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Sensitivity of UV detection in simultaneous separation and detection of B-vitamins using HPLC

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Abstrak. Satu teknik HPLC menggunakan media fasa balikan (C18) berserta reagen pasangan-ion telah dibangunkan bagi menganalisis secara serentak kumpulan vitamin B dalam makanan, seperti tiamin (B1), asid nikotinik (B3), riboflavin (B2), piridoksal (PL) dan piridoksin (PN). Pengesan UV telah digunakan kerana kos yang rendah, kestabilan yang baik dan kepekaan yang tinggi, berbanding dengan pengesan yang lain. Kaedah ini telah dinilai dari segi kejituan, kepekaan dan kepekaan, berdasarkan kepada had pengesanan (LOD) dan had penentuan nilai (LOQ). Analisis HPLC telah dilaksanakan dengan menggunakan turus pendek (C18, 150mm x 3.9mm; padatan 3µm, dengan 15% muatan karbon) dan fasa bergerak methanol : air (30:70) dengan 0.1% TEA dan mengandungi 5mM natrium oktansulfonat sebagai agen pasangan-ion. Pemisahan lengkap kumpulan vitamin B ujian telah dapat dicapai dalam masa 20 minit. Masa retensi bagi setiap vitamin B adalah 2.34 minit (B3), 3.46 minit (PL), 4.12 (PN), 7.51 (B1) dan 13.59 (B2). Dengan menggunakan pengesan UV pada 254nm, had pengesanan bagi kumpulan vitamin B adalah 0.06µg/ml (B3), 0.09 µg/ml (PL), 0.35 µg/ml (PN), 0.17 µg/ml (B1) dan 0.22 µg/ml (B2). Pekali variasi untuk kesemua analisis adalah kurang dari 2%. Julat pengesanan yang linear bagi pengesan UV 254 adalah 1- 100 µg/ml. Walaupun kepekaan untuk B1 adalah lebih rendah berbanding dengan vitamin yang lain, secara keseluruhan pengesanan dengan UV254 mampu memberikan kepekaan yang cukup tinggi untuk digunakan bagi analisis kesemua kumpulan vitamin B dalam makanan.

Abstract. A HPLC technique using reversed phase media (C18) with ion-pair reagents was developed to simultaneously separate the common B-vitamins, namely, thiamin (B1), nicotinic acid (B3), riboflavin (B2), pyridoxal (PL) and pyridoxin (PN), in food. UV detection was employed due to its low-cost, high stability and sensitivity when compared to other mode of detection. The method was tested for its precision, sensitivity and accuracy based on limit of detection (LOD) and limit of quantification (LOQ). Employing a short column (C18, 150mm x 3.9mm; 3µm particle, 15% carbon loading) and addition of ion pairing reagent (5mM sodium salt of octanesulfonic acid) in the mobile phase (methanol : water (30:70) and 0.1% TEA), complete separation were achieved after 20 minutes. The retention times for each vitamins were 2.34 min (B3), 3.46 min (PL), 4.12 min (PN), 7.51 min (B1) and 13.59 min (B2). Using UV 254, the detection limit for B-vitamins were 0.06µg/ml for B3, 0.09µg/ml for PL, 0.35µg/ml for PN, 0.17µg/ml for B1 and 0.22 µg/ml for B2. The coefficient of variation for all analyses were less than 2%. The UV254 gave linear detection within the range of 1 to 100 µg/ml. Although the sensitivity for B1 was slightly lower than the others, the overall results showed that the UV254 detection was sufficiently sensitive to measure all the vitamin Bs in food.

Keyword: Vitamin B, HPLC analysis, UV detection, limit of detection, limit of quantification

Introduction

Vitamin Bs are crucial for the maintenance of good health, due to its roles in the metabolism of carbohydrate and proteins. Due to their importance, various instrumental methods have been devised to determine their presence, qualitatively and quantitively. These methods include the spectrophotometry [1], fluorimetric methods [2], selective membrane electrode [3] and chromatographic methods [4]. The official methods are based on microbiological and chemical methods. However, with the rapid developments in chromatographic methods, especially in column packing materials, tremendous advances have been made in vitamin B analyses using HPLC. Several types of detectors have been employed, but the most common have been the UV and fluoresence detectors. Lack of fluoresence has led to derivatisation reactions to increase sensitivity [5, 6, 7, 8]. More often than not, this has led to complicated pre-column reaction steps and/or more expensive equipment for post-column derivatisation. UV detectors are known for its wide availability, low-cost (initial purchase and operation), stability and sensitivity. Numerous papers have been published for the analysis of B-vitamins in pharmaceutical preparations and model systems and food analysis [9, 10, 11, 12, 13]. Thus, the objective of this paper is to investigate the application of HPLC in the analysis of B-vitamin in food, with special emphasis on the suitability and sensitivities of UV detectors for such purpose.

