Separation and determination of some water-soluble vitamins in pharmaceutical preparation by IP RP-HPLC

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Abstract

A reversed phase(RP) HPLC with ion-pair reagent is used for simultaneous determinations of water-soluble vitamins (nicotineamide, nicotinic acid, folic acid, riboflavin (B_2) and thiamin (B_1)) in pharmaceutical preparations. The vitamins were analyzed using ZORBAX C_8 (25cm x 4.6mm i.d) analytical column, 6-8µm particle size, by isocratic elution with a mobile phase consisted of 20%methanol, buffered with 0.05M KH_2PO_4 (pH 3.5) , and $3x10^{-3}$M 1-octanesulfonic acid as an ion-pair reagent at a flow rate of (1.0ml/min.). The UV-Visible detection of the separated vitamins was made at 254nm for all water-soluble vitamins. The precision and accuracy of method were checked by calculating RSD% and relative error E%, which where found to be reasonable. A RSD% between (0.5-4.76) % and relative error between (0.05-4.93)%.The limit of detection range was found to be (0.0244, 0.0436µg/ml).

فصل و تعيين بعض الفيتامينات الذائبة بالماء في مستحضرات صيدلانية بكروماتوغرافية السائل ذات الاداء العالي(الطور العكوس)- المزدوج الايوني

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المستخلص

فصلت وقدرت خمسة فيتامينات ذائبة بالماء(حامض النيكوتينيك و نيكوتين أمايد و حامض الفوليك و رايبوفلافين B_2 و الثايمين B_1) بشكل آني بطريقة RP-HPLC على عمود من نوع ZORBAX C_8 (25 سم x 4.6 ملم قطر داخلي) وذو حجم حشوة تتراوح اقطارها بين (6 – 8 µm). تمت الازاحة الازوكراتية لمزيج المكونات بطور متحرك مكون من 20% ميثانول ومحلول بفر KH_2PO_4 بتركيز (0.05 مولاري) لضبط الدالة الحامضية عند pH= 3 ويحتوي 1 – اوكتان حامض السلفونيك بتركيز (3.0×10^{-3} مولاري)كعامل مكون للمزدوج الآيوني وكان معدل سرعة الجريان مساوية لـ 1.0 ml/min. تم الكشف عن الفيتامينات المستردة من العمود باستخدام مجس نوع UV-Vis. عند طول موجي مقداره 254 nm. لقد اظهرت نتائج الدراسة ضبط ودقة جيدين وذلك من خلال حساب مقدار النسبة المؤية للانحراف القياسي النسبي الذي تراوح بين (0.5 – 4.76 %) وحساب النسبة المؤية للخطأ الذي تراوحت قيمته بين(0.05 – 4.96 %) تراوحت قيمته الحد الادنى للكشف بين (0.0244 – 0.0436 مايكروغرام\ مللتر).

Key words: water soluble vitamin analysis, HPLC

Introduction

Vitamins as a biologically active compounds which are needed in relatively small amounts for the sustain of life and a good health so, they are essential dietary components[1]. Increasingly, many diverse food products are being fortified with vitamins to enhance their nutritional value [2]. In addition, because vitamins might be lost during processing and storage of food through chemical reactions, it is important to take extraneous vitamins (e.g. multivitamin tablets) so as to compensate the possible lack of the vitamins in our diet [3]. Therefore, pharmaceutical products, which contain vitamins, are most interesting for analysis, because of their complex composition. It is special provocation for the investigators[4].

Vitamins, depending on their solubility, are classified into fat-soluble vitamins (A, D, E and K) and water-soluble vitamins (B-complex, folic acid, pantothinic acid, nicotinic acid (nicotineamide), biotin (H))[5], among water-soluble vitamins, the B-group are the most important. They are not toxic and stored in a human body in scanty amount, and when they exceed the body needs, they are excreted into urine so they must be continually supplied in the diet [6].

In the literature, several methods have been proposed for the determination of vitamins including Spectrophotometry [7,8], Fluorometry [5,9], Chemiluminescence [10], Flow-injection analysis (FIA) [11,12], Electrochemical [13], Capillary electrophoresis [14], and High-Performance liquid chromatography (HPLC) [15-17]. Among all mentioned methods, HPLC technique was found to be rapid, simple, versatile, precise and specific. The success of the HPLC method depends on a good selection of a number of factors such as flow rate, temperature, type and concentration of mobile phase and column dimension[18], which were applied in this paper for the simultaneous determinations of five water-soluble vitamins using a developed HPLC system by optimizing thermodynamic and kinetic parameters that effect the separation.

Experimental

a- Solvent and reference compounds.

All chemicals used were of analytical grade reagents except otherwise mentioned, and deionized water was used throughout. Methanol, HPLC grade (Hayman, England); 1-octansulfonic acid ion-pair (Aldrich, England); and potassium dihydrogen phosphate (BDH, England) were used to prepare the mobile phase. Hydrochloric acid (Riedel-deHaën) was used for adjusting the pH values.

