Controlling the retention and separation of water–soluble vitamins on zwitterion column by hydrophilic interaction echanism

- **Nguyen Ngoc Vinh**
- **Nguyen Thi Thuy Luyen**
- **Nguyen Tien Giang**
- **Nguyen Huy Du**
- **Nguyen Anh Mai**

University of Science, VNU–HCM

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ABSTRACT

Chromatographic conditions including pH, type and concentration of buffer for separation of five B vitamins on ZIC-HILIC column were investigated. Ammonium formate, ammonium acetate and ethylendiamonium chloride were used as buffer salts in the study. The results revealed that pH and buffer identity had significant effects on charged analytes. Indeed, ethylenediamonium competed very well with positively-charged analytes when the excessive retention was the problem while pH could control the charge state of the vitamins. The quanlity of acetonitrile was also employed to control the elution power in general. The mobile phase containing 10 % 40 mM ethylendiamonium chloride at pH 2.5 and 90 % ACN by volume was found to be the most suitable to separate the vitamins. The calibration curves had the R2 > 0.997, % RSD of retention time and peak areas in most of the cases were lower than 1% and 3.2 %, respectively. The developed method was applied to determine the B vitamins in Trivita BF, a commercial pharmaceutical product.

***Keywords**: hydrophilic interaction chromatography, water-soluble vitamin, ZIC-HILIC column*

INTRODUCTION

Water-soluble vitamins including C and B vitamins (B1, B2, B3, B6, B9, B12…) are of biological importance. They have polarity from medium to high with negative $\log K_{o/w}$ thanks to polar functional groups eg. hydroxyl, amine, carboxylic acid in the molecules. Some B vitamins eg. B1 and B12, carry permanent charge.

The analysis of the water-soluble vitamins has been performed in reversed-phase chromatography mode. However, due to the high polarity most of them were eluted very early on C18 columns and that could cause problems when analyzing samples with complex matrices [1-3]. Ion-pairing reagents have been used to increase the retention and better separations could be achieved [4]. Though normal-phase chromatography with polar stationary phase has been used for polar analytes it cannot be used for water-soluble vitamins due to their low solubility in the non-aqueous mobile phases [5].

Hydrophilic interaction chromatography (HILIC) has become a separation mode of choice for very hydrophilic analytes. The mobile phase containing up to 40 % water promotes the dissolution of these compounds in comparison to non-aqueous mobile phases of normal-phase chromatography. HILIC stationary phases possess polar groups (eg. diol, cyanopropyl, amide, ionic groups…) grafted onto silica or organic polymeric

supports and mobile phases consist of acetonitrile (ACN) in most of the case and an aqueous buffered solution. In HILIC analytes partition is occurred between the bulk organic-rich mobile phase and the water-rich layer immobilized on the stationary phase. Other interactions eg. hydrogen bonding, electrostatic attraction, dipole-dipole interaction can also involve in the retention mechanism [6]. ZIC-HILIC stationary phase with sulfobetain functional groups, a zwitterion, is able to interact with all type of polar compounds from electrically neutral to anions and cations (Fig.1). Therefore, it was expected a suitable column for water-soluble vitamins.

In this work, an analytical procedure was developed using ZIC-HILIC column and UV detection for the determination of five B vitamins including B1 (thiamine), B2 (riboflavin), B3 (nicotinamide), B6 (pyridoxine), and B9 (folic acid) (Fig. 2)