Materials and Methods

Standard Solution

Individual vitamin B standards, comprising of anhydrous riboflavin (B2), pyridoxal monohydrochloride (PL), pyridoxin monohydrochloride (PN), thiamine monohydrochloride (B1) and nicotinic acid (B30), were obtained from Sigma Chemicals Co., USA. Prior to solubilisation, the vitamins were dried overnight over P_2O_5 in a dissicator. Individual standards solution of each vitamin was prepared at 100µg/ml in 0.1N HCl while standard solution vitamin mixture was prepared at 50µg/ml for each vitamins.

HPLC System

The HPLC analyses was performed using a Metaphase reversed phase C18 column (3.9mm x 150mm; 3µ particle, endcapped) connected to a Rheodyne 7125 manual injector (Rheodyne Inc., Cotati, CA) with 20µl sample loop on Waters 501 HPLC pump running at 1.0 ml/min. The detector was Waters 484 (Waters Corp., Milford, MA 01757) variable wavelength detector set at 254nm.

Mobile Phase

The mobile phase comprised of methanol : water (30:70). However, the water component was actually buffer solutions at pH 5.5 containing 5mM sodium salt of octanesulfonic acid (PIC-B8) paired-ion reagent and 0.1% triethanolamine (TEA) base modifier.

Standard Curve

Each vitamin was injected (8 times) over a concentration range of 1µg/ml to 100µg/ml, and the peak area were determined. From the data obtained, a standard curve of peak area against concentration was plotted. The linear equation was determined.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The estimates were based on method proposed by an earlier investigator [14]. To determine the LOD, the vitamins were diluted to a range from 0.1µg/ml to 8.0µg/ml while for LOQ determinations, the concentration range were 1µg/ml to 20µg/ml. LOD was reached when signal/noise (S/N) ratio is 3, while LOQ was defined as the point where S/N = 10.

Extraction of Vitamin B

The method was tested to determine the vitamin B contents in several food systems. Vitamin Bs were extracted from pharmaceutical preparation (capsule), solid food (sliced bread, breakfast cereal) and liquid food (malt drink). Extraction method from solid food was according to Chase et al.,[8] while extraction from liquid food was according to Gennaro [12].

The efficiency of the extraction method was first determined by extracting known amount of vitamin B spiked into vitamin B capsules.

Results dan Discussion

Resolution (R_s) is related to column efficiency (N), selectivity (α) and capacity (k') and all these factors can be expressed in the following equation.

$$R_s = \frac{\sqrt{N}}{4}\left[\frac{\alpha - 1}{\alpha}\right]\left[\frac{k'}{1 + k'}\right]$$

The classic equation for determination of resolution showed that resolution can increased by increasing the capacity factor, k'. The k' factor is related to solvent polarity. However excessive increase in R will cause an unacceptable increase in retention time (t_R) and peak broadening. For isocratic separation, $1 < k' < 20$ are most suitable [15].

The solvent system formulated for these analyses, even running under isocratic condition, gave very good separation, with resolution between each peak is always higher than 5 with k' ranging from 1.68 to 20.32 (Table 1). The combination was obtained after exhaustive testings of various types of solvents, modifiers and paired-ion reagents.

Table 2 shows that the detector a very linear response at concentration range of 1µg/ml to 100µg/ml, for each vitamin analysed. The standard deviation for eight independent determinations were very low. The sensivity shown by the UV detection is more than adequate to determine the concentration of these vitamins in food systems. However the method may no be adequate for determinations of vitamin B in nanogram levels.

Table 1 : The Effect of capacity factor (k') on the resolution of vitamin B separation

Vitamin	Capacity Factor (k')	Retention time (t_R)	Resolution (R_s)
Nicotinic acid (B3)	1.68	1.82	5.9
Pyridoxal (PL)	3.54	3.07	8.1
Pyridoxin (PN)	5.98	4.72	21.9
Thiamine (B1)	14.57	10.19	10.0
Riboflavin (B2)	20.32	14.43	

