the recovery of vitamin B₂ in the present study (98.9%) was better than the result by Moreno and Salvado (2000). The recoveries of vitamins in the present study were higher than the recoveries by Albalá-Hurtado et al. for determination of water-soluble vitamins in infant milk as well (1997).

4) System Suitability

System suitability was estimated by calculating the relative standard deviation of peak area, the tailing factor of peak and resolution in chromatogram (Table 2). The relative standard deviation of peak area ranged from 0.32 to 0.54%, the tailing factor

of peak ranged 0.91 to 1.86. The resolution was ranged from 3.22 to 19.68 which were satisfied according to USP and ICH standard (over than 2.0) (ICH 1994).

The data previous described demonstrated that the proposed HPLC method was suitable for application to analysis of B group vitamins and vitamin C.

3. Assessment of Sample Extraction

The extraction procedure of vitamins from foods was necessary for the use of successive acid or enzymatic hydrolysis steps by viñas et al. (2003) and Ndaw et al. (2000). But vitamins in dietary supplements are easily extracted without specific preparation such as enzymatic or acid hydrolysis because vitamins are added as an isolation type into dietary supplements. Therefore, this study considered both resolution time of ultra sonic and the type of extraction solvent for fast and accurate extraction of vitamins, rather than extraction based on an enzymatic or acid hydrolysis. Mobile phase, ethanol, methanol and water were used as an extraction solvent. Vitamins from dietary supplements were extracted for 1, 10, 20 and 30 minutes using ultra sonic processor in each extraction solvent. Table 3 - Table 5 show the obtained results. Even though extracts were various by product type, use of water and mobile phase as extraction solvent in the same preparation showed higher recovery than others such as ethanol and methanol. As far as extraction time concern, the best time was observed

20 minutes by using ultra sonic processor for all solvent ($p < 0.05$).

Vitamin B₁ was extracted better in mobile phase than other solvents for all product types (tablet, capsule, powder and liquid). Because the extraction of vitamin B₁ from foodstuffs normally requires the use of acid or enzymatic hydrolysis steps (Ndaw et al. 2000; Viñas et al. 2003), better result might be obtained by using mobile phase adjusted pH 3.5 with acid. Regardless of vitamin types, capsule type dietary supplements had better extraction efficiency in mobile phase than other solvents. Because oils are used as subsidiary ingredient of soft capsule product, mobile phase adding acid seems to be the best extraction solvent. Whereas the best extraction efficiencies of vitamin B₂, B₃ and B₆ from tablet sample were achieved by using water as extraction solvent. It might be considered that hydro-soluble dextrin and lactose are generally used as subsidiary ingredient in tablet product.

Based upon the above mentioned results as a conclusion, when the simultaneous determination of vitamin B₁, B₂, B₃ (nicotinic acid, nicotine amide), B₆ and vitamin C from dietary supplements was carried out, even though extraction efficiencies were various by product type, water and aqueous (the same as mobile phase) prepared to mix 0.02% triethylamine, 17.5% methanol and 5 μ M sodium hexane-sulfonic acid in water adjusted to pH 3.5 with acetic acid as extraction solvent were better solvent than ethanol and methanol. In comparison of extraction time, the best was 20 minutes by ultra sonic extraction.

Table 3. Effect of product type and extraction condition on vitamin B₁ and Vitamin B₂ content

