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Simultaneous Determination of the Water Soluble Vitamins in Multi-Nutrient Tablets by Reversed-Phase High-Performance Liquid Chromatography

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Abstract

Simultaneous determination of nine water-soluble vitamins contained in multi-nutrient tablets was carried out by reversed phase high-performance liquid chromatography (RP-HPLC) equipped with analytical C_{18} column and UV (270 nm) detector. Those standard vitamins were successfully separated within 23 minutes by gradient elution with solvent A (0.5 M potassium phosphate monobasic) and solvent B (0.25 M potassium phosphate monobasic-methanol, 1 : 1). Calibration curves showed good linealities with correlation coefficients (>0.92) in tested range, respectively. The detection limits were considered to be 2.1 ng for ascorbic acid, 60 ng for Vit B_6, 3 ng for *p*-aminobenzoic acid, 9 ng for niacinamide, 9 ng for thiamin, 5.0 ng for folic acid and 1.5 ng for riboflavin at 0.05 a.u.f.s. Solid phase extraction through Sep-Pak (C_{18}) cartridge was successfully applied for purification of water soluble vitamins in commercial multi-nutrient tablets.

Key words: RP-HPLC, water soluble vitamins, C_{18} Sep-Pak solid phase extraction

INTRODUCTION

Vitamin analysis was generally carried out by measuring chemical reactions, physical changes, or biological metabolism with microorganisms or animals. Analytical methods of vitamins by microorganisms or animals have the advantage of high sensitivity for quantitative analysis. Also, it is possible to analyze special vitamins without purification steps. However, it takes a long time: 20 hours or more (1). Compared with these biological methods, chemical or physical methods have advantages such as a short time for analysis procedural ease. For the chemical methods, colorimetry, fluorescence and spectrophotometry were mainly used. A disadvantage of these methods is the exclusion of interfering materials inhibiting the quantitative analysis. To remove the interfering materials, sample purification steps with tubular chromatogram and others before the analysis were required (1). Therefore, the choice of an analytical method for vitamin analysis depends on kinds of samples, variety of inhibitors contained in the samples, instrumental accuracy and sensitivity.

Recently, vitamin analysis was mainly done by HPLC. Vitamin analysis by HPLC is reported to be the most suitable method for quantitative analysis of several vitamins including water-soluble vitamins (2). The advantage of vitamin analysis by HPLC was to simplify purification steps of samples because of high separation ability, the maximum protection from air, high temperature or light, and high sensitivity of detection by electrochemical detector or fluorescence spectrophotometry. However, it is impossible to simultaneously analyze all of water-soluble vitamins by a single method of sample preparation, because water-soluble vitamins have diverse chemical properties and several forms in foods, and because they are easily broken by being exposed to air.

Vitamin analysis by HPLC with fluorescence spectrophotometry detector is possible with high sensitivity, but the fluorescence spectrophotometry detector has a disadvantage that several vitamins at the same time can not be detected because of the differing wavelengths of each vitamin. Another disadvantage in measuring water-soluble vitamins by fluorescence spectrophotometry is that the vitamins without fluorescent light should be changed to the compounds with fluorescent light by chemical reactions in order to be detected. During the chemical reaction, a lot of vitamin loss occurs (3-6). Therefore, it is very effective to directly analyze the sample with high concentration or multi-nutrient tablets from a drug store if several vitamins are simultaneously analyzed by using an optimum wavelength (7-10) with even a little less sensitivity.

Although vitamin extraction is diverse and complex according to kinds of samples, nutrient tablets from a local market can be easily extracted and purified by a relatively simple method because it exists as free forms. Water-soluble vitamins extracted by metaphosphoric acid solution can be purified by strong anion exchange column (7) or

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C_{18} Sep-pak solid extractor (3,4,6,11). The column for the analysis of water-soluble vitamins by HPLC is changed from the initial use of silica gel column to the recent use of reverse-phase column. Also, polar column or ion exchange column are often used.

The objectives of this study were to purify the water-soluble vitamins of free forms from store-bought nutrient tablets after extraction with a Sep-Pak C_{18} cartridge, to find the method of analyzing at one time a lot of possible water-soluble vitamins by reverse-phase column and UV detector, and finally to apply this analytical method to the analysis of multi-nutrient tablets from a local market.