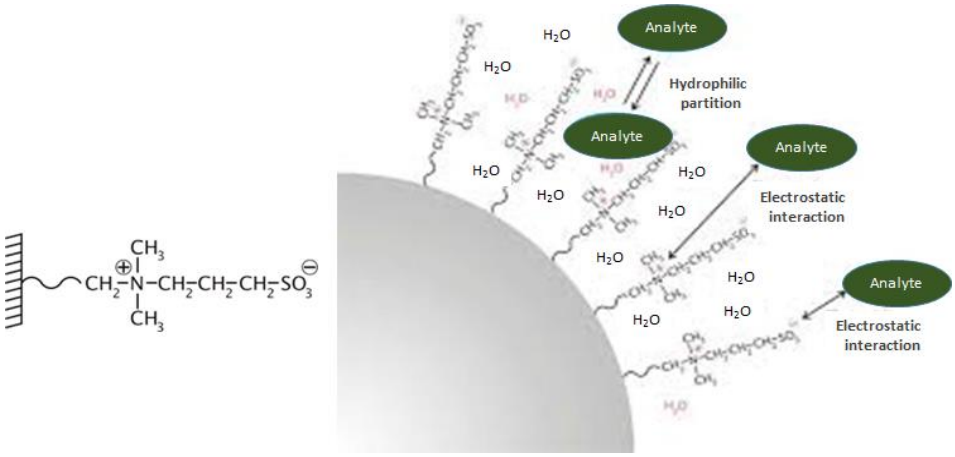

Fig. 1. Sulfobetain functional group and types of interactions of the stationary phase

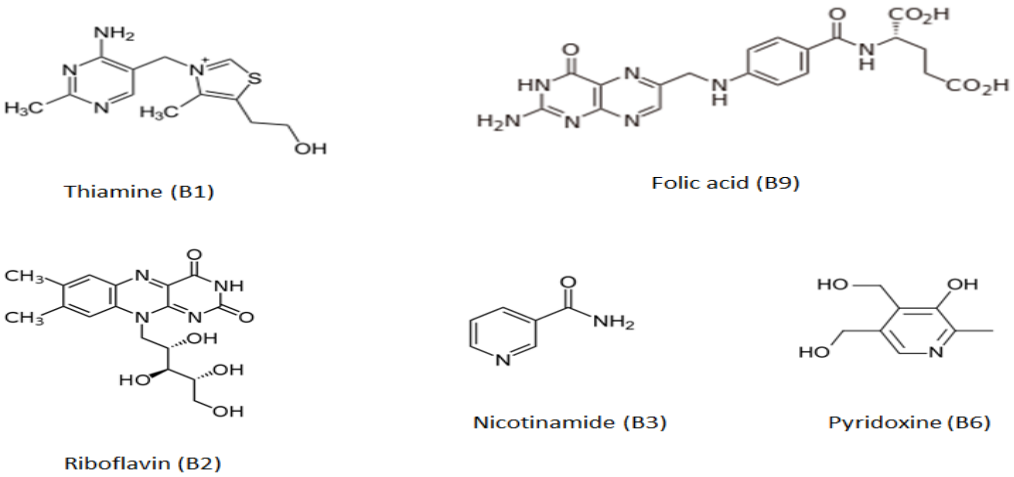

Fig. 2. Chemical structures of B vitamins involved in this study

MATERIALS AND METHOS

Chemicals and reagents

All chemicals and reagents were of analytical grade and used as obtained. Ammonium acetate, ammonium formate solutions were prepared from acetic acid, formic acid (Merck) and ammonia (Merck). Ethylenediammonium dichloride solution was prepared by adding HCl (Prolabo) to ethylenediamine (Merck) to the desired pH values. B vitamins namely, thiamine (B1) (95.6 %), riboflavin (B2) (99.3 %), nicotinamide (B3) (97.5 %), pyridoxin (B6) (99.7 %), folic acid (B9) (91.5 %), cyanocobalamin (B12) (92.3 %) were purchased from Institute of Drug Quality Control, Ho Chi Minh City, Viet Nam. Acetonitrile (ACN) of HPLC grade was a product of Labscan, Thailand. Double-distilled water filtered through a 0.45 µm filter membrane was used throughout the study.

Standard solutions and sample preparation

Stock solutions of the vitamins B (~400 mg/L) were prepared by dissolving appropriate amounts of the analytes in ACN:H_2O (1:1, v/v). For vitamin B2 and B9, ammonia was added to aid the dissolution. Stock solutions were stored in brown flasks at 4–8 ^oC for not more than 4 days. Working solutions were prepared daily by diluting the stock solutions with mobile phase.

For determination of B vitamins in trivita BF, 20 pills were weighed to calculate average mass of a pill before being ground and mixed thoroughly. An amount corresponding to a pill was weighed in a 100 mL flask, the dissolution in the mobile phase was aided by sonication for 15 min. The sample solution was further diluted to appropriate concentrations for chromatographic determination.

