

Development of a HPLC method for the simultaneous determination of several B-vitamins and ascorbic acid

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ABSTRACT

In cognizance of the difficulties involved in the colorimetric and titrimetric methods for the determination of individual vitamins, this laboratory has been carrying out a series of studies into the use of HPLC for improved analysis of these nutrients. Preliminary studies have been carried out for the determination of four B-vitamins. The present paper reports on further improvements made to enable the simultaneous determination of eight vitamins i.e. B₁, B₂, B₆, B₁₂, C, niacin, niacinamide and folic acid. Trials were carried out to determine the most suitable chromatographic system include changing the proportion of methanol in the mobile phase, the use of different ion-pairing reagents and other additives such as triethylamine and ammonia. Three sets of HPLC mobile phase systems are proposed to enable successful separation of all eight vitamins in less than 20 minutes, varying slightly with the type of ion-pairing reagent and mobile phase additive. This laboratory is currently carrying out trials to determine if the developed methods could be used for the determination of pharmaceutical products and food samples.

INTRODUCTION

The manual methods of the AOAC widely used for the estimation of water soluble vitamins i.e. vitamins B₁, B₂, B₆, B₁₂, C, niacin, niacinamide and folic acid involved extraction procedures and chemical reactions, followed by fluorimetry (B₁ and B₂), spectrophotometry (niacin and folic acid), titration (ascorbic acid and pyridoxine) and microbiological (B₁₂) (Deutsch, 1984). This laboratory has been using these tedious and time-consuming procedures for the development of the Malaysian food composition database (Tee *et al.*, 1987). The need for the use of hazardous chemicals such as cyanogen bromide for the estimation of niacin poses additional difficulties. Of late, it has been difficult to obtain this toxic and carcinogenic chemical.

In recent years, high-pressure liquid chromatography (HPLC) has been shown to be a powerful tool for the determination of various compounds including water-soluble vitamins (Wills *et al.*, 1977; Amin and Reusch, 1987a, 1987b; Dong *et al.*, 1988; Speck, 1989). Some of these publications dealt with the simultaneous separation of several B-vitamins whereas the majority reported the separation of only a few vitamins. HPLC methods have the advantage of being more specific, non-destructive and are capable of differentiating between different forms of a vitamin with varying biological activity.

This laboratory has been carrying out a series of studies into the development of improved methods for the determination of vitamins in food and serum using HPLC. It would obviously be

advantages if several vitamins can be determined simultaneously from a single food extract. Preliminary studies have been carried out for the determination of four B-vitamins (Tee and Khor, 1993). The present paper reports subsequent work carried out to enable the simultaneous determination of eight vitamins i.e. B₁, B₂, B₆, B₁₂, C, niacin, niacinamide and folic acid.

MATERIALS AND METHOD

Apparatus

The liquid chromatography system used consist of a Gilson 305 piston pump connected to a Gilson 805S manometric module, a model 7125 Rheodyne injector with a variable sample loop. A stainless steel 30 cm by 3.9 mm I.D. 10 mm mBondapak C₁₈ column was used for the chromatographic separation. This was preceded by a Supelco guard column holder containing a 2 cm disposable guard column insert which was packed with the same material as that in the analytical column. The detector used was a Gilson 116 UV detector with dual wavelength detection at 265 nm at 0.2 absorbance unit full scale which was switched to 0.05 absorbance unit full scale at 4.8 minutes. The second wavelength was fixed at 290 nm at 0.02 absorbance unit full scale for better detection of pyridoxine. This detector was connected in series with a Waters 440 fixed-wavelength detector at 340 nm at 0.005 absorbance unit full scale to detect cyanocobalamin (B₁₂). The pump and detector was controlled by the Gilson 714 system controller software which saved, viewed and enabled reintegration of all stored chromatographic data. All chromatograms and results analysed were then recorded on a Panasonic KX-P1081 printer. The characteristic absorption spectrum of cyanocobalamin, ascorbic acid, niacinamide and folic acid vitamins was determined using a Perkin-Elmer 550S model UV/VIS Spectrophotometer with 561 model recorder. The absorption spectra of the other vitamins were similarly determined as reported in Tee & Khor (1993).

Chemicals and reagents

Methanol used was of HPLC grade (BDH) and glacial acetic acid and ammonia (Merck) were of analar grade. Hydrochloric acid and sodium hydroxide for standard preparation were obtained as standard vials of 0.1N (BDH) and triethylamine was obtained from Sigma Chemicals. The ion pairing reagents were from two sources, ie (1) Waters, Division of Millipore, packed in vials of 25 ml each, and comprising 1-Pentane Sulphonic Acid (PIC B-5), 1-Hexane Sulphonic Acid (PIC B-6), 1-Heptane Sulphonic Acid (PIC B-7) and 1-Octane Sulphonic Acid (PIC B-8), and (2) Sigma Chemicals, as the sodium salt of 1-Pentane Sulphonic Acid and 1-Hexane Sulphonic Acid. Ultra pure water of resistivity of around 18 megohms centimetres (MΩ - cm) was used for mobile phase, standard preparation and sample extraction. Nicotinic acid (niacin), niacinamide (nicotinamide), pyridoxine hydrochloride (B₆), thiamine hydrochloride (B₁), riboflavin (B₂), cyanocobalamin (B₁₂) and folic acid (pteroylglutamic acid) were all Sigma products. L-ascorbic acid was BDH analar grade.