MATERIALS AND METHODS

Samples and reagents

The nutrient tablets used in this study were the commercial product of 'D' company and they were purchased from a local drug store. These multi-nutrient tablets mainly contained of several vitamins and other nutrients including minerals and dried-yeasts. The standard materials of such water-soluble vitamins, that is, ascorbic acid, *p*-aminobenzoic acid, pyridoxal, pyridoxine, pyridoxamine, niacinamide, thiamin, folic acid and riboflavin were purchased from Sigma Inc. (St. Louis, MO, USA). Column for sample purification was C_{18} Sep-Pak solid phase extraction column (5 cm × 1 cm I.D. Merck Inc., Darmstadt, Germany). The methanol for HPLC solvent was purchased from Merck Inc. (Darmstadt, Germany). Other chemicals were analytical grades.

Preparation of standard solution of water-soluble vitamins

The standard solution of concentrations of 0.0025 mg/mL of ascorbic acid, 0.1 mg/mL of each pyridoxal, pyridoxine, pyridoxamine (Vit B_6), 0.005 mg/mL of *p*-aminobenzoic acid, 0.017 mg/mL of niacinamide, 0.012 mg/mL of thiamin, 0.01 mg/mL of folic acid and 0.002 mg/mL of riboflavin was added to deminerized water.

Standard curves for water soluble vitamins

The standard curve between the concentration of water-soluble vitamins and the peak area of HPLC was drawn by injecting the diluted standard solutions with 2, 3, 4 and 5 times. Also, the correlation coefficients were calculated.

Measurement of detection sensitivity

The baseline of detector was established at 0.05 a.u.f.s. that with the highest sensitivity little affected by changes of the composition of solvent during analysis. And, then the established minimum detection limit of S/N ratio for all the water-soluble vitamins was over twofold.

Vitamin analysis of multi-nutrient tablets

Multi-nutrient tablets of 'D' company were purchased from a local drug store. The tablets were ground and put into 10 mL of 0.1% metaphosphoric acid. And, then it was treated for 5 min with ultrasonic-cleaner. The treated sample was centrifuged for 20 min at 10,000 rpm. The supernatant was put into a 100 mL mass-flask. The precipitant was added to 10 mL of 0.1% metaphosphoric acid and was centrifuged as above. This process was repeated four times and the collected solution was adjusted to 100 mL with 0.1% metaphosphoric acid into a 100 mL mass-flask. Ten mL of this solution was passed through a C_{18} Sep-Pak column (5 cm × 1 cm I.D.) which was conditioned by passing through with 5 mL of methanol. The passed solution was collected into 25 mL of mass flask. The solutions of 5 mL of 1% phosphate, 7 mL of 0.1 N NaOH and 3 mL of the mixed solution of methanol and H_2O (1 : 1) were passed through a C_{18} Sep-Pak column in order and the passed solutions were collected into the above same mass flask. The volume of collected solutions was adjusted to 25 mL with 0.1% metaphosphoric acid. After the measured solution was filtrated by 0.25 μm membrane filter, 10 μL of the filtrated solution was injected to HPLC. The recovery yields of the added water-soluble vitamins to multi-nutrient tablets were analyzed by the same method as the analysis of water-soluble vitamins in multi-nutrient tablets. The standard solution of each water-soluble vitamin (0.5 mL) was added to 10 mg of the ground multi-nutrient tablets. To the above solution, 5.5 mL of 0.2% metaphosphoric acid was added. The other steps were the same as the analysis of water-soluble vitamins of the multi-nutrient tablets. The recovery yield was calculated in every experiment by measuring the amount of each standard vitamin of the added group and the amount of no added group. The recovery yield on the added amount of each standard vitamin was calculated by abstracting the peak area of no added group from that of the added vitamin group.

High-performance liquid chromatography (HPLC)

HPLC was that of Physics Inc. with 8800 ternary pump (San Jose, California, USA) and the detector was Spectra 200 of UV detector with programmable wavelength. It was detected at 270 nm. Detector sensitivity was established at 0.05 a.u.f.s. Nova-Pak C_{18} column (300 × 3.9 mm I.D., 4 μm dimethyloctadecylsilyl-bonded amorphous silica, Waters) was used and the column temperature was fixed at 30°C. Solvent was continuously degassed by the degasing apparatus with He gas (Spectra-Physics). Solvent A of Table 1 was 0.5 M potassium phosphate monobasic solution and solvent B was the mixture of 0.25 M potassium phosphate monobasic solution and methanol with the ratio of 1 and 1. Solvents A and B were controlled to pH 6.7 with NH_4OH. Flow rate was 1.0 mL/min and the solvent gradient is shown in Table 1.