All solutions were filtered through a 0.45-µm filter membrane before injection.

Instrumentation and chromatographic conditions

A Shimadzu 20A HPLC system equipped with LCsolution software, UV detector at 254 nm and manual injection of 20 µL was used in all experiments. ZIC-HILIC column (150 mm × 4.6 mm, 5 µm particle size) from Merck-SeQuant was used for the separation of the vitamins.

The mobile phases consisted of (1) solvent A: aqueous buffer salt (ammonium formate, ammonium acetate or ethylenediamonium chloride) at various concentrations and pH values and (2) solvent B: ACN. The mobile phases were filtered through a 0.45 µm membrane and degased by sonication prior to use. All chromatographic runs were conducted under isocratic condition.

RESULTS AND DISCUSSION

Effect of buffer and pH on the retention of B vitamins

From our previous studies the identity and concentration of buffers in the mobile phase play an important role in the retention of analytes on the sulfobetain stationary phase [7]. Cations and anions originated from the buffers tend to concentrate around sulfonate and tertiary amine groups of the stationary phase. This result in a thicker water-rich layer which promotes the retention. In the other hand these ions decrease electrostatic interactions between charged analytes and the fixed charges of the stationary phase. Besides buffers, pH can also affect the retention by altering the charge state of analytes. In this study, therefore, buffer and pH were the two main factors to be focused on.

Mobile phase containing ammonium formate and ammonium acetate

The development of analytical method for B vitamins was started with typical HILIC mobile phase containing volatile buffers namely, ammonium formate and ammonium acetate.

Initial experiments with ammonium formate were conducted in isocratic mode with various concentration of the buffer and pH while the

volume ratio of ACN to aqueous phase was kept constant at 80:20. Conclusions on the impacts of pH and buffer on the retention mechanism of the target analytes could be drawn as follows:

- B2 (pK_a = 9.9) was retained somewhat stronger than B3 (pK_a = 3.3) though the mobile phase with 20 % aqueous phase was too strong for both B2 and B3 because their retention times were only of ca. 3.2–3.6 min. This could be a result of their charge state in the pH range of 3–7. Indeed, B2 had a positive charge (+1) while B3 was almost neutral. It was hardly seen the effect of pH and buffer concentration on the retention of B2 and B3, that would be properly because they were eluted too fast and their charge state do not change under the investigation conditions (Fig. 3). In order to separate B2 and B3 mobile phases with weaker elution power were desired (ie. lower % ACN).

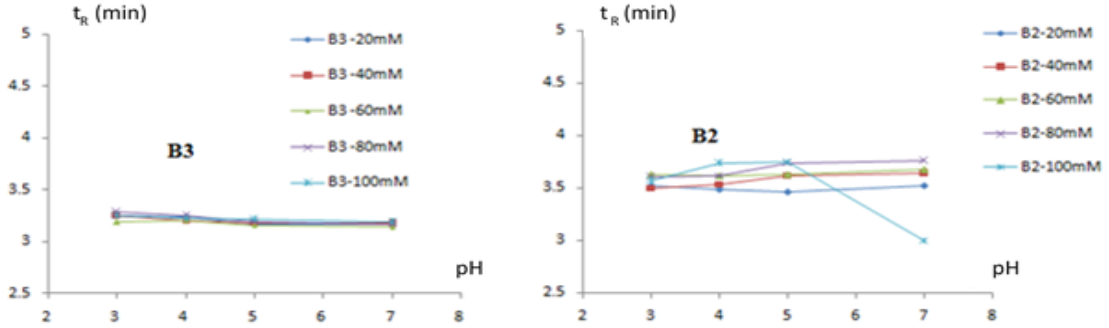

Fig. 3. Effect of pH and $HCOONH_4$ concentration on the retention of vitamin B2 and B3 (ACN:buffer = 80:20, v/v)