Standard vitamins; nicotinic acid, nicotineamide, and folic acid were obtained from BDH (England), while riboflavin (B_2) was purchased from Schuch ardt (Germany) and thiamin (B_1) from Fluka (Switzerland).

b- Mobile phase and standard solution preparation.

A mobile phase consisting of 20% methanol was prepared by mixing 200ml of methanol with 800ml of deionized water containing 0.64g of 1-octanesulfonic acid (i.e. $3x10^{-3}$M) and 6.8g of KH_2PO_4 (i.e. 0.05M). The pH of the mixture was adjusted to 3.5 with (0.1M) HCl. After preparation, it was filtered and degassed in an ultrasonic bath prior to use.

Stock solutions (100µg/ml) of nicotinic acid, nicotineamide, and thiamin were prepared by dissolving 0.025g of each vitamin in deionized

water. The volumes of the resulted solutions were then completed to 250 ml with deionized water and stored in dark containers.

Riboflavin and folic acid stock solutions (100µg/ml) were prepared by dissolving 0.025 g of each compound in about 10 ml of (3:1 v/v) deionized water : (1M) ammonium hydroxide solution. The resulted mixtures were then diluted in 250 ml volumetric flask with the same mentioned solution [19].

Test mixtures were prepared by using equal parts of each of the prepared standards solutions and stored in a refrigerator.

c- Preparation of the sample solution.

1- *Vitamin B-compound (I) (HM-Generics - Netherlands)*: Each tablet contains 1 mg thiamin, 1 mg riboflavin, 15 mg nicotineamide and 0.05 mg folic acid. A number of vitamin tablets were grounded into fine powder, sieved and then a precise portion of the powder, equivalent to about 188.5 mg (the average weight of 10 tablets),was dissolved in about 100 ml deionized water. The solution then warmed to 40°C with shaking for 45 min. to dissolve riboflavin [20], filtered and the volume was made to 500ml with deionized water.

2- *B-Complex tablet (II) (LORESTAN CO.-Iran)*: Each tablet contains 5 mg thiamin, 2 mg riboflavin and 20 mg nicotineamide. A number of vitamin tablets were grounded into powder, sieved and the portion of the powder, equivalent to 240 mg (average weight of 10 tablets),was dissolved in about 100 ml deionized water. The solution was warmed to 40°C with shaking for 45 min. to dissolve riboflavin, then filtered and diluted to *500 ml.*

3- *Folic acid tablets (III) (PRISM International-India)*: Each tablet contains 5 mg folic acid. A number of tablets was ground into fine powders, sieved and then a portion of powder (200 mg), equivalent to the average weight of 10 tablets, was dissolved in about 100 ml of deionized water and 2 ml of 0.1N Na_2CO_3 was then add [21]. The resulted mixture was vigorously shaked for 45 min., and the solution was filtered before dilution to 1L with deionized water.

4-*Nicotinic acid tablets (IV) (SOBHAN pharm. Co.- Iran)*: Each tablet contains 25 mg nicotinic acid. A number of vitamin tablets were grounded into fine powders, sieved and then 135.0 mg portion of the powder, equivalent to of the average weight of 10 tablets, was dissolved in about 100 ml deionized water. The solution was then vigorously shaked for 45 min., filtered and diluted to 1L with deionized water. The working solution was freshly prepared by diluting 16 ml aliquot of the mother solution to 50 ml with deionized water.

d- HPLC equipment.

The chromatographic system consists of: a pump (type waters Model 501) with a high-pressure range 6000 psi; a model 481 UV-Visible detector (waters Assoc.); a ZORBAX-C_8 column (25 cm x 4.6 mm. i.d) with 6-8 µm particle size. A sample injector model U6K (water Assoc.) with 10 µl injection loop; a water bath (Tecon Cambridge, England) to keep the column temperature at constant level.

Result and discussion

a- Effect of flow rate of the mobile phase.

To investidate the effect of the flow rate on the retention times (t_R) of the vitamins test mixture (3 µg/ml for each vitamins), the composition of the mobile phase was held constant during *isocratic elution with 20% methanol* (pH 5.0) at 30 °C. The results on Fig. (1) show that the retention time of the tested vitamins decreased with increasing the flow rate. This could be attributed to the molecular size of the

vitamins and their ability to form stable complexes with the ion pairing reagent, and to the relative lipophilicity of the ion-pair formed within the system [22]. The aim of choosing the optimum flow rate is to obtain a short analysis time, which in turn prevents solute band broading; this finally leads to increasing column efficiency [23]. A flow rate of 1 ml/min. was selected to obtain maximum resolution in a suitable analysis time.

b- Percentage of organic modifier of the mobile phase.