Table 1. Solvent gradients for water-soluble vitamin analysis by HPLC

Running time (min)	0	6	13	15	17	20
Solvent A[1)] (%)	100	100	80	80	20	0
Solvent B[2)] (%)	0	0	20	20	80	100

[1)]Solvent A is 0.5 M potassium phosphate monobasic solution.
[2)]Solvent B is the mixture of 0.25 M potassium phosphate monobasic solution and methanol with the ratio of 1 and 1.

RESULTS AND DISCUSSION

HPLC chromatogram and calibration curve for water-soluble vitamins

Nine standard vitamins contained in relatively high amounts in foods among water-soluble vitamins were separated by C_{18} Nova-Pak column and the chromatogram on the separated vitamins is shown in Fig. 1. Both the separation of pyridoxamine from *p*-aminobenzoic acid, and the separation of niacinamide from thiamin were not completely carried out, but the standard vitamins were separated with a relative satisfaction of the resolution value of 1.2. In the case of water-soluble vitamins, reverse-phase column was mainly used (12-15). Other columns for vitamin analysis were Zorbak CN polar column (7,16) for thiamin, ion-exchange column (17) for riboflavin, Aminex A-5 (18) or Bio-Rad A-25 (19) ion-exchange columns for vitamin B_6, Nucleosil-5 NH_2 (20) polar column for niacin, Pellionex SAX (21) anion-exchange column for folic acid and Alltech NH_2 (22) or μ-Bondapak NH_2 (23) polar columns for vitamin C.

Two kinds of solvents of nonion pairs (14,15,24-28) and ion pairs can be applied for vitamin analysis (3-5,8,29-32). The case of ion pair solvents has a problem that reproducibility of retention time was unstable because of the complex composition of mobile phase. In this study, the reproducibility of vitamin analysis by the ion pair solvent of 1-hexanesulfonic acid sodium salt was unstable. In the case of quantitative analysis of water-soluble vitamins, fluorescence spectrophotometry detector is commonly used to increase detection sensitivity (2), but a fluorescence spectrophotometry detector has the limitation that the detector can detect only one or two vitamins by a wavelength because each vitamin has its own wavelength. Another limit is that because thiamin, ascorbic acid, niacin and folic acid have no fluorescence, they should be oxidized by fluorescent material (3,4,22,33), and that a lot of vitamin loss occurred during their oxidation because their pretreatment is complex. Although the sensitivity of UV detection was inferior to that of fluorescence spectrophotometry detection, it has several advantages of simultaneous detection of several vitamins, instrumental convenience and diverse use. The wavelengths of UV detection for water-soluble vitamins were 245 nm for thiamine (7,16), 254 nm for riboflavin (4), 280 nm for vitamin B_6 (14), 254 nm for niacin (20,34,35), 280 nm (21,26) and 365 nm for folic acid (27), and 254 nm (20,36), 250 nm (37) and 244 nm (23) for ascorbic acid. In this study, 270 nm was used because the wavelength had the high sensitivity for nine vitamins and the baseline was most stable for solvent gradient analysis. Standard curves of riboflavin and ascorbic acid showing the lowest values of their correlation coefficients are shown in Fig. 2. The correlation coefficient of riboflavin was 0.927, the lowest value among the 9 vitamins but the significantly accepted value in $p<0.001$. The reason of the low correlation co-

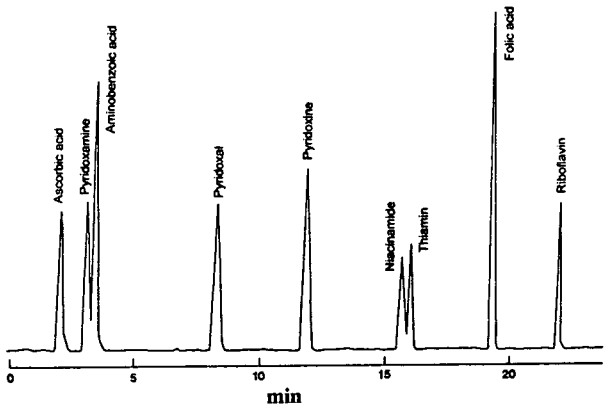

Fig. 1. Chromatogram of standard water soluble vitamins resolved on Nova-Pak (300×3.9 mm I.D.) C_{18}-column. Injection amounts were 12.5 ng ascorbic acid, 500 ng pyridoxamine, 25 ng *p*-aminobenzoic acid, 500 ng pyridoxal, 500 ng pyridoxine, 85 ng niacinamide, 60 ng thiamin, 50 ng folic acid, 10 ng riboflavin.