Procedure for preparation of mobile phase

The mobile phases used comprised a mixture of methanol, glacial acetic acid and water with different concentrations of ion-pairing reagents and additives such as triethylamine or ammonia, where necessary. All mobile phases solutions were filtered with a 47 mm 0.45 µm Schleicher & Schuell RC 55 membrane filter paper utilising a millipore filtration set and subsequently degassed for 15 minutes with an ultrasonic bath. The column was washed with at least 100 to 150 mls of mobile phase and then equilibrated at a flow rate of 1 ml per minute before injecting 10 µl of the standard mixture. After chromatography, the column was flushed with 5 column volumes of 1:1 methanol/water to prevent the ion-pairing reagent from remaining in the pump or column before shutting down.

Procedure for preparing standards

Niacin, niacinamide, B₁ and B₆ solutions of 5 mg per ml were prepared in 0.1N hydrochloric acid. Vitamin C of 5 mg/ml and cyanocobalamin of 10 mg/ml were prepared in ultra-pure water, B₂ standard solution of 40 µg per ml was prepared in the mobile phase and folic acid of 5 mg/ml in 0.1N sodium hydroxide. These standard solutions were kept refrigerated in ambered containers and were stable for several months except for vitamin C which was prepared fresh for each assay. Vitamin B₂ standard solution in mobile phase was found to be stable for at least two weeks.

Fresh standard solutions of mixtures containing 100 µg/ml each of ascorbic acid, niacin and niacinamide, 25 µg/ml each of pyridoxine and thiamine, 12.5 µg/ml folic acid, 50 µg/ml cyanocobalamin and 20 µg/ml of riboflavin were prepared in the appropriate mobile phase and filtered with a 13 mm 0.45 µm Schleicher & Schuell RC 55 membrane filter paper using a Water's sample filtration kit. For each solvent system being studied, each vitamin was injected separately to determine the individual retention times and then multivitamins mixtures were injected to determine the degree of resolution of the components.

Absorbance readings of each standard except vitamin C, was also taken before each assay and the appropriate absorptivities (extinction coefficients) of Sober (1970) were used to calculate the exact amounts (Table 1).

Table 1. Wavelength maxima and extinction coefficients of the vitamins studied

Vitamin	Solvent	Wavelength maxima	Extinction coefficients [E (1% / 1 cm)]
Thiamine	0.1 N hydrochloric acid	246 nm	410
Riboflavin	0.1 N hydrochloric acid	266 nm	870
Pyridoxine	0.1 N hydrochloric acid	290 nm	422
Cyanocobalamin	Ultra pure water	360 nm	204
Niacin	0.1 N hydrochloric acid	260 nm	432
Niacinamide	0.1 N hydrochloric acid	260 nm	435
Folic acid	0.1 N sodium hydroxide	282 nm	600

According to Sober (1970), the wavelength maxima for ascorbic acid in aqueous solution (pH 2.8) is 245 nm with an extinction coefficient value of 560. However when a 10 µg/ml standard

was prepared in ultrapure water, a scan of the standard show maxima at 263 nm and a pH of approximately 5.0. For this reason the amount of vitamin C was obtained directly from the weight i.e. 0.5 g per 100 ml for preparing a stock solution of 5 mg/ml, instead of calculating from its extinction coefficient.

A trial was also performed to determine the stability of ascorbic acid in ultrapure water and different strengths of glacial acetic acid. Stock solutions of 5 mg/ml were prepared in ultrapure water, 1% glacial acetic acid, 5% glacial acetic acid and 10% glacial acetic acid and kept refrigerated overnight when not in used. A working solution of 5 µg/ml in its solvent was prepared and monitored for 10 days by taking its absorbance reading at its maxima and another working solution in mobile phase for HPLC chromatography. All stock solutions were found to be stable for at least 7 days.

Riboflavin prepared in 0.1 N hydrochloric acid was only soluble below 50 µg/ml strength. This would require pipetting of amounts above 1 ml to prepare the standard mixture solution. To omit interference due to solvent peak attributed by the acid, it was found preferable to prepare the stock standard in the mobile phase of methanol: glacial acetic acid: water 26.5:0.5:73 (v/v/v) with 10 mM sodium pentane sulfonic acid and 0.09% triethylamine. It was found that over a period of 3 days, duplicate weighings of 0.004 g of riboflavin to 100 ml 0.1 N hydrochloric acid or mobile phase (40 µg/ml) did not show much difference in the absorbance reading of a working solution of 5 µg/ml preparation in both solvents. There was also hardly any difference in the wavelength maxima (266 nm in 0.1 N hydrochloric acid, compared to 267 nm in the mobile phase). The extinction coefficient value for B₂ in acid was thus used to calculate the actual amount of the standard preparation in mobile phase.

