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ORIGINAL RESEARCH

Simultaneous determination of nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin in multivitamin with minerals tablets by reversed-phase ion-pair high performance liquid chromatography

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ABSTRACT: A reversed-phase ion-pair high performance liquid chromatographic method (HPLC) has been developed and validated for the routine analysis of nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin in multivitamin with minerals tablets. HPLC separation of the vitamins was performed on a Hypersil C_{18} column and detected by ultraviolet absorbance at 280 nm. The use of methanol-aqueous 0.5% acetic acid solution (18:82, v/v; containing 2.5 mM sodium hexanesulfonate, pH = 2.8) as the mobile phase at a flow-rate of 1.2 mL/min enables the baseline separation of the four analytes free from interferences with isocratic elution at 30°C. The analysis time was 17 min per injection. The method was linear in the ranges of 5–90, 2.5–90, 5–95 and 25–450 μg/mL for thiamine mononitrate, riboflavin, pyridoxine hydrochloride and nicotinamide, respectively. The average coefficients of variation of within- and between-day assays were 2.2 and 3.6% for thiamine mononitrate, 1.8 and 2.4% for riboflavin, 1.3 and 1.7% for pyridoxine hydrochloride and 1.0 and 1.5% for nicotinamide, respectively. The average recoveries of thiamine mononitrate, riboflavin, pyridoxine hydrochloride and nicotinamide were 97.0, 97.2, 98.9 and 100.4% for the tablets, respectively. The method has been successfully applied to the simultaneous determination of nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin in multivitamin with minerals tablets.

INTRODUCTION

As its well known, vitamins are vital to human life. The human metabolism is not able to synthesis these compounds, which have a catalytic function in anabolic and catabolic pathways. Therefore, a supplementation of these substances, through food or multivitamin tablets is necessary. These compounds can be classified into two main groups: water-soluble and fat-soluble vitamins. Among water-soluble vitamins, the B group is the most important. They play different specific and vital functions in metabolism, and their lack or excess produces specific diseases. Due to their function, vitamins are involved in developmental and fast-reproducing processes like blood formation, maintenance of epithelial tissue, ossification of bone, eye functions, as well as in the metabolic pathways of the central nervous system.

Many techniques for determining vitamins, including spectrophotometric, polarographic, fluorimetric, enzymatic and microbiological procedures have been described. The methods are tedious, sometimes non-specific and time-consuming, and involve pre-treatment of the sample through complex chemical, physical or biological reactions to eliminate interferences, followed by individual methods for each different vitamin. In recent years several papers have been published concerning the separation and quantification of vitamins by more simple methodologies. Among them the techniques most widely used are chromatographic, capillary electrophoresis (Dinelli and Bonetti, 1994; Fotsing *et al.*, 1997), and, in particular, high performance liquid chromatography (HPLC; Blanco *et al.*, 1994; Barua *et al.*, 1993; Craft *et al.*, 1988; Chase *et al.*, 1995; Chu and Tin, 1998; Delgado *et al.*, 1992; Gamiz-Gracia and Luque de Castro, 1997; Lee *et al.*, 1992; Li *et al.*, 1995; Moreno and Salvado, 2000; MacCrehan and Schonberger, 1995; Papadoyannis *et al.*, 1997; Rizzolo and Polesello, 1992; Sharpless and Duewer, 1995; Zaman *et al.*, 1993), which provides rapid, sensitive and accurate methods for vitamin determination.

HPLC determination of vitamins can be done either by normal phase chromatography or by reversed phase chromatography. Individual vitamins, as well as certain combinations of two or three vitamins, can be determined isocratically. Most of published HPLC methods involved the use of complex buffered mobile phases (Zaman *et al.*,

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1993), a gradient elution program (Blanco *et al.*, 1994; Papadoyannis *et al.*, 1997; Moreno and Salvado, 2000), electrochemical (Delgado *et al.*, 1992) or fluorimetric (MacCrehan and Schonberger, 1995; Sharpless *et al.*, 1995) detector, two or three different detectors (Craft *et al.*, 1988; Chase *et al.*, 1995), or changing the detection wavelength during the run (Lee *et al.*, 1992; Li *et al.*, 1995). Sometimes, diode-array detectors (Blanco *et al.*, 1994; Barua *et al.*, 1993; Chu and Tin, 1998) are used for the detection of vitamins. The complexity of these HPLC conditions, no doubt, effectively improves the selectivity and sensitivity of vitamin detections. Nevertheless, they are considered complicated and costly for routine analysis.