Product type	Extraction solvent	Extraction time(min)							
		Vitamin B ₁				Vitamin B ₂			
		1	10	20	30	1	10	20	30
Tablet	Solvent ¹⁾	22.03±0.21 ^{Ab2)}	22.23±0.21 ^{Ab}	23.37±0.21 ^{Aa}	21.80±0.56 ^{Ab}	271.27±0.38 ^{Bd}	298.87±0.32 ^{Bb}	324.27±0.31 ^{Ba}	282.53±1.81 ^{Bc}
	Ethanol	10.53±0.15 ^{Cc}	14.90±0.70 ^{Cb}	14.53±0.91 ^{Cb}	18.90±0.66 ^{Ba}	171.40±1.30 ^{Cd}	213.63±1.89 ^{Cc}	271.57±0.15 ^{Ca}	225.40±1.13 ^{Cb}
	Methanol	13.07±0.72 ^{Bb}	17.43±0.60 ^{Ba}	18.60±0.10 ^{Ca}	17.33±1.17 ^{Ca}	133.17±2.11 ^{Dd}	176.70±2.66 ^{Dc}	208.73±0.71 ^{Da}	196.60±2.03 ^{Db}
	Water	21.23±0.59 ^{Ab}	21.50±0.53 ^{Ab}	22.10±0.56 ^{Ba}	21.90±0.70 ^{Ab}	313.83±0.60 ^{Ab}	314.00±0.46 ^{Ab}	328.63±0.38 ^{Aa}	289.77±0.81 ^{Ac}
Powder	Solvent	11.03±0.21 ^{Aa}	11.57±0.49 ^{Aa}	11.73±0.21 ^{Aa}	11.60±0.17 ^{Aa}	303.77±2.02 ^{Ad}	317.53±1.07 ^{Ac}	353.40±0.36 ^{Aa}	346.90±0.90 ^{Ab}
	Ethanol	1.67±0.12 ^{Cb}	1.87±0.15 ^{Cb}	1.60±0.10 ^{Cb}	2.00±0.20 ^{Ca}	47.90±0.53 ^{Cd}	85.33±2.50 ^{Bc}	107.37±1.66 ^{Ca}	94.40±1.21 ^{Cb}
	Methanol	0.73±0.06 ^{Dc}	0.87±0.06 ^{Dc}	1.43±0.15 ^{Ca}	1.10±0.10 ^{Db}	41.33±1.22 ^{Db}	65.10±2.27 ^{Ca}	67.63±0.32 ^{Da}	65.87±0.55 ^{Da}
	Water	8.57±0.31 ^{Bc}	9.57±0.21 ^{Bb}	10.63±0.06 ^{Ba}	9.47±0.45 ^{Bb}	245.13±1.89 ^{Bc}	315.03±5.35 ^{Ab}	322.67±0.87 ^{Ba}	235.60±4.70 ^{Bd}
Capsule	Solvent	383.17±1.27 ^{Ac}	388.00±1.47 ^{Ac}	419.07±0.40 ^{Aa}	405.80±1.00 ^{Ab}	247.00±0.62 ^{Ad}	388.00±1.47 ^{Ac}	419.07±0.40 ^{Aa}	405.80±1.00 ^{Ab}
	Ethanol	262.17±5.43 ^{Cc}	341.43±0.55 ^{Ca}	346.50±2.62 ^{Ca}	318.10±0.26 ^{Cb}	262.17±5.43 ^{Cc}	341.43±0.55 ^{Ca}	346.50±2.62 ^{Ca}	318.10±0.26 ^{Cb}
	Methanol	186.03±0.78 ^{Dc}	226.03±0.45 ^{Db}	250.83±3.85 ^{Da}	249.33±1.97 ^{Da}	186.03±0.78 ^{Dc}	226.03±0.45 ^{Db}	250.83±3.85 ^{Da}	249.33±1.97 ^{Da}
	Water	359.23±0.57 ^{Bd}	375.87±0.85 ^{Bc}	395.87±2.75 ^{Ba}	385.83±1.16 ^{Bb}	359.23±0.57 ^{Bd}	375.87±0.85 ^{Bc}	395.87±2.75 ^{Ba}	385.83±1.16 ^{Bb}
Liquid	Solvent	13.00±0.17 ^{Aa}	11.83±0.12 ^{Ab}	12.67±0.25 ^{Aa}	11.60±0.35 ^{Ab}	8.47±0.21 ^{Aa}	8.67±0.15 ^{Ac}	15.43±0.38 ^{Aa}	14.33±0.64 ^{Ab}
	Ethanol	2.43±0.12 ^{Cc}	3.50±0.35 ^{Cb}	4.57±0.15 ^{Ca}	4.37±0.06 ^{Ca}	3.07±0.21 ^{Cc}	4.27±0.06 ^{Cb}	4.70±0.10 ^{Ca}	4.47±0.12 ^{Cb}
	Methanol	1.33±0.06 ^{Dd}	2.70±0.17 ^{Dc}	6.77±0.15 ^{Da}	4.00±0.35 ^{Cb}	1.70±0.10 ^{Dd}	2.67±0.21 ^{Db}	3.03±0.06 ^{Da}	2.77±0.06 ^{Db}
	Water	4.63±0.29 ^{Bc}	8.40±0.17 ^{Ba}	8.70±0.10 ^{Ba}	8.10±0.35 ^{Bb}	5.97±0.31 ^{Bc}	10.50±0.20 ^{Ba}	10.70±0.26 ^{Ba}	10.00±0.66 ^{Ba}

¹⁾ The same as mobile phase,

²⁾ Different small letters indicate significant differences according to the extraction times at $p < 0.05$ level,

Different capital letters indicate significant differences according to the extraction solvent of product type at $p < 0.05$ level.