RESULTS AND DISCUSSION

Absorption spectra of individual vitamins

The characteristic absorption spectra of the 4 vitamins in the solvent in which they were prepared were obtained using a Perkin-Elmer UV/VIS spectrophotometer in order to determine the position of the main absorption maxima of the individual vitamins. From the absorption spectra of each vitamin, the main absorption maxima was found to be 360 nm for cyanocobalamin, 263 nm for ascorbic acid, 260 nm for niacinamide and 282 nm for folic acid. Since the λ maxima were 246 nm for thiamine, 266 nm for riboflavin, 260 nm for niacin and 290 nm for pyridoxine as reported by Tee & Khor (1993), therefore the wavelength of 265 nm, 290 nm and 340 nm were selected for monitoring HPLC runs.

Separation of vitamin mixture using previously developed systems (Tee & Khor, 1993)

Arising from an earlier study (Tee & Khor, 1993), the following chromatography systems have been found to be satisfactory for the separation of vitamin B₁, B₂, B₆ and niacin:

1. methanol-glacial acetic acid-water, 24.5: 0.5: 75 (v/v/v), with 5 mM PIC B-5,
2. methanol-glacial acetic acid-water, 34.5: 0.5: 65 (v/v/v), with 5 mM PIC B-6,

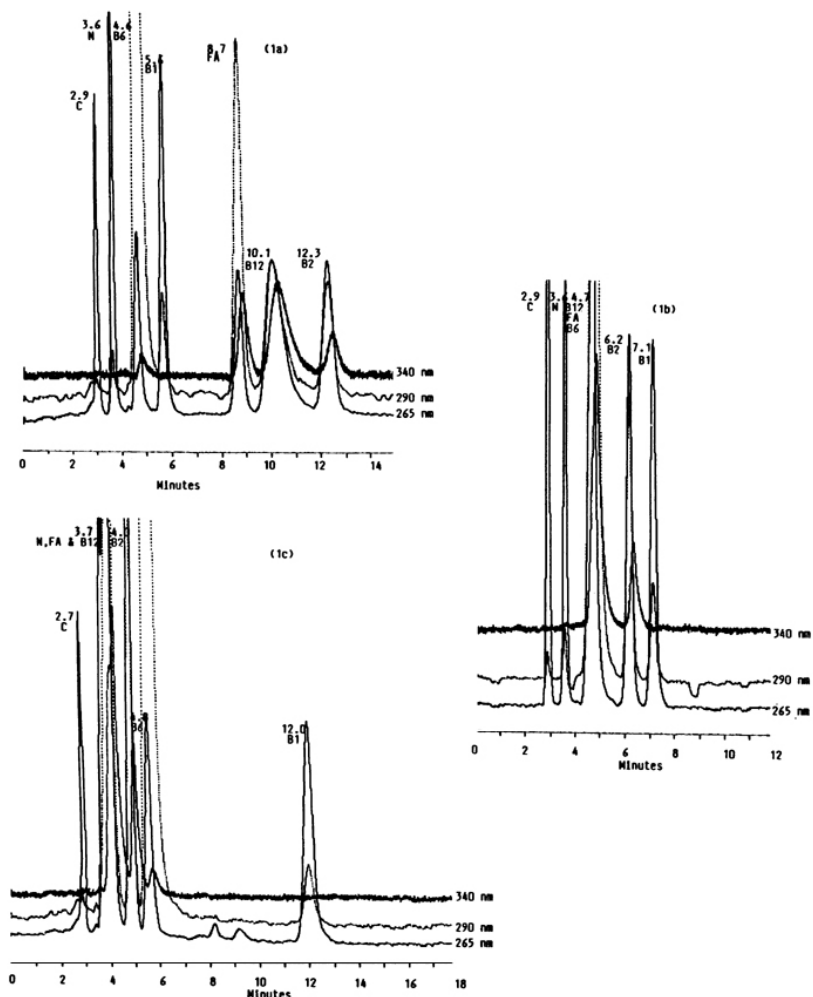
Determination of B-vitamins and ascorbic acid

3. methanol-glacial acetic acid-water, 39.5: 0.5: 60 (v/v/v), with 7 mM PIC B-7,
4. methanol-glacial acetic acid-water, 22.5: 0.5: 77 (v/v/v), with 3 mM PIC B-5 + PIC B-7, and
5. methanol-glacial acetic acid-water, 44.5: 0.5: 55 (v/v/v), with 2 mM PIC B-8

The ion-pairing reagents used were from Waters which were available as standard vials of 25 ml but the exact composition of the reagents was not known.