Methanol was used as a typical mobile phase modifier for this study. It was mixed with (0.05M) KH_2PO_4 buffer (pH 5) and ($3x10^{-3}$M) 1-octansulfonic acid as an ion-pairing reagent at 30°C. The results obtained indicate that the retention times of the studied vitamins decreased as the percentage of methanol raised from 10 to 30% as a consequence of decreasing of the stability of the complex in the ion-pairing mechanism, due to a weakening in the hydrophobic interaction between the ion-pair forming species and the separated species, which in turn decreases the retention time (t_R) value [24] (Fig. (2). Thiamin (B_1), which is the more polar among the separated vitamins, is directly affected by altering this factor, its retention time was changed from 23.38 min. to 7.58 min.. The results obtained indicate that the suitable percentage of the methanol as organic modifier for separation of the studied vitamins is (20%).

c- Effect of pH and buffer concentration.

The studied water-soluble vitamins, are greatly affected by changing the pH values in the range of 3 to 5. The plots of the experimental data of capacity factor (K′) as a function of pH of the mobile phase (Fig. (3)) show that at pH 3 folic acid eluted after nicotineamide, while at pH 5 the order of elution is reversed. This reveals the fact that under the acidic condition, basic vitamins eluted earlier due to protonation and increasing water solubility, while the acidic vitamins eluted later due to ion suppression, while lower pH values were found to give better overall results of the two (3.6 ± 0.1) [19,26].

In general, the t_R value can be correlated with the pKa and pKb of the solute molecule. Vitamins with pK value below 3.0 and above 5.0 showed little change in t_R with pH change of the mobile phase. The mobile phase with pH 3.5 was chosen for subsequent work because it gives good separation in the overall results.

On the other hand, a definite buffer content in the mobile phase is required to maintain equilibrium constancy within the column. Phosphate buffer (at pH > 3) is preferred over others because of the superior column efficiency often obtained with phosphate in the eluent. In general, buffer components compete with solute to form complexes with the ion pairing reagent, hence decreasing the retention times of the vitamins [27]. Fig. (4) shows that there is no significant difference in (K′) value for the studied vitamins except that for thiamin, which is an ionizable form. These results also indicate that 0.05M KH_2PO_4 buffer was an optimal concentration to give good peak resolution with suitable t_R for all vitamin bands.

d- Concentration of the ion-pair reagent.

Ion-pair chromatography is a technique used with a reversed-phase system, in which ionized compounds can be made in favor to the organic stationary phase by using suitable counter ion to form ion pair [28]. 1-octanesulfonic acid was chosen as an ion-pairing reagent because it is well suited to form ion-pair complexes with

solutes having basic functional groups such as amine groups [29]. In general, the t_R values of the studied vitamins are increased with increasing the concentration of 1-octansulfonic acid (Fig.(5)) . A $3x10^{-3}$M of the ion-pair was found to give an excellent analysis time and capacity factor (K′), while higher concentrations were found to increase analysis time and cause band broadening.

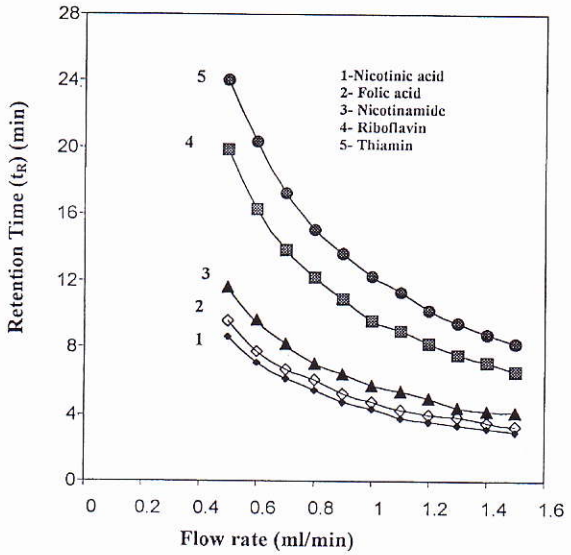


Fig.(1): Flow rate effect on the chromatographic separation.

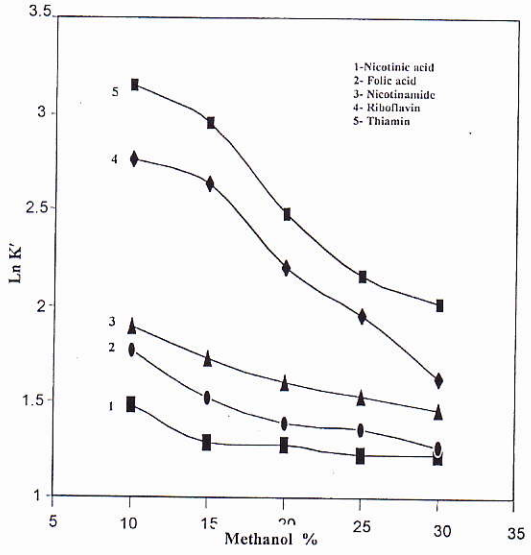


Fig.(2): Effect of organic modifier percentage (methanol%) on the capacity factor (K′) for each vitamin.