- B6 and B1 had higher affinity to the stationary phase in comparison to B2 and B3. While retention times of B6 were of 4–6 min, those of B1 were of 12–25 min depending on the pH. The result could be accounted by a permanent positive charge of the tertiary amine and the protonation of B1 at low pH to further promote the attraction of B1 to the sulfonate group of the stationary phase (pKa = 5.5) (Fig. 4). In comparison to B1, the B6 retained much less since the maximal charge that B6 can carry is +1 originated from the protonation of the molecule (pKa = 5.6). The protonation of B1 and B6 molecules was suppressed as pH increased which resulted in the shorter retention times. Sharing a general trend, retention times of B6 and B1 decreased as the concentration of the buffer increased indicated that the electrostatic interaction was dominated.

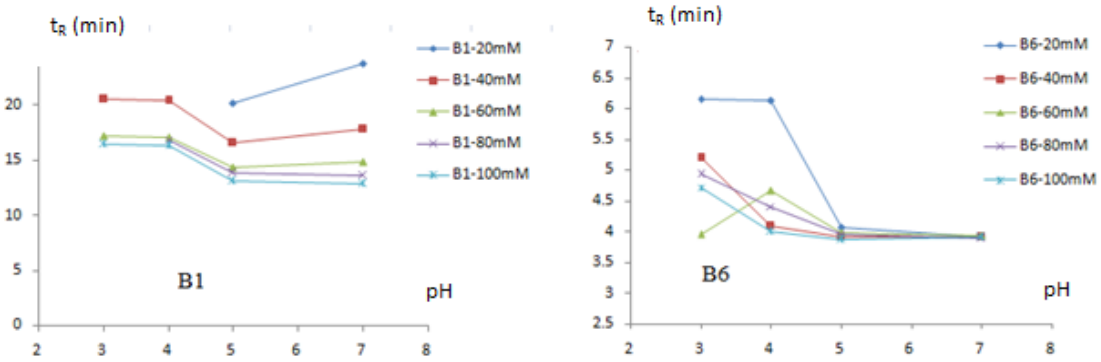

Fig. 4. Effect of pH and $HCOONH_4$ concentration on the retention of vitamin B1 and B6

- B9 experienced the strongest and complicated effect of pH and buffer concentration both on its peak shape and retention time. In general, the retention time of B9 increased significantly (Fig. 5) and the peak became much broader (Fig. 6) as pH increased. B9 can carry both negative charges from the dissociation of the two carboxylic groups (pK_{a1} = 4.7, pK_{a2} = 6.8) and a positive charge from the protonation of a basic group in the molecule (pK_{a3} = 9.0). These two type of charges are able to interact with the opposite charges of the zwitterion stationary phase. The peak shape distortion of B9 at pHs 5 or 7 could be a consequence of the interaction of the carboxylate groups with the positively charge site located in the inner space on the stationary phase which was more difficult to access because of the negatively-charged barrier of sulfonate.

With ammonium acetate as the buffer, similar effect and trends were observed for all B vitamins as ammonium formate (data not shown).

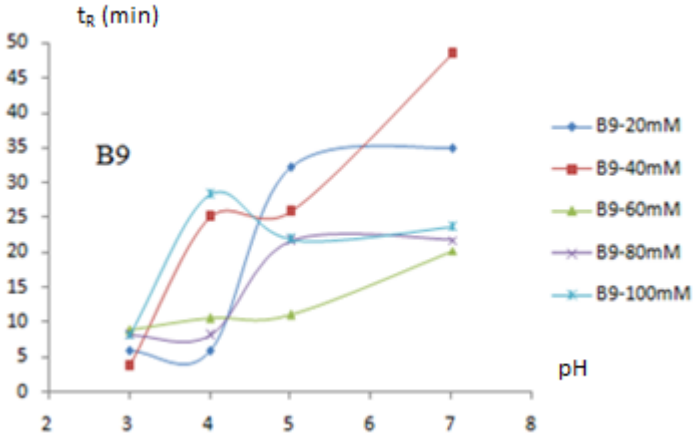

Fig. 5. Effect of pH and $HCOONH_4$ concentration on the retention of vitamin B9, ACN:buffer = 80:20, v/v