When three additional vitamins (B₁₂, C and folic acid) were added to the mixture and chromatographed using the above systems, only combination 1 was able to effectively separate all the vitamins studied:

1. In combination 1, all vitamins separated quite well in 14 minutes (Figure 1a);
2. In combination 2, folic acid, B₁₂ and B₆ did not separate (Figure 1b);
3. In combination 3, niacin, folic acid and B₁₂ resolution was only 0.2 (Figure 1c)
4. In combination 4, B₂ and B₁₂ did not separate with chromatographic runtime of 24 minutes which is rather long (Figure 1d); and
5. In combination 5, niacin, B₁₂ and folic acid did not separate and the chromatographic runtime was 16 minutes (Figure 1e)



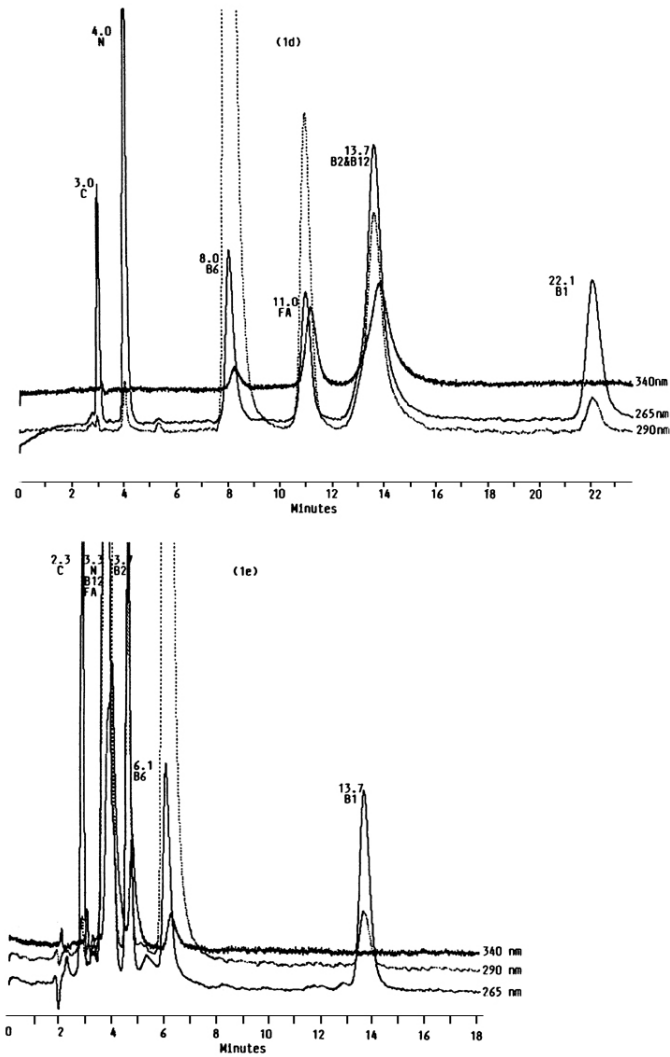


Figure 1. Chromatograms showing the separation of vitamin mixture using previously developed mobile phase systems (Tee & Khor, 1993):

(a) methanol-glacial acetic acid-water, 24.5: 0.5: 75 (v/v/v), with 5 nM PIC B5

(b) methanol-glacial acetic acid-water, 34.5: 0.5: 65 (v/v/v), with 5 nM PIC B6

(c) methanol-glacial acetic acid-water, 39.5: 0.5: 60 (v/v/v), with 7 nM PIC B7

(d) methanol-glacial acetic acid-water, 22.5: 0.5: 77 (v/v/v), with 3 mM PIC B-5 + PIC B-7

(e) methanol-glacial acetic acid-water, 44.5: 0.5: 55 (v/v/v), with 2mM PIC B-8

B₁-thiamine, B₂-riboflavin, B₆-pyridoxine, B₁₂-cyanocobalamin, C-ascorbic acid, N-niacin, FA-folic acid

Other chromatography condition are as given in the text

Trials were therefore carried out to determine the most suitable chromatography systems for the separation of all the vitamins in the mixture by modifying the proportion of methanol in the mobile phase and the addition of ion-pairing reagent and other additives. Although combination 1 appeared workable, it was felt desirable to investigate the use of other ion-pairing reagents and mobile phase additives.

Effect of different proportions of methanol and ion-pairing reagent

Waters PIC reagent

Since combinations 2 to 5 above did not give satisfactory separation for all the vitamins, the proportion of methanol in the mobile phase was changed and used in combination with various Waters PIC reagent, namely PIC B-6, PIC B-7, PIC B-5 plus B-7 and PIC B-8. An earlier paper by Tee & Khor (1993) reported minor differences in the separation of B₁, B₂, B₆ and niacin when glacial acetic acid was increased from 0.5% to 2%, the best being the 0.5% since absence lead to tailing of pyridoxine. Therefore the glacial acetic acid in the system was fixed at 0.5% for all the experiments. It was found that increasing the proportion of methanol in the mobile phase decreased the chromatographic runtime but separation of the 8 vitamins was not satisfactory. Using PIC B-6 at a concentration of 6 mM, the optimum mobile phase of 24.5% methanol did not resolve B₁ and B₂ completely. When 5 mM of PIC B-7 was used with 33.5 to 37.5% methanol, niacin and B₁₂ did not separate. Combination of PIC B-5 and B-7 at a concentration of 3 mM also gave poor separation for B₂ and B₁₂ with 20.5 to 24.5% methanol. As for the 2 mM PIC B-8, niacin, niacinamide and folic acid were not resolved when the proportion of methanol was increased from 41.5 to 44.5%. The series of trials carried out showed that except for PIC B-5, the other PIC reagents gave unsatisfactory separation with varying proportions of methanol.