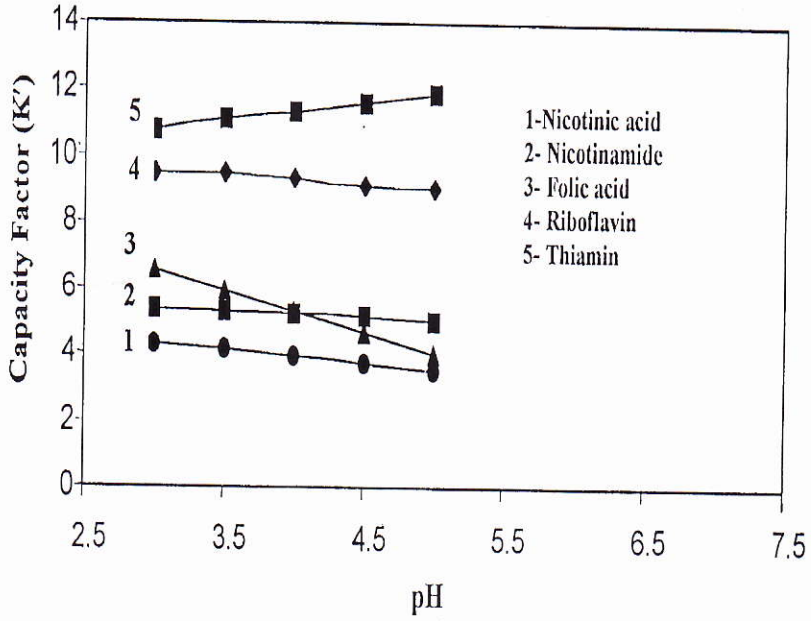


Fig.(3): Effect of pH of the mobile phase.

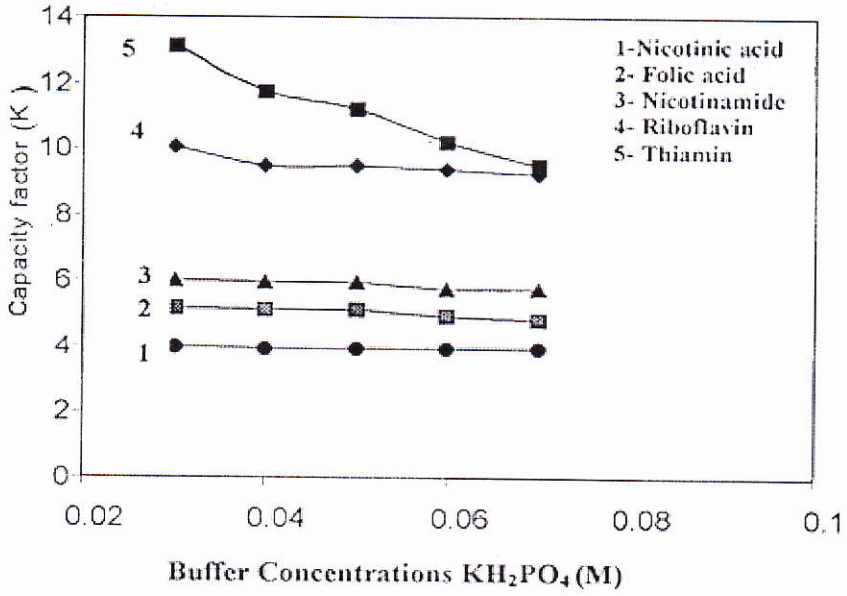


Fig.(4): Effect of buffer concentration (KH_2PO_4).

e- Column temperature.

The effect of column temperature in the range of 20 to 50°C on the t_R values of vitamins was investigated. Generally increasing column temperature in RP-chromatography decreases the t_R of the separated bands and increases column efficiency by decreasing mobile phase viscosity, which in turn lowers the column head pressure [30]. Fig. (6) shows the relation between ln K′ and 1/T. The plot shows that the optimum column temperature is 30°C. At this temperature good bands shape with a reasonable resolution of the separated vitamins were obtained.

f- Recommended analytical conditions.

According to the results obtained, optimum experimental conditions, tabulated in Table (1), were established for isocratic ion-pair reversed-phase HPLC for separation and quantitative determination of five water-soluble vitamins, which their structures are show in Table (2). The shapes and elution orders of the separated signal obtained for vitamins are shown in Fig. (6).

Calibration graphs and detection limit.