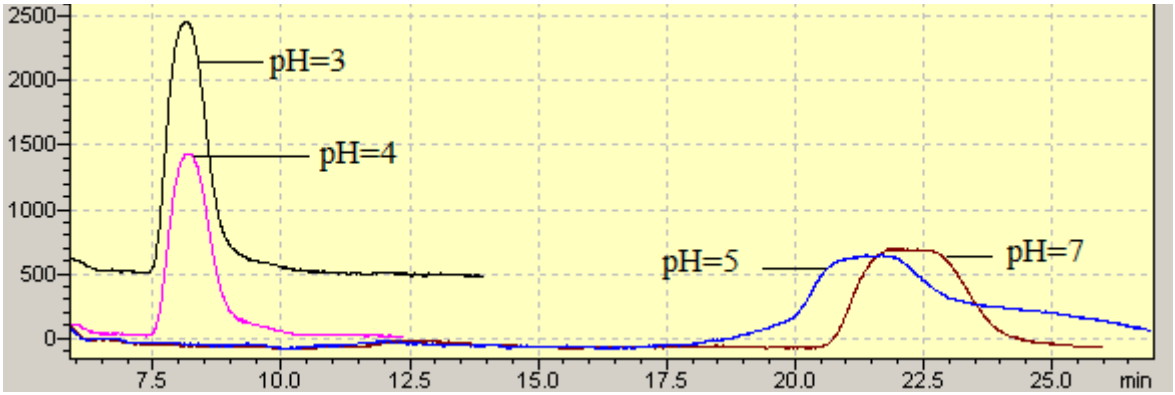

Fig. 6. Effect of pH on the peak shape of vitamin B9, ACN:80 mM $HCOONH_4$ = 80:20, v/v

The results from the above experiments suggested that appropriate mobile phases for the five B vitamins should had weaker elution power, ie. higher % ACN (> 85 %) to resolve the early eluting peaks of B2, B3 and low pH to decrease the retention of B9 as well as to improve its peak shape. However, B1 could not be eluted from the column with high % ACN mobile phase. To solve the problem cations of buffers must be highly charged (eg. +2) to compete with B1 for shorter analysis time.

Mobile phase containing ethylenediamine chloride

Mobile phase containing 10 % 40 mM ethylenediamine with pH adjusted to 3 by HCl and 90 % ACN was chosen to test the hypothesis. There was a better separation of the first 3 peaks B6, B3, and B2 although B6 and B3 were only partially overlapped (Fig.7a). Under this condition B1 was eluted at a reasonable retention time (34 min).

A closer look on the dependency of pH revealed that B6 was more sensitive to pH than B3. That could be resulted from the stronger basicity of B6 (pKa = 5.6) than B3 (pKa = 3.3). As can be seen from Fig.7b, at pH 3.8 B6 eluted later than B3 but the reversed elution order was appeared at pH as low as 3.0. We were a bit reluctant to further lower the pH because it was the lower limit usually recommended for silica-based columns. However, according to the producer the column can withstand alower pH in reversed-phase due to high content of ACN (90 %). Finally, at pH 2.5 a mobile phase contained 90 % ACN and 10 % 40 mM ethylenediamine aqueous solution was employed to completely separate all five B vitamins within 35 min (Fig.8).