Sigma ion-pairing reagent

With sodium pentanesulfonic acid fixed at 6 mM and glacial acetic acid at 0.5%, the proportion of methanol was increased from 24.5% to 29.5%. Increasing the proportion of methanol decreased the chromatographic runtime of the last eluting peak, B₂. Vitamin B₁₂ and folic acid did not separate well in most proportions of methanol studied except for 29.5% methanol where the chromatographic runtime was less than 14 minutes and the resolution of B₁₂ and folic acid was at least 0.8 (Figure 2).

Since sodium hexanesulfonic acid was most commonly used in the literature, trials were also carried out on this ion-pairing reagent. The proportion of methanol was varied with the strength of the ion-pairing reagent fixed at 5 mM and glacial acetic acid at 0.5%. The optimum mobile phase appeared to be 30.5% methanol, and separation for B₆, B₁₂ and folic acid was only partial.

Effect of different concentrations of Sigma ion-pairing reagents

Sodium pentanesulfonic acid

Since changing the proportion of methanol did not appear to resolve all peaks satisfactorily, another series of studies were carried out by varying the concentration of sodium pentanesulfonic acid but fixing the proportion of methanol at 24.5% and glacial acetic acid at 0.5%. Increasing the strength of sodium pentanesulfonic acid was found to improve the separation of B₁ and B₆, but separation of ascorbic acid, niacin, niacinamide, B₁₂ and folic acid was still poor. The retention time of the last eluting peak, B₂ was approximately 23 minutes in all concentrations of ion-pairing reagent (Figure 3).

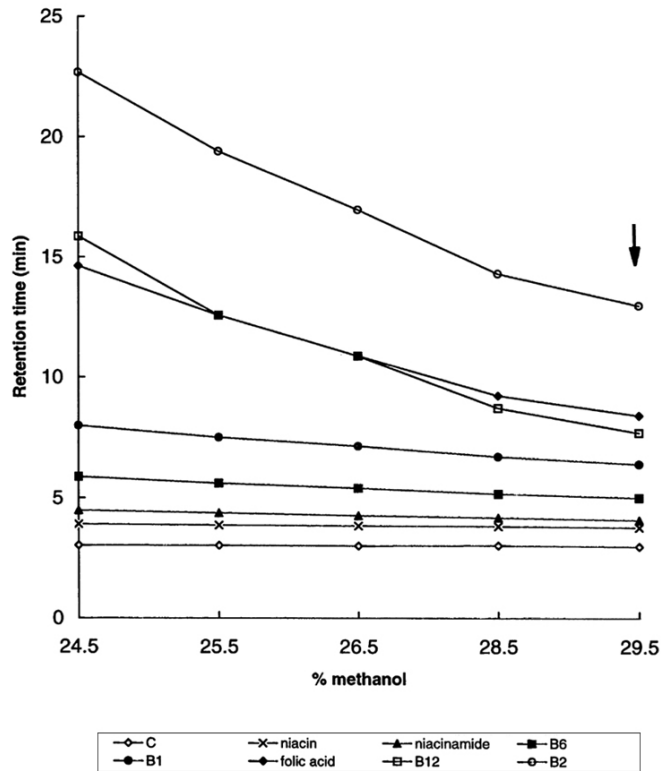


Figure 2. Effect of different proportions of methanol on the separation of water soluble vitamins in mobile phase of methanol - 0.5% glacial acetic acid - water with 6nM sodium pentanesulfonic acid

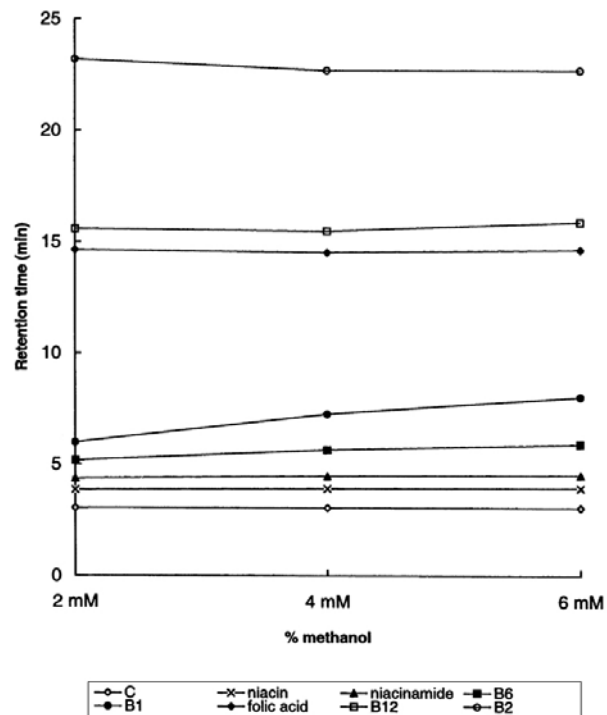


Figure 3. Effect of different concentrations of sodium pentanesulfonic acid on the separation of water soluble vitamins in a mobile phase of 24.5% methanol - 0.5% glacial acetic acid - 75.0% water