Calibration graphs (Fig. (7)) were constructed for the studied vitamins. Table (3) shows that the calibration graph data obtained for the analyses of nicotinic acid is linear in the concentration range (0.5-10.0 μg/ml) with correlation coefficient 0.9997; for nicotineamide in the range (0.4-10.0 μg/ml) with correlation coefficient 0.9997; for folic acid in the range (0.4-8.0 μg/ml) with correlation coefficient 0.9996; for riboflavin in the range (0.5-10.0 μg/ml) with correlation coefficient 0.9999 and for thiamin in the range (0.5-10.0 μg/ml) with correlation coefficient 0.9997. The detection limits (as peak height three times higher than the noise level) of the studied vitamins were in the range of (0.0244-0.0436 μg/ml), which were sufficient for this investigation.

Application

The proposed RP-HPLC system and the recommended procedure were applied for the determination of five B-group vitamins in four pharmaceutical preparations obtained from the local market by direct calibration graph and standard addition methods. The results obtained are recorded in Table (4), and Fig.(9) demonstrates the chromatogram of each sample.

1- *Vitamin B-compound* **(*I*)**

i- Direct calibration method: A 15ml of the prepared sample solution were diluted in a 50 ml volumetric flask with deionized water. 10 ml aliquot of the resulted solution was analyzed. The average peak height of five injections was recorded to determine the accurate amount of each individual vitamin depending on its corresponding calibration graph.

ii- Standard addition method: A 15ml aliquots of the prepared sample solution were added into a series of six 50ml volumetric flasks, followed by the addition of 0,4,6,8,10,12ml portions of standard solution (consists of 10μl nicotineamide, 30μg/ml folic acid, and 20μg/ml for both riboflavin and thiamin) to the flasks respectively. The resulted mixtures were then diluted with deionized water to the mark, 10μl of each was injected and the average peak height (n=5) for each mixture were recorded.

2- *B-complex tablet* **(*II*)**

i- Direct calibration method: A 25 ml of the prepared sample solution were transferred into 100ml flask and diluted to the mark, then directly injected to the system. The average peak height (n=5) was recorded.

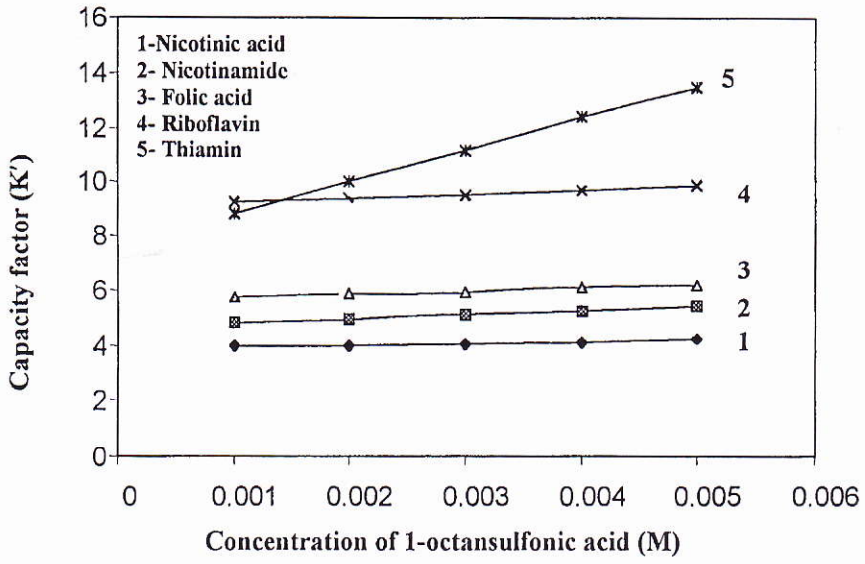


Fig.(5): Ion-pair concentration effects on the separation method.