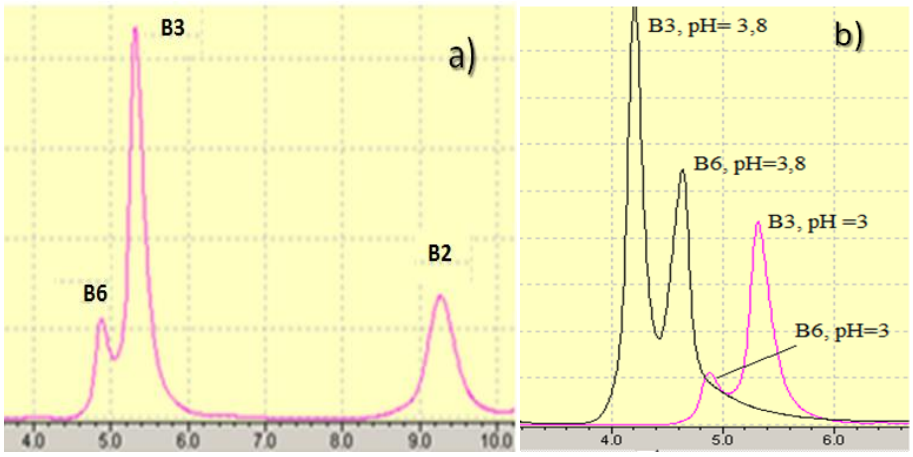

Fig. 7. The effect of pH on the retention of B3 and B6 mobile phase: 90 % ACN:10 % 40 mM ethylenediamine, pH adjusted to 3.0 (a); 3.8 (b)

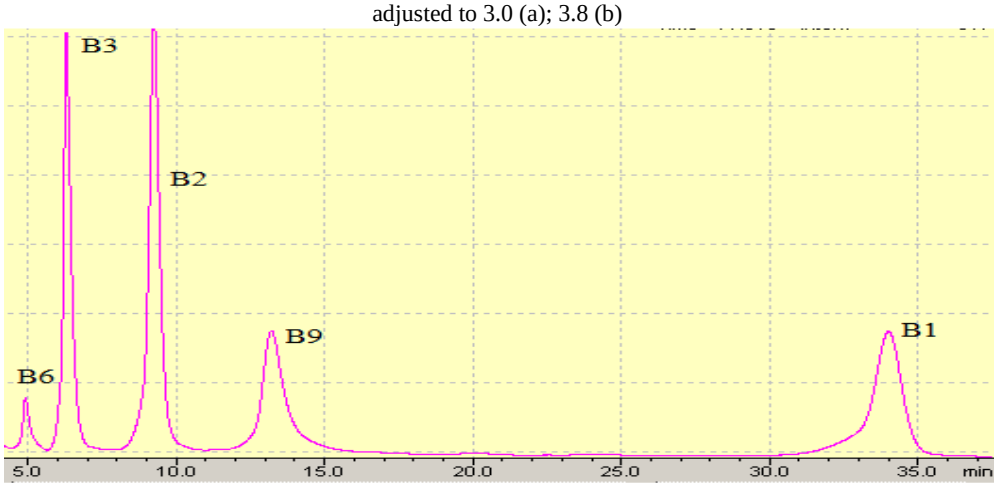

Fig. 8. Chromatogram of vitamins B1, B2, B3, B6, B9 with the mobile phase 40 mM EDA^{2+}:ACN = 90:10, v/v,

pH = 2,5

Method validation and application

Repeatability

A mixture of vitamin was analyzed six times under the optimum conditions to estimate the repeatability of retention time and peak area. Table 1 showed that the repeatability was fairly satisfactory except for B9 which could be a result of its low stability at low pH.

Calibration curves, limit of detection (LOD) and limit of quantitation (LOQ), Good linearity were obtained for all five B vitamins in the range ca. 0.5 – 100 ppm with $R^2 > 0.997$ (Table 2 and Fig. 9). LOD and LOQ were estimated as concentrations corresponding to the ratios of signal to noise of 3 and 10, respectively.

Table 1. Repeatability of retention times and peak areas of the B vitamins

Repeatability (% RSD)	B1	B2	B3	B6	B9
Retention time (t_R)	0.17	0.4	0.71	0.26	1.46
Peak area (A)	0.53	3.2	1.13	1.37	8.9

Table 2. Linearity, linear equation, LOD and LOD of the B vitamins

Vitamin	Regression equation	Correlation coefficient (R^2)	LOD (µg/mL)	LOD (µg/mL)
B1	y = 60547x + 64695	0.999	5.0	17.0
B2	y = 11629x + 64878	0.999	0.6	2.0
B3	y = 39128x + 60270	0.999	0.6	2.0
B6	y = 3795x + 3382	0.999	5.0	17.0
B9	y = 48873x - 19829	0.997	2.5	8.5