Sodium hexanesulfonic acid

With the proportion of methanol fixed at 30.5% and glacial acetic acid at 0.5%, the optimal strength of sodium hexanesulfonic acid was determined. Increasing the strength of the ion-pairing reagent from 1 to 7 mM increased the retention time of B₁ drastically but separation for B₆, B₁₂ and folic acid was only partial in most of the concentrations.

Use of mobile phase additives

Since changing proportion of methanol and strength of ion-pairing reagents did not give satisfactory separation, the effect of addition of mobile phase additives i.e. triethylamine (TEA) and ammonia was studied.

Addition of triethylamine (TEA)

Dong *et al.* (1988) described the use of triethylamine (TEA) as a mobile phase additive to reduce the peak tailing of basic solutes i.e. thiamine and pyridoxine. A study was carried out to determine the effect of different concentrations of TEA at varying proportions of methanol.

When 0.05% TEA was added to mobile phase containing methanol-glacial acetic acid-water 24.5:0.5:75 (v/v/v) with 6 mM of sodium pentanesulfonic acid, the chromatogram obtained was worse than without it. The resolution for B₆ and B₁ decreased and the retention time of the last eluting peak B₂ was still long i.e. 22 minutes (Figure 4a and 4b). Since increasing the proportion of methanol decreased the chromatographic runtime, the percentage of methanol was stepped up to 26.5%. The strength of ion-pairing reagent was also increased to 10 mM to improve the resolution of the early eluting peaks, i.e. ascorbic acid, niacin, niacinamide, pyridoxine and thiamine. To further improve separation between folic acid and B₁₂, TEA was also increased to 0.09% (Figure 5). Separation for all eight vitamins was now complete with chromatographic runtime of less than 18 minutes.

Addition of ammonia

Ammonia can also be used to improve the peak shape of thiamine (Dong *et al.*, 1988). Studies were carried out to compare the separation of the eight vitamins using analar grade ammonia obtained from two different sources.

When different concentrations of ammonia from the first source were used in combination with 29.5% methanol, 0.5% glacial acetic acid and 6 mM sodium pentanesulfonic acid, there was no improvement in the separation of B₆ and B₁. The chromatographic runtime was approximately 13 minutes for all concentrations of ammonia, ranging from 0.1 to 0.2%. Decreasing the proportion of methanol to 28.5% increased the chromatographic runtime to 16 minutes but the resolution for niacinamide, B₁ and B₁ was only 0.6 in the optimal concentration of 0.12% ammonia (Figure 6).

However when ammonia from the second source was used with combination of 28.5% methanol, 0.5% glacial acetic acid and 6 mM of sodium pentanesulfonic acid, the amount of ammonia required for optimum separation was much lesser i.e. 0.055% compared to 0.12% with ammonia

from the first source. To further improve the separation, the strength of sodium pentanesulfonic acid was increased to 8 mM. This appeared to be the optimum mobile phase for the separation of all eight vitamins using ammonia from the second source (Figure 7).