Replicates (n) = 8

Table 2 : Calibration Curve Characteristics

	Line Equation	R	SD
Nicotinic acid (B3)	Y= 32.05x – 0.001	0.9998	0.28
Pyridoxal (PL)	Y = 31.66x + 0.059	0.9999	0.73
Pyridoxin (PN)	Y =16.09x – 0.002	0.9998	0.19
Thiamine (B1)	Y = 39.35x + 0.14	0.9980	1.22
Riboflavin (B2)	Y = 34.72x – 0.082	0.9999	0.67

Replicates (n) = 8

Table 3 : Sensitivity of UV Detection at 254nm

	Peak area at S/N = 3 mVs^{-1} (n= 8)	Limit of Detection, LOD (μg/ml)	Peak area at S/N = 10 mVs^{-1} (n= 8)	Limit of Quantification, LOQ (μg/ml)
Nicotinic acid (B3)	1.82 ± 0.18	0.06	6.06 ± 0.56	0.19
Pyridoxal (PL)	2.73 ± 0.47	0.09	9.40 ± 1.30	0.32
Pyridoxin (PN)	5.64 ± 0.72	0.35	18.90 ± 2.56	1.18
Thiamine (B1)	5.79 ± 0.74	0.17	18.78 ± 2.11	0.54
Riboflavin (B2)	8.00 ± 0.82	0.22	26.52 ± 3.31	0.74

Table 4 : Estimation of Vitamin B Concentrations from Test Samples

	Actual Concentration (μg/ml)	Mean Value from analyses* (mg/ml)	Recovery (%)	Standard Deviation	Coefficient of Variation (% CV)
Nicotinic acid (B3)	10.00	10.05	100.05	0.07	0.74
Pyridoxal (PL)	10.00	10.01	100.01	0.13	1.19
Pyridoxin (PN)	10.00	10.10	100.10	0.16	0.84
Thiamine (B1)	10.00	10.03	100.03	0.12	0.97
Riboflavin (B2)	10.00	9.99	99.9	0.20	0.87

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for the method are shown in Table 3. The best LOD was for nicotinic acid (0.06 μg/ml) and the worst response was obtained for pyridoxin (0.35 μg/ml). The smallest variation in detection was shown in the determination of nicotinic acid. Hence, it can concluded that nicotinic acid can be reliably quantified (LOQ) at 0.19μg/ml, with the detector giving S/N ratio of 6.06 mVs^{-1}. Even so, the LOQ were still sufficiently low, as to make the UV detection a very useful and cheap method for the determination of vitamin Bs in foods and pharmaceutical preparations. Using optimum UV absorption for each vitamin, LODs of 0.05μg/ml for thiamine and 0.02μg/ml for the other vitamins can be obtained [10]. At microgram level, the low operation cost, stability and simplicity afforded by UV detection is unsurpassed. However, at nanogram level, UV detection can be sensitized by the formation of derivatives. If these steps are to be carried out, then it

is better to employ flouresence detector. The latest method [17] employing a sophisticated flow injection and solid phase on-line spectrofluorometric determination of vitamin B6 (pyridoxine) has obtained LOD value of 5.7ng/ml. However the system was developed for a high throughput on-line determination of vitamin B in pharmaceutical preparations. Using similar system and coupled with post-column derivatization with o-phthalaldehyde managed to determine thiamine and other pharmaceutical preparations at close to 0.1ng.ml^{-1}, and linearity in the range of 0.2 ± 6ng.ml^{-1} [18]

The critical part in the determination of vitamin B in a complex matrix, such as food, has always been the ability of extraction procedure to completely release all vitamin B bound in cellular matrices [16]. For the present study, extraction was only limited to pure vitamin solutions of pharmaceutical preparation. As shown in Table 4, the recoveries obtained were quite good, indicating that the extraction procedure employed was satisfactory. The coefficient of variation (%CV) of eight independent determinations showed less than 1%, with the exception of pyridoxal which showed a %CV of 1.19. Still, the value was accurate enough.

Conclusion

The solvent system made of methanol : water (30:70) with 0.1% TEA, 5mM PIC B8 (Na-salt of octane sulfonic acid), pH 5.5 gave good separation (Resolution > 5), under isocratic condition, with relatively short analysis time (16 minutes) for individual vitamins in a mixture of five B-vitamins. The column was a short 150mm x 3.9mm packed with reversed phase C18 3µ particle.

Using UV 254, the detection limit for B-vitamins were 0.06µg.ml for B3, 0.09µg/ml for PL, 0.35µg/ml for PN, 0.17µg/ml for B1 and 0.22 µg/ml for B2. The coefficient of variation for all analyses were less than 2%. The LOQ range from 0.19 – 1.18µg/ml. The detection system showed good linearity from 1µg/ml to 100µg/ml for the individual vitamin.

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