In the present paper, a simple and more reliable HPLC method has been developed for simultaneous determination of some water-soluble vitamins (nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin) in the multivitamin with minerals tablets. The analytes, after solution by water and filtration, can be directly injected into the HPLC system. The analysis time was 17 min per injection. It is therefore suitable for quality control and routine analysis of multivitamin products in the pharmaceutical and health food industries.

EXPERIMENTAL

Apparatus. A liquid chromatograph (Shimadzu, Tokyo, Japan) equipped with a Model LC-10 AT VP pump, a SPD-10A VP multiwavelength detector operated at 280 nm and a sensitivity setting of 0.05 absorbance units full scale (AUFS) and a C-R6A Chromatopac integrator was used to record chromatograms and to calculate peak areas. A Rheodyne (Berkeley, CA, USA) model 7725i injection valve with a 20 μL loop was used. Chromatographic separations were carried out on a Hypersil C_{18} column (150 × 4.6 mm i.d.; particle size 5 μm; Dalian Elite Scientific Instruments Co. Ltd, China) operated at 30°C.

Standards and reagents. Pyridoxine hydrochloride (99%) and riboflavin (99%) were supplied by Shanghai Chemical Reagents Co. Ltd. (Shanghai, China). Nicotinamide and thiamine mononitrate were supplied by Shanghai no. 2 Chemical Reagents Co. Ltd (Shanghai, China) and Tianjin Zhongjin Pharmaceutical Co. Ltd (Tianjin, China), respectively. Sodium hexanesulfonate was supplied by Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). HPLC-grade methanol was used to prepare the mobile phase. All chemicals, except where otherwise stated, were of analytical grade, and water used in this assay was doubly distilled. Multivitamin with minerals tablets were provided by the Nanjing Doyea Pharmaceutical Co. Ltd (Nanjing, China).

Mobile phase. The mobile phase consisted of methanol-aqueous 0.5% acetic acid solution containing 2.5 mM sodium hexanesulfonate (18:82, v/v). The mobile phase was filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and was then degassed before use. The flow rate of the mobile phase was 1.2 mL/min.

Preparation of solutions. The stock standard solution was prepared by transferring about 100 mg of nicotinamide reference standard, 15 mg of pyridoxine hydrochloride reference standard, 10 mg of thiamine mononitrate reference standard and 10 mg of riboflavin reference standard, each accurately weighed, to a 100 mL volumetric flask, and add about 80 mL of 65°C mobile phase to the flask. The flask was shaken in a hot water bath maintained at 65°C for approximately 15 min, until all the solid materials were dissolved. It was then chilled rapidly in a cold water bath to room temperature, diluted with the mobile phase to volume, and mixed. This standard stock solution was stored in a refrigerator.

Sodium thiosulphate solution was prepared by transferring 1 g of sodium thiosulphate to a 100 mL volumetric flask, dissolved and diluted with water to volume and mixed.

Analytical procedure. An accurately weighted and counted number of the tablets were ground to a fine power. An accurately weighed quantity of the powdered tablets, equivalent to 20 mg of nicotinamide, was transferred to a 250 mL flask. About 20 mL of sodium thiosulphate solution and 60 mL of water at 65°C were added to the flask and shaken in a hot water bath maintained at 65°C for approximately 15 min. After cooling to room temperature the contents of the flask were quantitatively transferred to a separate 100 mL volumetric flask. The flask was rinsed with water, and the rinsing was added to the volumetric flask, diluted with water to volume and mixed. A partion of the solution was filtered through a 0.45 μm membrane filter and the clear filtrate was used in as the HPLC assay.

RESULTS

Chromatographic separation

Figure 1 shows typical chromatograms of reference standard and sample. Under the chromatographic conditions described, nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin had retention times of approximately 2.4, 4.4, 12.3 and 15.2 min, respectively. It can be seen from Fig. 1 that good separation and detectability of the multivitamin with minerals tablets were obtained with baseline resolved peaks and chromatograms with minimal interference from the tablets in less than 17 min.