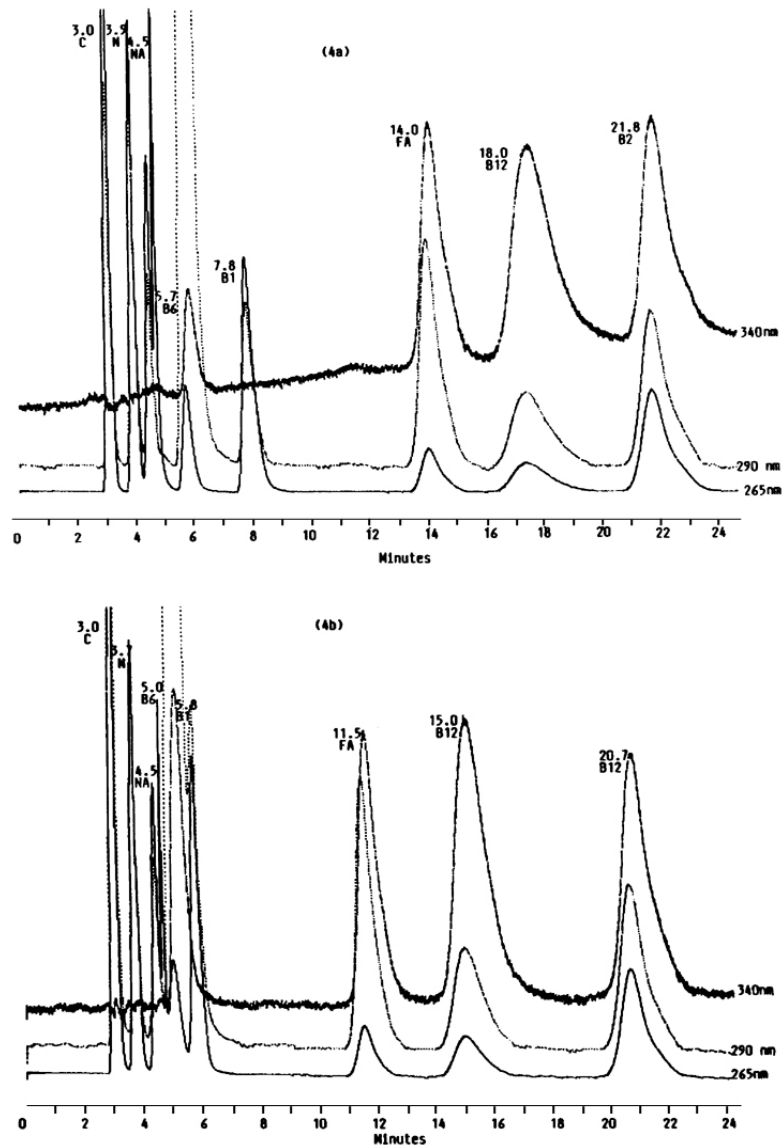


Figure 4. Chromatograms showing the effect of adding triethylamine (TEA) to the following mobile phases on the separation of vitamin mixture:

(4a) methanol-glacial acetic acid-water, 24.5: 0.5: 75 (v/v/v) with 6 mM sodium pentanesulfonic acid and without addition of triethylamine (TEA).

(4b) methanol-glacial acetic acid-water, 24.5: 0.5: 75 (v/v/v) with 6 mM sodium pentanesulfonic acid and 0.05% triethylamine (TEA).

B₁-thiamine, B₂-riboflavin, B₆-pyridoxine, B₁₂-cyanocobalamin, C-ascorbic acid, N-niacin, NA-niacinamide, FA-folic acid

Other chromatography conditions are as given in the text.

Determination of B-vitamins and ascorbic acid

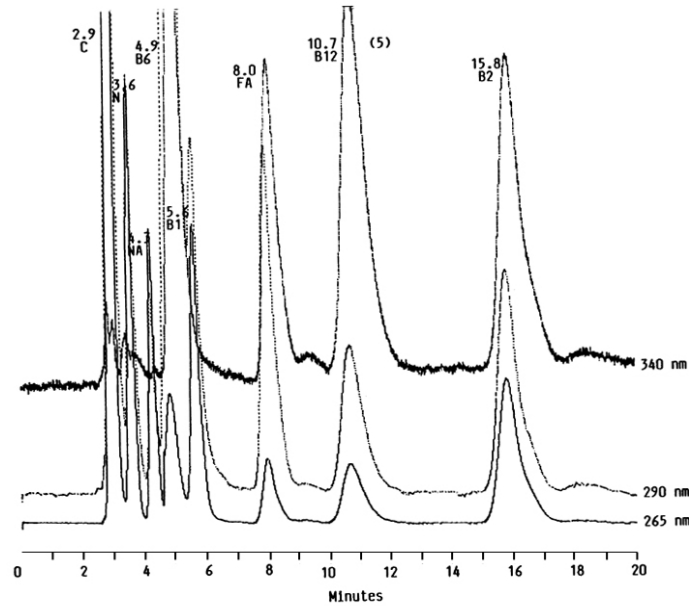


Figure 5. Chromatogram showing the separation of vitamin mixture using mobile phase containing methanol-glacial acetic acid-water, 26.5: 0.5: 73 (v/v/v) with 10 mM sodium pentanesulfonic acid and 0.09% triethylamine (TEA).
B₁ - thiamine, B₂ - riboflavine, B₆ - pyridoxine, B₁₂ - cyanocobalamin, C - ascorbic acid, N – niacin, NA - niacinamide, FA - folic acid
Other chromatography conditions are as given in the text.

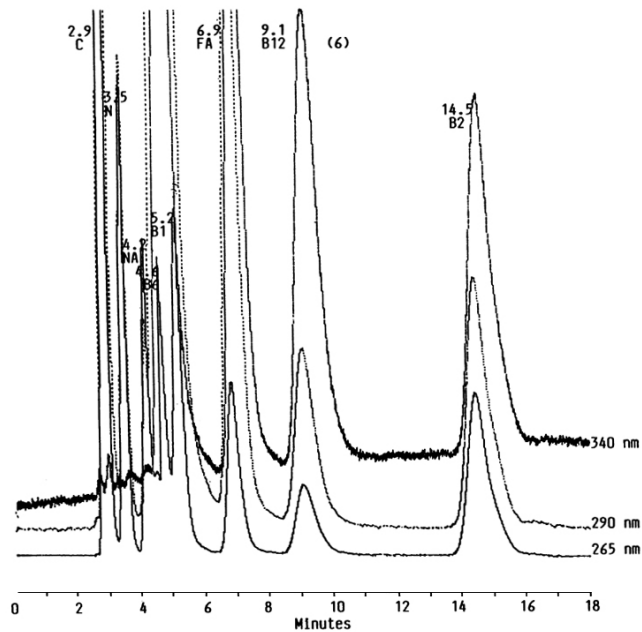


Figure 6. Chromatogram showing the separation of vitamin mixture using mobile phase containing methanol-glacial acetic acid-water, 28.5: 0.5: 71 (v/v/v) with 6mM sodium pentanesulfonic acid and 0.12% ammonia from first source.
B₁ - thiamine, B₂ - riboflavine, B₆ - pyridoxine, B₁₂ - cyanocobalamin, C - ascorbic acid, N - niacin, NA - niacinamide, FA - folic acid.
Other chromatography conditions are as given in the text.