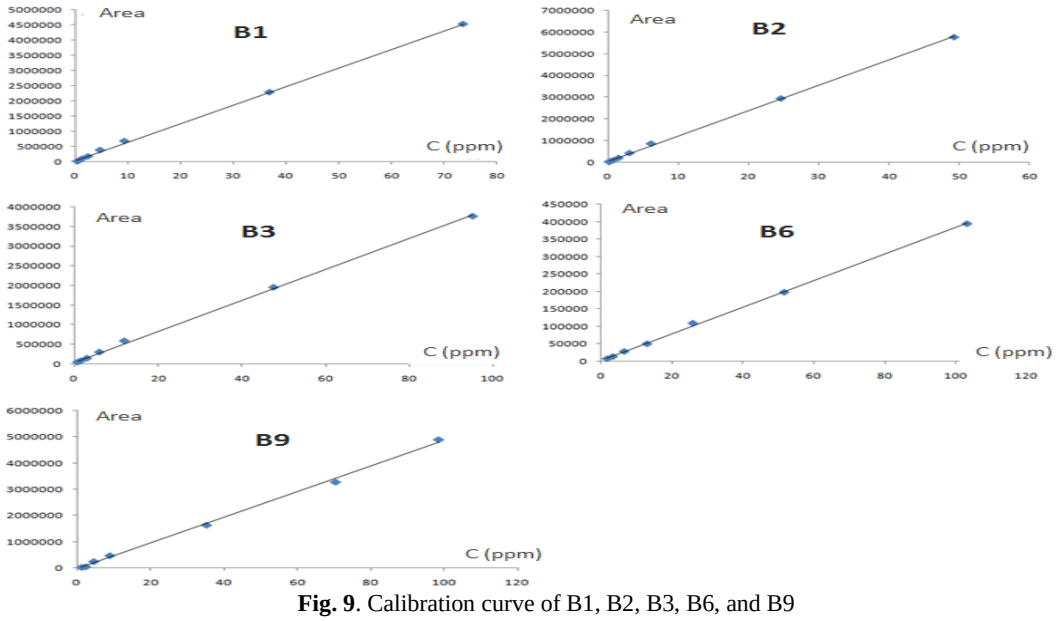

Fig. 9. Calibration curve of B1, B2, B3, B6, and B9

Application

The developed method was applied to the determination of vitamin B1, B2, and B6 in "Trivita BF" of Pharmedic company, Viet Nam. Chromatograms of the spiked sample with the vitamins are shown in Fig.10. The amounts of B1, B2 and B6 found in each pill were well agreed with those on the label (Table 3)

It should be noted that there were shifts in the retention times of the vitamins between the sample and the standard solutions, as can be seen in the case of B6 (Fig.10). From our experience with this column, the stationary phase is rather sensitive to the contaminants in the matrix, especially ions. With such a simple sample treatment ie. only dissolution and dilution with the mobile phase, the stationary phase might be contaminated with the matrix and change its property. In order to confirm the peak identity, spiked samples were chromatographed together with the sample.