Table 4. Effect of product type and extraction condition on vitamin B₃ content

Product type	Extraction solvent	Extraction time (min)							
		Nicotinic acid				Nicotinamide			
		1	10	20	30	1	10	20	30
Tablet	Solvent ¹⁾	294.13±4.10 ^{Bd2)}	319.83±2.15 ^{Bb}	343.73±2.01 ^{Ba}	302.53±1.00 ^{Bc}	42.73±0.42 ^{Bb}	43.53±0.80 ^{Bb}	48.37±1.00 ^{Ba}	42.43±0.50 ^{Bb}
	Ethanol	29.13±0.72 ^{Cd}	33.33±0.45 ^{Cc}	45.57±0.93 ^{Ca}	43.87±0.72 ^{Cb}	7.77±0.45 ^{Cc}	8.47±0.15 ^{Cc}	10.53±0.35 ^{Da}	9.57±0.57 ^{Db}
	Methanol	27.53±1.00 ^{Cc}	29.97±0.76 ^{Cb}	33.47±0.25 ^{Da}	30.80±0.60 ^{Db}	8.43±0.32 ^{Cc}	9.17±0.25 ^{Cc}	12.30±0.40 ^{Ca}	10.77±0.65 ^{Cb}
	Water	352.27±4.55 ^{Ad}	395.43±3.73 ^{Ab}	480.60±2.59 ^{Aa}	374.47±3.81 ^{Ac}	44.87±0.65 ^{Ac}	52.17±0.71 ^{Aa}	52.93±0.25 ^{Aa}	49.00±0.50 ^{Ab}
Powder	Solvent	118.03±0.78 ^{Ac}	132.20±1.31 ^{Ab}	146.73±0.70 ^{Aa}	132.60±1.61 ^{Ab}	91.27±0.81 ^{Bc}	104.67±0.93 ^{Ab}	114.80±0.82 ^{Aa}	104.07±1.12 ^{Ab}
	Ethanol	73.87±0.65 ^{Cd}	90.93±1.29 ^{Cc}	106.47±1.00 ^{Ca}	102.63±2.76 ^{Bb}	21.27±1.00 ^{Dd}	48.47±1.08 ^{Cc}	84.03±0.91 ^{Ca}	78.23±1.68 ^{Cb}
	Methanol	43.43±0.85 ^{Dd}	55.27±0.67 ^{Dc}	67.67±0.64 ^{Da}	58.23±0.59 ^{Cb}	34.17±1.27 ^{Cd}	39.50±0.80 ^{Dc}	44.97±0.76 ^{Da}	42.40±0.98 ^{Db}
	Water	110.93±0.55 ^{Bd}	122.80±1.25 ^{Bc}	142.47±0.67 ^{Ba}	135.53±1.70 ^{Ab}	95.63±0.75 ^{Ab}	101.33±1.02 ^{Ba}	102.27±1.03 ^{Ba}	94.70±0.82 ^{Bb}
Capsule	Solvent	207.33±1.25 ^{Ad}	214.73±1.56 ^{Ac}	244.57±1.10 ^{Aa}	217.73±0.67 ^{Ab}	11.30±0.72 ^{Aa}	11.63±0.15 ^{Aa}	12.57±0.67 ^{Aa}	11.83±0.40 ^{Aa}
	Ethanol	123.17±1.91 ^{Cd}	163.40±1.74 ^{Cc}	178.13±1.16 ^{Ca}	166.87±1.39 ^{Cb}	1.60±0.20 ^{Cc}	1.77±0.15 ^{Bc}	2.43±0.12 ^{Ba}	2.13±0.06 ^{Bb}
	Methanol	114.20±2.42 ^{Dd}	122.47±0.80 ^{Dc}	172.83±1.04 ^{Da}	167.13±1.52 ^{Cb}	1.30±0.10 ^{Cb}	1.37±0.15 ^{Bb}	1.73±0.06 ^{Ca}	1.63±0.06 ^{Ca}
	Water	196.67±0.76 ^{Bd}	207.73±1.06 ^{Bb}	216.40±0.46 ^{Ba}	201.93±0.55 ^{Bc}	8.60±0.20 ^{Bc}	11.37±0.35 ^{Ab}	12.50±0.26 ^{Aa}	12.07±0.21 ^{Aa}
Liquid	Solvent	17.90±0.60 ^{Aa}	18.57±0.21 ^{Aa}	18.93±0.50 ^{Aa}	18.97±0.55 ^{Aa}	3.43±0.21 ^{Ac}	3.63±0.06 ^{Ab}	4.23±0.15 ^{Aa}	3.83±0.06 ^{Ab}
	Ethanol	10.93±0.21 ^{Bc}	11.47±0.21 ^{Bb}	12.77±0.25 ^{Ba}	12.37±0.25 ^{Ca}	1.70±0.10 ^{Cb}	2.07±0.06 ^{Ca}	2.23±0.15 ^{Ca}	2.30±0.26 ^{Ca}
	Methanol	10.27±0.51 ^{Bc}	11.30±0.26 ^{Bb}	13.33±0.25 ^{Ba}	13.47±0.31 ^{Ba}	1.57±0.12 ^{Cc}	1.67±0.15 ^{Db}	2.60±0.17 ^{Ba}	1.93±0.15 ^{Db}
	Water	18.53±0.15 ^{Aa}	18.60±0.72 ^{Aa}	19.33±0.31 ^{Aa}	18.73±0.21 ^{Aa}	2.37±0.12 ^{Bb}	2.63±0.06 ^{Ba}	2.83±0.12 ^{Ba}	2.63±0.15 ^{Ba}