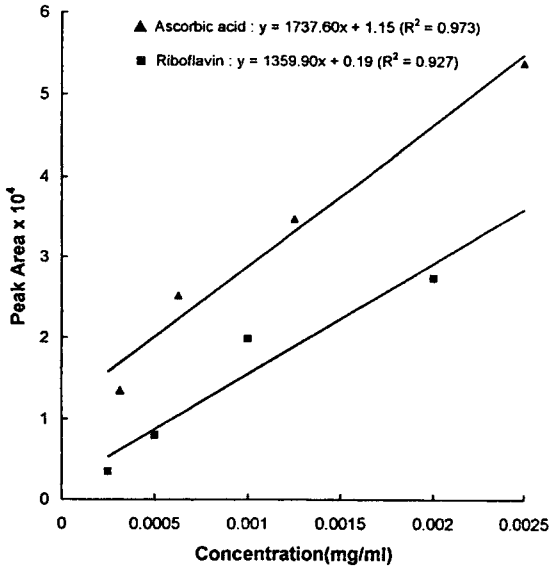

Fig. 2. Representative standard calibration curves of ascorbic acid and riboflavin. Each point is the mean of three measurements.

efficient of riboflavin was thought that riboflavin was easily destroyed by green light and ultraviolet (2,38). Based on these data, it is possible to do quantitative analysis for vitamins by these standard curves.

Detection sensitivity

Detection sensitivities of standard water-soluble vitamins are shown in Fig. 3. In the stable baseline setting on 0.05 a.u.f.s., the limited concentrations of vitamin detection were 2.1 ng for ascorbic acid, 60 ng for pyridoxal, pyridoxamine and pyridoxine, 3 ng for *p*-aminobenzoic acid, 9 ng for niacinamide, 9 ng for thiamine, 50.1 ng for folic acid, and 1.5 ng for riboflavin. It was possible to detect the decreased amounts than above values with increasing the detection sensitivity of detector, but it was impossible to do the actual quantitative analysis because of an unstable baseline by increasing the detection sensitivity more than 0.05 a.u.f.s. In thiamin, it was reported that its detection limit by fluorescence spectrophotometry after oxidation with thiochrome was 0.5 ng (24) showing about 18 times higher sensitivity than that of this study.

Kamman et al. (8) reported that the detection limits of thiamin and riboflavin were 30 ng and 5 ng on 0.008 a.u.f.s. of UV detector, respectively. This was more sensitive than that of this study. The detection limit of riboflavin with fluorescence spectrophotometry was reported to be about 50 pg in several reports (2,25,39). It was about 30 times more sensitive than that of this study. The detection limits of vitamin B_6 with fluorescence spectrophotometry were 1.24 ng for pyridoxal, 0.79 ng for pyridoxamine and 1.26 ng for pyridoxine in other reports (39). It showed higher detection sensitivity than that of this study by 48 times to 76 times. If all of the nine vitamins are analyzed by isocratic method, much higher sensitivity can be obtained because a stable baseline below 0.05 a.u.f.s. is achieved.

Contents of water-soluble vitamins in multi-nutrient tablets

Multi-nutrient tablets of 'D' company were purchased from a local drug store and water-soluble vitamins of the tablets were extracted. The extracted vitamins were purified by C_{18} cartridge. The analytical chromatograms of the purified water-soluble vitamins and of the extracted vitamins without purification are shown in Fig. 4 and Fig. 5, respectively. As shown in Fig. 5, although water-soluble vitamins in nutrient tablets existed in the free form state, chromatograms for the qualitative and quantitative analyses can not be obtained without purification steps of the mixture if the vitamins are mixed with other nutrients. It is very efficient to purify the extracted water-soluble vitamin by C_{18} cartridge because the vitamins exist in the free form state with inorganic nutrients and the powders of yeast extracts in the multi-nutrient tablets and because the other nutrients with vitamins could be extracted with water soluble vitamins during extraction procedure.