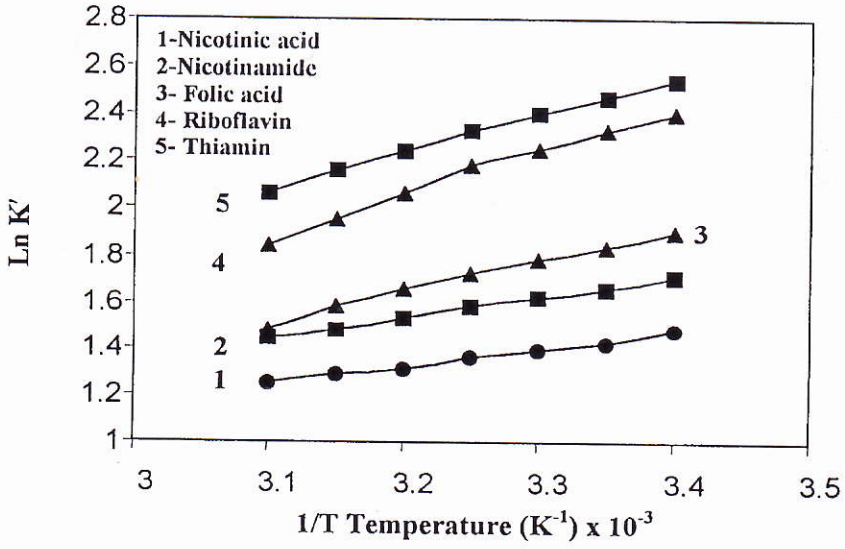
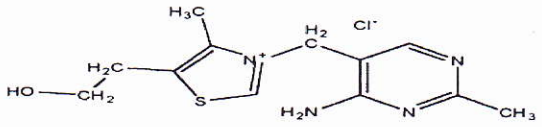
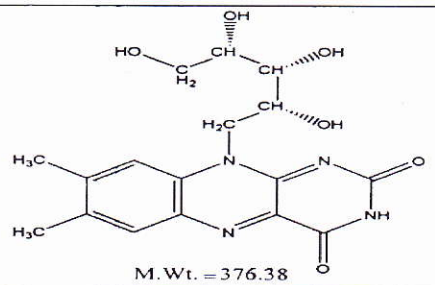
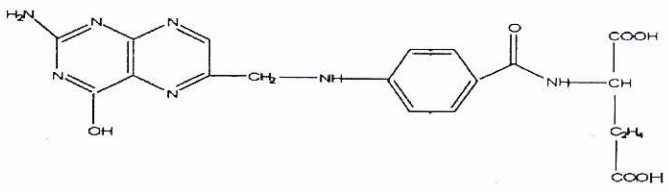
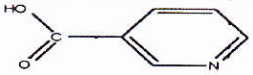
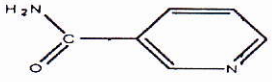


Fig.(6): Effects of column temperature.

Table (1): The optimum working conditions for the determination of vitamins.

Parameters	Value
Sample volume	10 µl
Column	ZORBAX C_8 (25 cm x 4.6 mm i.d)
Organic modifier	20% methanol
Ion-pair reagents	$3x10^{-3}$M 1-octanesulfonic acid
Buffer	0.05 M KH_2PO_4
pH	3.5
Flow rate	1.0 ml/min.
Column temperature	30°C
$\lambda_{maximum}$	254 nm

Table (2): Structures of five water-soluble vitamins.

Vitamins	Synthetic form	Formula
thiamin (B_1)	thiamin hydrochloride	 M.Wt. =337.27
riboflavin (B_2)	riboflavin	 M.Wt. =376.38
folic acid	pteroyl glutamic acid	 M.Wt. =441.91
niacin (B_3)	nicotinic acid	 M.Wt. =123.11
	nicotinamide	 M.Wt. =122.11

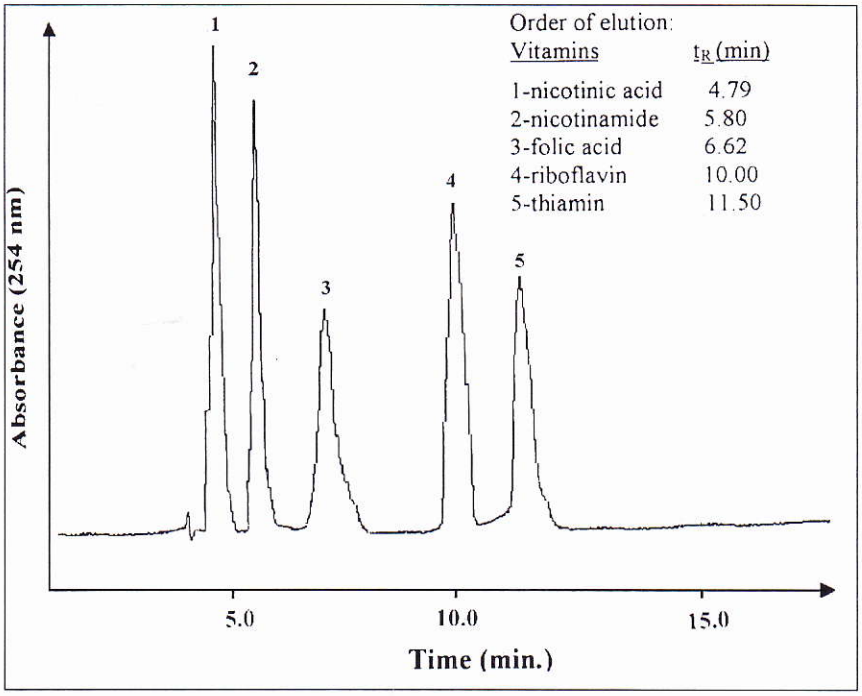


Fig. (7): Separation chromatogram for five water soluble vitamins under recommended conditions