¹⁾ The same as mobile phase,

²⁾ Different small letters indicate significant differences according to the extraction times at $p<0.05$ level,
Different capital letters indicate significant differences according to the extraction solvent of product type at $p<0.05$ level.

Table 5. Effect of product type and extraction condition on vitamin B₆ and Vitamin C content

Product type	Extraction solvent	Extraction time(min)							
		Vitamin B ₆				Vitamin C			
		1	10	20	30	1	10	20	30
Tablet	Solvent ¹⁾	198.27±1.60 ^{Bd2)}	255.67±2.48 ^{Ac}	285.30±3.12 ^{Ba}	272.57±1.07 ^{Bb}	1,841.07±2.83 ^{Ac}	1,843.97±3.64 ^{Bc}	2,423.30±9.8 ^{Aa}	1,967.30±15.68 ^{Ab}
	Ethanol	22.23±0.47 ^{Cc}	25.17±0.59 ^{Cb}	30.57±0.90 ^{Ca}	13.87±0.60 ^{Dd}	277.93±2.24 ^{Cd}	304.60±0.92 ^{Dc}	327.57±1.61 ^{Ca}	315.57± 5.25 ^{Cb}
	Methanol	22.50±0.53 ^{Cc}	24.70±0.50 ^{Cb}	27.27±0.50 ^{Da}	25.43±0.50 ^{Cb}	263.17±2.54 ^{Dc}	314.00±2.21 ^{Cb}	335.10±2.04 ^{Ca}	316.20± 1.14 ^{Cb}
	Water	318.67±5.38 ^{Ad}	392.33±3.98 ^{Bb}	400.70±0.72 ^{Aa}	368.63±0.87 ^{Ac}	1,653.67±6.54 ^{Bc}	1,906.70±3.08 ^{Aa}	1,914.83±7.27 ^{Ba}	1,814.67± 9.29 ^{Bb}
Powder	Solvent	13.20±0.36 ^{Ac}	13.67±0.15 ^{Ac}	33.63±0.23 ^{Ab}	34.90±0.70 ^{Aa}	2,678.87±3.00 ^{Ad}	2,691.93±5.98 ^{Ac}	2,378.60±2.51 ^{Aa}	2,846.23± 1.19 ^{Ab}
	Ethanol	25.90±1.08 ^{Cc}	25.07±0.97 ^{Cc}	26.77±0.06 ^{Bb}	29.30±0.40 ^{Ca}	813.67±4.23 ^{Cd}	1,296.87±9.47 ^{Cc}	479.17±4.02 ^{Ca}	326.50±18.06 ^{Cb}
	Methanol	25.13±0.38 ^{Ca}	28.67±0.76 ^{Bb}	26.27±1.03 ^{Bb}	29.60±0.70 ^{Ca}	524.10±6.22 ^{Dd}	793.83±5.55 ^{Dc}	1,133.03±2.81 ^{Da}	944.40± 7.96 ^{AD}
	Water	28.87±0.67 ^{Bd}	31.97±0.47 ^{Ac}	34.33±0.25 ^{Aa}	32.93±0.45 ^{Bb}	2,379.10±0.36 ^{Bd}	2,413.17±7.13 ^{Bc}	774.00±2.33 ^{Ba}	2,634.77± 4.15 ^{Bb}
Capsule	Solvent	641.47±1.26 ^{Ad}	643.30±0.61 ^{Ac}	693.27±0.31 ^{Aa}	688.03±0.75 ^{Ab}	3,190.63±802 ^{Ac}	3,243.07±9.51 ^{Ab}	3,558.33±0.95 ^{Aa}	3,244.00±11.45 ^{Ab}
	Ethanol	182.47±0.80 ^{Cc}	185.20±1.95 ^{Cc}	235.40±1.97 ^{Ca}	192.33±2.08 ^{Cb}	1,068.47±5.05 ^{Dd}	1,259.57±4.50 ^{Dc}	1,533.90±5.07 ^{Ca}	1,385.93± 5.67 ^{Cb}