The contents of water-soluble vitamins and the recovery yields of the added standard vitamins are shown in Table 2. All of the labelled water-soluble vitamins were detected and showed little interfere from other peaks. The recovery yields for all standard vitamins showed the substantially

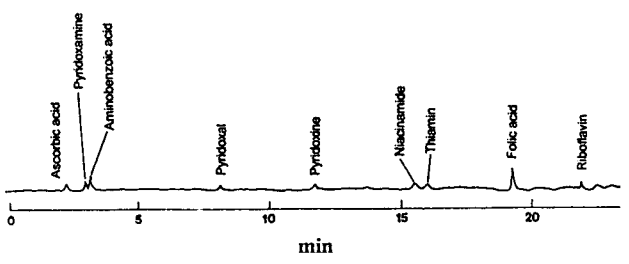

Fig. 3. Chromatogram of standard water-soluble vitamins showing the sensitivity. Injection amounts were 2.1 ng ascorbic acid, 60 ng pyridoxamine, 3 ng *p*-aminobenzoic acid, 60 ng pyridoxal, 60 ng pyridoxine, 9 ng niacinamide, 50.1 ng folic acid, 1.5 ng and riboflavin in the range of 0.05 a.u.f.s.

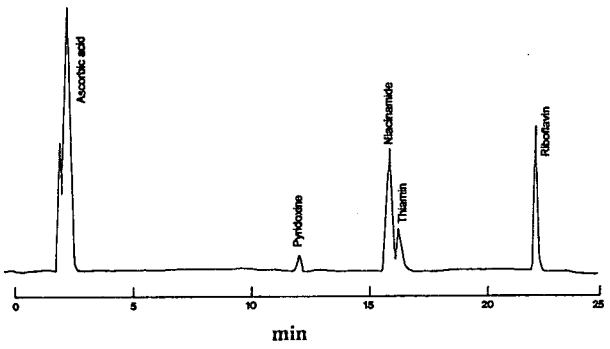

Fig. 4. Chromatogram of water-soluble vitamins in a commercial multi-nutrient tablets with clean-up procedure by Sep-Pak C_{18} cartridge.

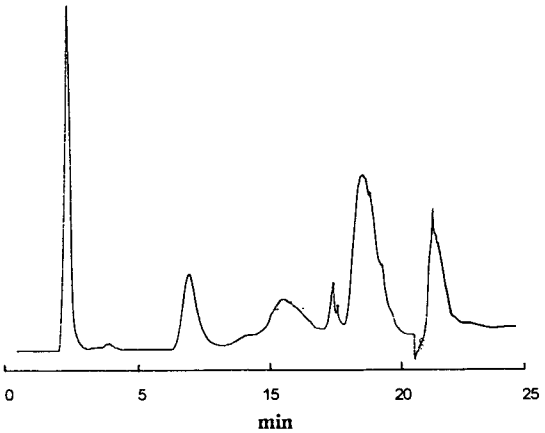

Fig. 5. Chromatogram of water soluble vitamins in a commercial multi-nutrient tablet without clean-up procedure by Sep-Pak C_{18} cartridge.

Table 2. Water-soluble vitamin contents in a commercial multi-nutrient tablet and recoveries of standard vitamins supplemented to the tablet mg/tablet (1.07 g)

Vitamins	Analytical values	Labelled values	Recoveries[1)]
Ascorbic acid	731.33±30.60	618.6	104.45±3.21
Pyridoxine	12.84±3.34	5.2	99.89±1.54
Niacinamide	90.95±0.01	100	95.98±2.01
Thiamine	18.37±0.81	15.5	97.64±0.87
Riboflavin	8.03±0.92	10	101.87±0.95

n=3.

[1)]Recoveries of standard water-soluble vitamins supplemented to the multi-nutrient tablets.

accurate values of the range between 95% and 105%. As the results of this study, the purification process by C_{18} Sep-Pak column was thought to be very effective for the analysis of vitamins of free form. The actual amounts of ascorbic acid, pyridoxine and thiamin by these analytical studies were higher than their labelled amounts of these vitamins, while the analytical amounts of niacinamide and riboflavin were lower than their labelled amounts. The reason why the amount of ascorbic acid was high was thought that incomplete separation of another peak before the peak of ascorbic acid affected the content of ascorbic acid. The differences between the amounts of other vitamins and the labelled amounts are thought to be due to inaccuracy of the labelled amounts, based on the accuracy of recovery yield of the added standard vitamins.

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