Precision

The reproducibility of the present method was assessed by analyzing the tablet samples seven times a day for five consecutive days. The results showed that the average coefficient of variations of within-day and between-day assays were 2.2 and 3.6% for thiamine mononitrate, 1.8 and 2.4% for riboflavin, 1.3 and 1.7% for pyridoxine

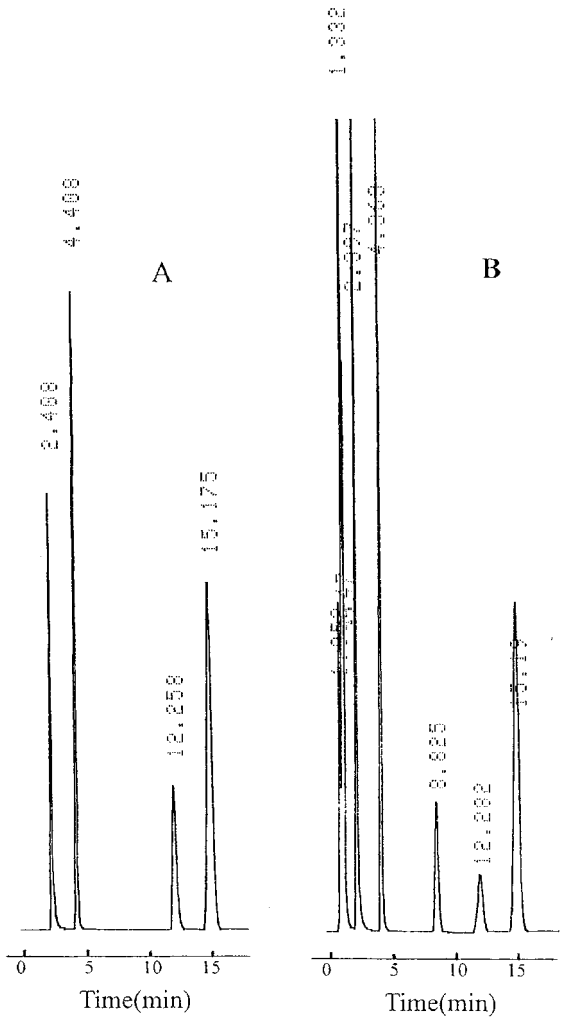

Figure 1. Chromatograms of (A) mixture of nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin. (B) multivitamin with minerals tablets. Nicotinamide (2.4′); pyridoxine hydrochloride (4.4′); folic acid (8.8′); thiamine mononitrate (12.3′); riboflavin (15.2′).

hydrochloride and 1.0 and 1.5% for nicotinamide analysis, respectively.

Linearity

A series of samples containing 5, 15, 30, 45, 60, 75 and 90 μg/mL of thiamine mononitrate, 2.5, 15, 30, 45, 60, 75 and 90 μg/mL of riboflavin, 5, 20, 35, 50, 65, 80 and 95 μg/mL of pyridoxine hydrochloride and 25, 75, 150, 225, 300, 375 and 450 μg/mL of nicotinamide were prepared to study the relationships between the peak area and the concentrations of the vitamins under selected conditions. The results showed that the peak area was linearly related to the thiamine mononitrate for the range of 5–90 μg/mL. The linear equation for the concentration vs peak area was $y = 3503.5\,x + 1071.1$ with a correlation coefficient of 0.9999. For the riboflavin, the linear range was 2.5–90 μg/mL and the linear equation was $y = 12313\,x + 4637.2$ with a correlation coefficient of 0.9997. For pyridoxine hydrochloride, the linear range was 5–95 μg/mL and the linear equation was $y = 5717.3\,x + 432.9$ with a correlation coefficient of 0.9999. For nicotinamide, the linear range was 25–450 μg/mL and the linear equation was $y = 722.5\,x + 4609.8$ with a correlation coefficient of 0.9998.

Recovery

In order to determine the accuracy of the method recovery studies were carried out. Known amounts of each vitamin were added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence. All analyses were carried out in five samples at three concentration levels. The results are given in Table 1.

Applications

The performance of the proposed method was tested by applying it to the assay of nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin in multivitamin with minerals tablets each obtained from 10 tablets from five different batches of commercial tablets. Samples were prepared as described previously and injected in duplicate, The results was summarized in Table 2.