	Methanol	102.10±0.66 ^{Dd}	104.77±0.81 ^{Dc}	145.03±0.78 ^{Da}	125.23±1.37 ^{Db}	1,351.27±8.04 ^{Cd}	1,370.17±4.14 ^{Cc}	1,525.57±2.85 ^{Ca}	1,390.90± 6.66 ^{Cb}
	Water	447.67±2.75 ^{Bd}	546.80±7.02 ^{Bc}	628.50±0.46 ^{Ba}	606.47±1.75 ^{Bb}	1,635.83±922 ^{Bd}	1,920.40±7.07 ^{Bb}	2,035.40±9.04 ^{Ba}	1,734.90±10.31 ^{Bc}
Liquid	Solvent	10.30±0.17 ^{Ac}	10.63±0.25 ^{Ac}	12.17±0.50 ^{Aa}	11.47±0.15 ^{Ab}	1,085.00±1.78 ^{Ba}	1,091.53±2.82 ^{Aa}	1,094.67±0.91 ^{Ba}	1,086.57± 7.70 ^{Aa}
	Ethanol	4.17±0.12 ^{Cc}	5.23±0.25 ^{Bb}	6.77±0.15 ^{Ca}	5.23±0.21 ^{Cb}	434.70±2.74 ^{Cd}	619.60±1.76 ^{Bc}	725.00±6.12 ^{Ca}	671.03± 4.82 ^{Cb}
	Methanol	1.57±0.12 ^{Dc}	1.67±0.21 ^{Cc}	4.87±0.15 ^{Da}	3.83±0.31 ^{Db}	389.77±2.19 ^{Db}	482.47±3.69 ^{Ca}	484.93±2.32 ^{Da}	482.43± 8.91 ^{Da}
	Water	9.70±0.17 ^{Bb}	10.27±0.25 ^{Aa}	10.63±0.15 ^{Ba}	9.43±0.40 ^{Bb}	1,091.33±2.27 ^{Ab}	1,094.57±0.81 ^{Ab}	1,154.33±4.03 ^{Aa}	1,037.70± 1.90 ^{Bc}

¹⁾ The same as mobile phase,

²⁾ Different small letters indicate significant differences according to the extraction times at $p<0.05$ level,
Different capital letters indicate significant differences according to the extraction solvent of product type at $p<0.05$ level.

CONCLUSION

Capcell-Pak C₁₈ column and UV/VIS detector were applied to the simultaneous determination of five water-soluble vitamins in dietary supplements using reversed-phase liquid chromatography. The pH, the concentration of ion-pairing reagent and the proportion of methanol in mobile phase were properly proposed to determine of the water-soluble vitamins in dietary supplements. Precision, accuracy, linearity and system suitability for simultaneous determination of HPLC method were validated individually, in which showed the good results being eligible for USP and ICH standards. The procedure was applied to the determination of water-soluble vitamins in dietary supplements with great results. We found that water and elution were suitable for extraction and recovery of water-soluble vitamins in dietary supplements. As far as extraction time concern, the best time was observed 20 minutes by using ultra sonic.

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