Summary of mobile phase systems

The series of studies carried out showed that the best chromatography systems for the B-vitamins and ascorbic acid are as shown in Table 2. Waters PIC reagent required no further additives for complete separation of the vitamins and ascorbic acid, but additives such as ammonia and triethylamine is a necessity when using ion-pairing reagents from Sigma.

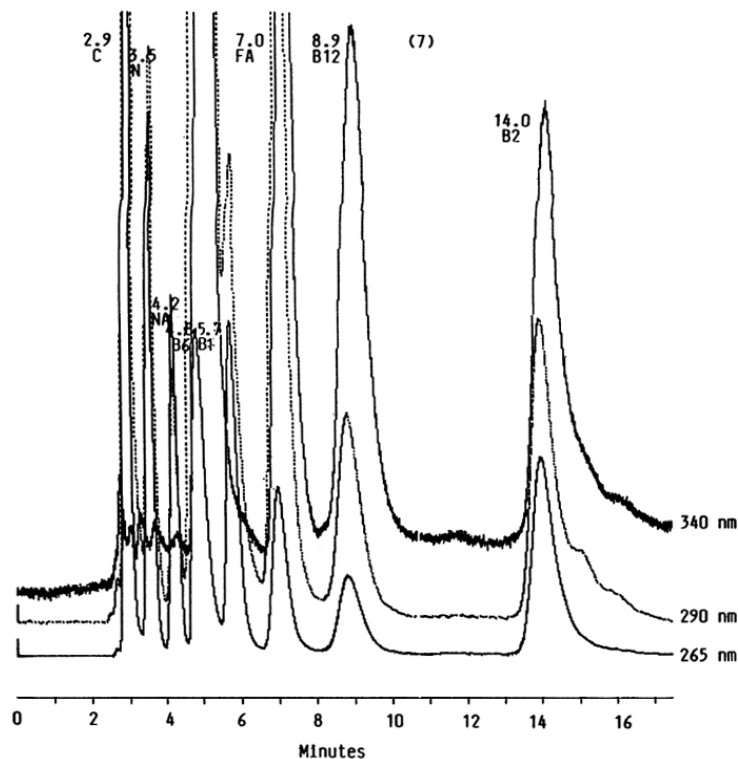


Figure 7. Chromatogram showing the separation of vitamin mixture using mobile phase containing methanol-glacial acetic acid-water, 28.5: 0.5: 71 (v/v/v) with 6mM sodium pentanesulfonic acid and 0.055% ammonia from second source.

B₁ - thiamine, B₂ - riboflavine, B₆ - pyridoxine, B₁₂ - cyanocobalamin, C - ascorbic acid, N - niacin, NA - niacinamide, FA - folic acid.

Other chromatography conditions are as given in the text.

Table 2. Mobile phase systems suitable for the separation of vitamin B₁, B₂, B₆, B₁₂, C, niacin, niacinamide and folic acid

Mobile phase system	Methanol (%)	Glacial acetic acid (%)	Water (%)	Ion pairing reagent	Additives	Chromatogram
1	24.5	0.5	75	5mM Waters' PIC B-5	none	Figure 1a
2	26.5	0.5	73	10mM sodium pentanesulfonic acid	0.090% triethylamine	Figure 5
3	28.5	0.5	71	8mM sodium pentanesulfonic acid	0.055% Mercek ammonia	Figure 7

CONCLUSION

The simultaneous determination of the eight water soluble vitamins i.e. vitamins B₁, B₂, B₆, B₁₂, C, niacin, niacinamide and folic acid can be satisfactorily performed on a 10 mm μ Bondapak C₁₈ column using the above 3 mobile phase systems. There is no necessity to have a gradient HPLC system, but the detectors used should be capable of simultaneous detection and quantitation in at least 3 wavelengths. It would be advantageous to have a computer software to handle the chromatographic data since several vitamins are being analysed.

The suitability of the chromatography system developed for the determination of multivitamin preparation and foods will be examined in subsequent studies. Prior to separation in the HPLC system, samples will require suitable extraction and purification procedures which will necessarily vary for different test materials. It would be advantageous to be able to simultaneously determine several vitamins in a single chromatography run. It is however necessary to ensure that accurate results are obtainable. Therefore, there has to be a compromise between the number of vitamins that can be simultaneously determined and the validity of the results.

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