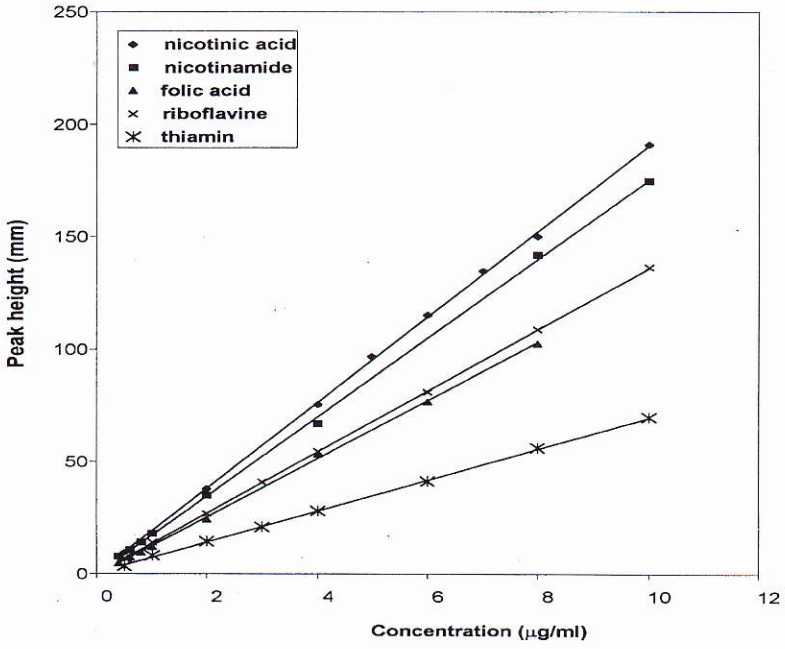


Fig. (8): Calibration graph for the water soluble vitamins.

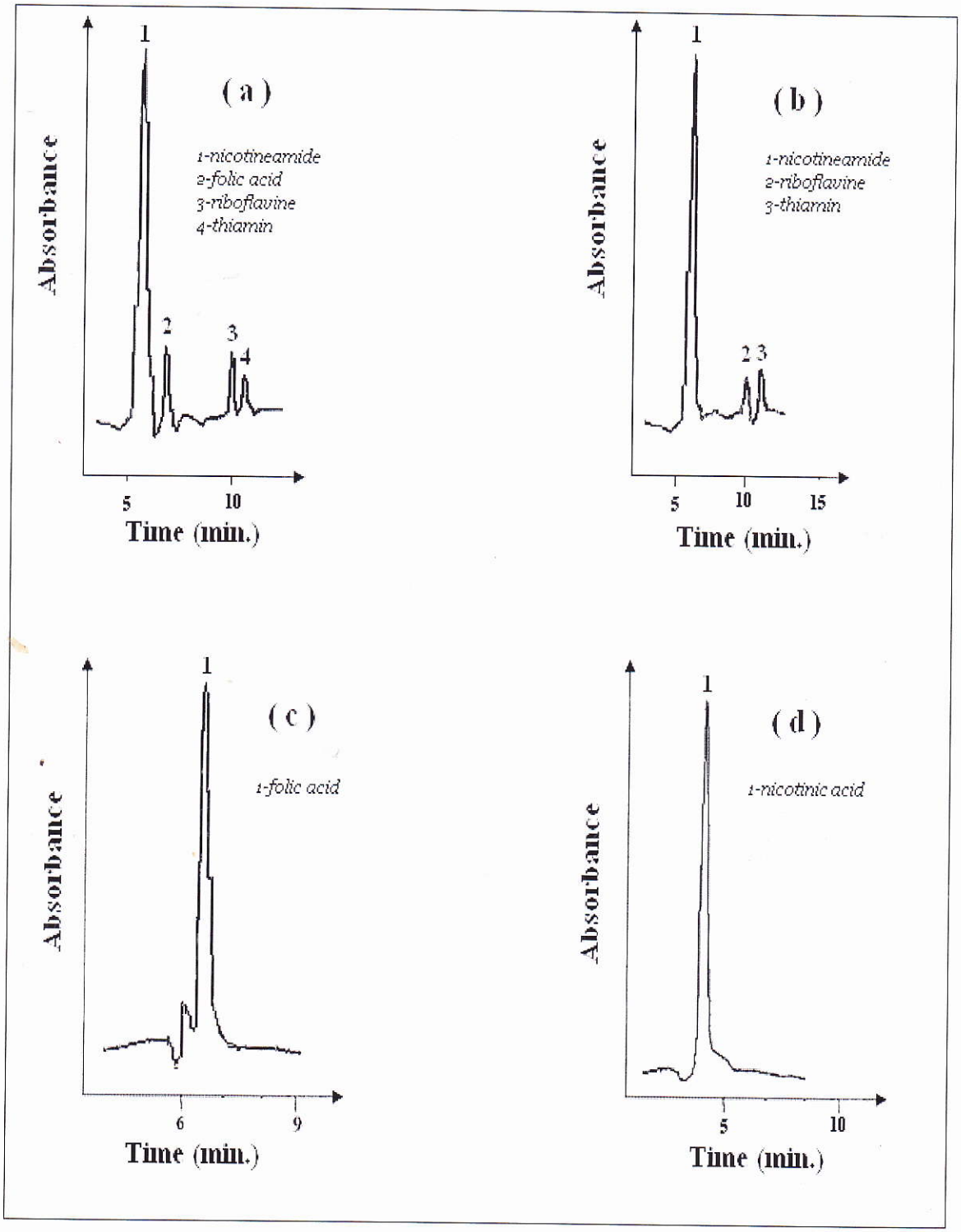


Fig. (9): Isocratic reversed phase HPLC chromatogram for:

- **a- Vitamin B-compound (*I*)**
- **b- B-complex tablets (*II*)**
- **c- Folic acid tablets (*III*)**
- **d- Nicotinic acid tablets (*IV*)**

Table (3):The statistical treatment of the calibration results and detection limits of vitamins.

Vitamins	Linear range (µg/ml)	Slope	Intercept	Detection Limit (µg/ml)	Correlation coefficient
nicotinic acid	0.5 – 10.0	19.0497	0.1732	0.0340	0.9997
nicotineamide	0.4 – 10.0	17.5367	-0.0730	0.0380	0.9997
folic acid	0.4 – 8.0	12.9676	-0.5827	0.0436	0.9996
riboflavin	0.5 – 10.0	13.6205	-0.0386	0.0326	0.9999
thiamin	0.5 – 10.0	6.9408	0.3426	0.0244	0.9997

Table (4): Results for analysis of water-soluble vitamins in four pharmaceutical formulation samples.

Sample No.	Available vitamins	Labeled vitamins mg/tablet	Found vitamins mg/tablet*					
			Direct method	RSD %	E %	Standard addition	RSD %	E%
I	thiamin (B_1) riboflavin (B_2) folic acid nicotineamide	1.00 1.00 0.05 15.00	1.090 0.980 --** 15.070	6.09 6.45 -- 1.00	9.00 - 2.00 -- 0.46	0.985 0.968 0.033 14.952	2.17 3.20 5.08 0.33	-1.50 -3.20 - 34.00 -0.32
II	thiamin (B_1) riboflavin (B_2) nicotineamide	5.0 2.0 20.0	5.040 1.910 19.800	2.55 1.2 0.41	0.80 - 4.50 - 1.00	4.981 1.987 19.827	2.39 0.80 0.27	-0.38 -1.07 -0.86

Table (4): continue

Sample No.	Available vitamins	Labeled vitamins mg/tablet	Found vitamins mg/tablet*					
			Direct method	RSD %	E %	Standard addition	RSD %	E%
III	folic acid	5.0	4.830	1.9	-3.40	4.985	0.79	-0.3
IV	nicotinic acid	25.00	24.930	2.01	-0.28	24.953	0.90	-0.188

*Average of five determinations.

** Folic acid cannot be detected by direct method.

ii- Standard addition method: To a series of six 50ml volumetric flasks, each containing 10ml of sample solution, 0,4,6,8,10,12ml of standard vitamin solution mixture (15µg/ml nicotineamide,30µg/ml riboflavin and 20µg/ml thiamin) aliquots were added. The volume of the resulted mixture was then diluted to the mark before analysis.

3- *Folic acid tablets* **(*III*)**

i- Direct calibration method: A 10 µl of the prepared sample solution was injected into the system, then the amount of vitamin was measured.

ii-Standard addition method: A standard solution of vitamin was prepared (20µg/ml). 0,4,6,8,10,12ml aliquots of this solution were added into a series of six 50ml volumetric flasks, each contains 20ml of the prepared sample solution, the volume of each of the resulted solutions was then diluted to the mark, and directly injected to the system.

4- *Nicotinic acid tablets* **(*IV*)**

i- Direct calibration method: A 10µl sample solution was injected into the system, and then the amount of vitamin was determined.

ii- Standard addition method: A standard solution of 5µg/ml was prepared and 0,4,6,8,10,12ml aliquots of it were transferred into six volumetric flasks, each contains 20ml of the sample solution prepared. The volumes of the resulted mixtures wrer then diluted to the mark with deionized water. A 10µl portion of each solution was then injected into the system.

Conclusion

Although the determination of vitamins differs from other classes of biochemical compounds, because these compounds do not represent a structurally or functionally related series, RP-HPLC system appears to offer significant advantage in determination of water-soluble vitamins in pharmaceutical formulations, with good results, high

accuracy and precision. Moreover, the simplicity of the procedure should make it highly desirable for quality control of multivitamin products in the pharmaceutical industries. On the other hand, vitamins in pharmaceutical preparations show some interesting problems, upon their analyses, because their responses are not limited to a single wavelength detection and the vitamins are not found in sample solution within a uniform concentration range (as in sample (III)), when folic acid vitamin is present only in trace in comparison with the amount of other vitamins.

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