DISCUSSION

To achieve the optimum resolution of the vitamins, the effects of the composition and the acidity of the mobile phase, and sodium hexanesulfonate concentration in the

Table 1. Recovery studies of vitamins in multivitamin with minerals tablet samples ($n = 5$)

Vitamin	Amount added (μg/mL)	Amount found (μg/mL)	Average recovery (%)	RSD (%)
Vitamin PP	160.0	161.2	100.8	1.4
	200.0	201.9	101.0	1.1
	240.0	238.9	99.5	1.2
Vitamin B_6	12.0	11.8	98.3	1.7
	24.0	24.1	100.4	1.1
	36.0	35.3	98.1	1.0
Vitamin B_1	7.5	7.3	97.3	3.6
	15.0	14.5	96.7	3.4
	22.5	21.8	96.9	2.1
Vitamin B_2	8.5	8.1	95.3	3.2
	17.0	16.6	97.6	2.6
	25.5	25.2	98.8	2.2

Vitamin PP, nicotinamide; vitamin B_6, pyridoxine hydrochloride; vitamin B_1, thiamine mononitrate; vitamin B_2, riboflavin.

Table 2. Application of the proposed method to the analysis of multivitamin with minerals tablets from different batches

Batch no.	Nicotinamide (mg/tablet)	Pyridoxine hydrochloride (mg/tablet)	Thiamine mononitrate (mg/tablet)	Riboflavin (mg/tablet)
1	23.26	2.51	1.56	1.89
2	21.44	2.31	1.43	1.73
3	20.22	2.17	1.32	1.60
4	23.41	2.54	1.64	1.94
5	22.58	2.20	1.39	1.62

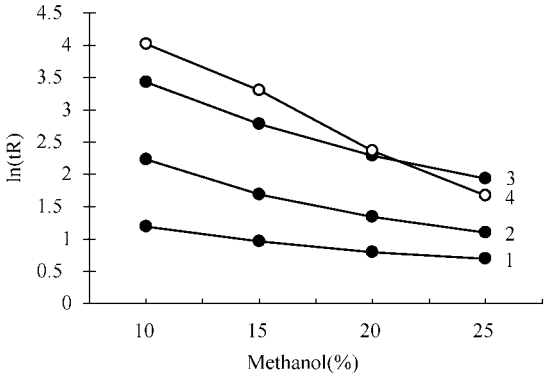

Figure 2. Dependence of the retention times of nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin on the methanol composition. **1**, nicotinamide; **2**, pyridoxine hydrochloride; **3**, thiamine mononitrate; **4**, riboflavin.

mobile phase on the chromatographic separation were investigated. The results indicated that optimum resolution for nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin in the multivitamin tables was obtained when the mobile phase was composed of methanol and 0.5% acetic acid solution (18:82, v/v; containing 2.5 mM sodium hexanesulfonate, pH = 2.8). The retention times of the thiamine mononitrate and riboflavin were obviously prolonged with decreasing methanol content. Moreover, the responses of peak area tended to decrease, too. However, the retention times of nicotinamide and pyridoxine hydrochloride changed only slightly with varying methanol content (Fig. 2). On the other hand, the retention times of pyridoxine hydrochloride, thiamine mononitrate and riboflavin changed noticeably with varying acidity of the mobile phase from pH 2.5 to 3.3. However, the effect of acidity of the mobile phase on the retention time of nicotinamide was not obvious.

A study was made on the effect of sodium hexanesulfonate concentration in the mobile phase on the chromatographic peaks at a given acidity and composition. The results indicated that the retention time of riboflavin was reduced with a decrease in the salt concentration. In contrast, the retention time of thiamine mononitrate was reduced with an increase in the salt concentration. On the other hand, the retention times of nicotinamide and pyridoxine hydrochloride were unaltered.

On the basis of these results the chromatographic parameters recommended together with the 150 × 4.6 mm i.d. Hypersil C_{18} column for this procedure were as follows: the concentration of sodium hexanesulfonate solution was selected as 2.5 mM and the pH was adjusted to 2.8. The ratio of methanol and aqueous 0.5% acetic acid solution was selected as 18:82 (v/v). Under the selected chromatographic condition, nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin can be separated isocratically within 17 min with baseline resolved peaks.

CONCLUSION

The method provides excellent recovery and good precision, and is simple and reliable in both chromatographic condition and sample preparation. Furthermore, the analytical procedure is easy to handle and is very suitable for routine determination of a large number of samples. It has been successfully applied to the simultaneous determination of nicotinamide, pyridoxine hydrochloride, thiamine mononitrate and riboflavin in multivitamin with minerals tablets.

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