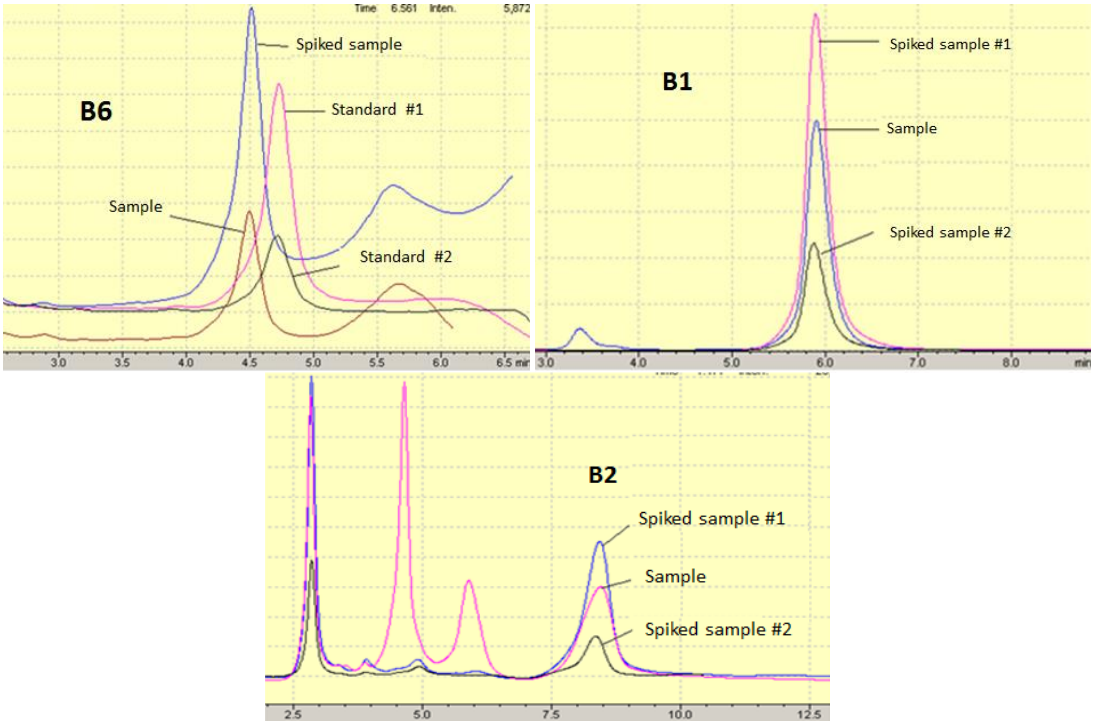

Fig. 10. Chromatograms of Trivita BF sample and the sample spiked with the standards corresponding to 150 % (#1) and 50 % (#2) of the labeled amounts

Table 3. Labeled and found amounts of three B vitamins in Trivita BF

Vitamin	Labeled amount (mg/pill)	Found amount (mg/pill)
B1	250	255
B6	250	266
B2	200	2.47

CONCLUSION

A method for determination of five B vitamins using ZIC-HILIC column was successfully developed. pH and buffer identity were found to be the most important factors to control the separation of the vitamins. The mobile phase consisted of 90 % ACN and 10 % 40 mM ethylendiamine with pH adjusted to 2.5 by HCl was found to be the most suitable to separate the vitamins. The calibration curves had R^2 > 0.997, % RSD of retention time and peak area in most of the cases were lower than 1 % and 3.2 %, respectively. The developed method was applied to determine the B vitamins in Trivita BF, a pharmaceutical products. However, to the sample treatment required further study to obtain more reliable results.

Điều chỉnh sự lưu giữ và tách các vitamin tan trong nước bằng cột zwitterion theo cơ chế tương tác ưa nước

- **Nguyễn Ngọc Vinh**
- **Nguyễn Thị Thùy Luyên**
- **Nguyễn Tiến Giang**
- **Nguyễn Huy Du**
- **Nguyễn Ánh Mai**

Trường Đại học Khoa học Tự nhiên, ĐHQG–HCM

TÓM TẮT

Điều kiện phân tích bằng sắc ký bao gồm pH, loại và nồng độ của dung môi giải ly để tách năm vitamin B trên cột ZIC-HILIC đã được khảo sát trong bài báo này. Khảo sát pH với các chất đệm như ammonium acetate, ammonium formate và ethylenediamonium chloride cho thấy chúng có ảnh hưởng mạnh đến các chất phân tích mang điện tích. Ethylenediamonium chloride có khả năng cạnh tranh với các vitamin mang điện tích dương bị lưu giữ rất mạnh trên cột trong khi thay đổi pH để kiểm soát điện tích của các vitamin. Pha động chứa 10 % ethylendiamonium chloride 40 mM ở pH 2,5 và 90 % acetonitrile là hệ pha động thích hợp nhất để tách các vitamin này. Đường chuẩn có R2 > 0,997, %RSD của thời gian lưu và diện tích mũi sắc ký trong hầu hết các trường hợp đều nhỏ hơn 1 % và 3,2 %. Phương pháp phân tích đã được áp dụng để xác định các vitamin B trong dược phẩm Trivita BF.

Từ khóa: *Sắc ký tương tác ưa nước, vitamin tan trong nước, cột ZIC-HILIC*

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