

## **Simultaneous determination of B-vitamins and ascorbic acid in multi-vitamin preparations by reversed-phase HPLC**

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### **ABSTRACT**

The tedious and time consuming methods employed for the analysis of individual B-vitamins can now be replaced by ion-pair reversed-phase high-performance liquid chromatographic (HPLC) methods. This laboratory has previously reported the simultaneous determination of eight water-soluble vitamin standards that is, B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, B<sub>12</sub>, C, niacin, niacinamide and folic acid. The proposed isocratic HPLC method, employing 3 channels of detection, adequately separated all eight vitamins in less than 20 minutes. This study reports another phase of the project whereby the method was employed for the analysis of pharmaceutical preparations. Different extraction procedures were first evaluated, namely acid, acid plus enzyme and alkaline hydrolysis methods, using vitamin standards, individual vitamin tablets and multivitamin preparations. The amounts obtained from the analysis were compared with the declared values. Recovery studies were also carried out. The method of acid hydrolysis with 0.1N sulphuric acid was found suitable for use and was thus adopted as the extraction procedure for the analysis of 10 multivitamin preparations obtained from various pharmaceutical outlets. For most of these preparations, the amount obtained were close to the declared values, except for folic acid and cyanocobalamin. Further trials on folic acid showed that the problem could be resolved by omitting the filtration step in the final extract after acid hydrolysis and diluting with 0.01N sodium hydroxide before processing for chromatography. Vitamin B<sub>12</sub> was not detectable using the present chromatography system probably because of its low concentration in the samples studied.

### **INTRODUCTION**

There has been various attempts to improve the procedures for the determination of B-vitamins and ascorbic acid. These vitamins have traditionally been analysed using the manual methods of the AOAC. These methods involve extraction procedures and chemical reactions, followed by fluorimetry (B<sub>1</sub> and B<sub>2</sub>), spectrophotometry (niacin and folic acid), titration (ascorbic acid and -pyridoxine) and microbiological (B<sub>12</sub>) (Deutsch, 1984). These methods are usually tedious and time-consuming, and may require the use of hazardous chemicals such as cyanogen bromide for niacin determination. 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; Dong *et al.*, 1988; Speek, 1989). 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. Several investigators have reported the use of HPLC methods for the determination of B-vitamins in foods and pharmaceutical preparations (Toma & Tabekhia, 1979; Wehling & Wetzal, 1984; Amin & Reusch, 1987a, b; Rees, 1989; Hollman *et al.*, 1993). Some of these publications dealt with the simultaneous separation of

several B-vitamins whereas the majority reported the separation of only a few vitamins.

This laboratory has embarked on a project to develop improved methodologies for the analysis of water-soluble vitamins. It would obviously be advantageous if several vitamins can be determined simultaneously in a single chromatographic run. Preliminary studies have been carried out for the determination of four B-vitamins (Tee & Khor, 1993). Further work carried out led to the establishment of a method that enabled the simultaneous determination of eight water-soluble vitamins i.e. B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, B<sub>12</sub>, C, niacin, niacinamide and folic acid (Khor & Tee, 1996). This paper reports the application of the developed method to the determination of these vitamins in pharmaceutical products.

## **MATERIAL AND METHODS**

### **Apparatus**

A Gilson liquid chromatography system was used with a 714 system controller software. A stainless steel 30 cm by 3.9 mm I.D. 10 mm mBondapak C<sub>18</sub> column was used for the chromatographic separation. 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. 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 (B<sub>12</sub>). The characteristic absorption spectra of the vitamins analysed were determined using a spectrophotometer. Full details of the apparatus set up are as given in Khor and Tee (1996).

### **Chemicals and reagents**

The solvents and chemicals used for the analysis are as described in Khor and Tee (1996). The ion pairing reagents were obtained from Sigma Chemicals and ultra pure water of resistivity of at least 18 megohms centimeters (MΩ-cm) was used for mobile phase, standard preparation and sample extraction.

### **Procedure for preparation of mobile phase**

The mobile phase used has been previously found to be give satisfactory separation and comprised a mixture of methanol:glacial acetic acid:water 26.5:0.5:73 with 10 mM sodium pentanesulphonic acid with 0.09% triethylamine (Khor & Tee, 1996). Five ml of glacial acetic acid, 20 ml of 500 mM sodium pentanesulphonic acid and 0.9 ml triethylamine were added to 265 ml of methanol and make up to 1 litre with ultrapure water. This was then filtered with a 47 mm 0.45 (m Schleicher & Schuell RC 55 membrane filter paper utilising a millipore filtration set and subsequently degassed for 15 minutes with an ultrasonic bath. The HPLC column was washed with at least 100 to 150 ml of mobile phase and then equilibrated at a flow rate of 1 ml per minute before injecting 10 (1 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**

The vitamin standards used for the study were thiamine (B<sub>1</sub>), riboflavin (B<sub>2</sub>), niacin, niacinamide, pyridoxine (B<sub>6</sub>), folic acid, cyanocobalamin (B<sub>12</sub>) and ascorbic acid. Procedures for the preparation of these standards are as described by Khor and Tee (1996).

### **Procedures for preparing test samples (pharmaceutical preparations)**

#### *Acid hydrolysis*

The weight of 10 tablets or capsules was taken for calculations of vitamin content in each tablet. The tablets were finely ground with pestle and mortar or an electric mill and a 1-g sample was weighed accurately into a 125 ml amber erlenmeyer flask before adding 32.5 ml of 0.1N sulphuric acid. The sample was heated for half an hour over a boiling waterbath and stirred frequently with a glass rod. For capsule samples, glass rods were found to be unsuitable for use as the samples tended to adhere to the rods. The sample was next cooled in a basin of water and the pH adjusted to 4 - 4.5 with 2.5M of sodium acetate using pH paper. The extract was then transferred into a 50 ml volumetric flask and made up to the mark with ultra pure water. After shaking well, a portion of the extract was filtered with a Whatman 541 filter paper. For samples containing folic acid, the extract was diluted 1:1 with 0.01N sodium hydroxide before filtration to increase the solubility of folic acid since the vitamin is insoluble in solutions with a pH below 5.0. All extracts were filtered with 0.45 µm membrane filter and diluted with mobile phase, if necessary, before chromatography.

#### *Acid and enzyme hydrolysis*

The method of acid plus enzyme hydrolysis utilized the same hydrolysis procedure as described above except that after adjusting pH to 4.0 - 4.5 with pH paper, 2.5 ml of a 10% aqueous solution of Taka-diastase was added and then incubated at 45-50° for 3 hours. The extract was then cooled and made up to 50 ml with volumetric flask before filtration with Whatman 541 paper. All extracts were filtered with 0.45 µm membrane filter and diluted with mobile phase, if necessary, before chromatography.

#### *Alkaline hydrolysis*

The procedure for alkaline hydrolysis was as described above for acid hydrolysis, except that 0.1N sodium hydroxide was used in place of hydrochloric acid. The step of pH adjustment to 4-4.5 with a 2.5M of sodium acetate was omitted and all dilutions, if necessary, were made in mobile phase before chromatography.

### **Recovery studies**

For recovery of individual vitamin standards, known amounts of each standard as stated in Table 1 were added to the extraction flask before the introduction of reagents as mention in the procedure above for each hydrolysis step. The recovery of each vitamin when mixed with other vitamin standards was also studied. Known amounts of each vitamin as mention in Table 2 were

mixed together in the extraction flask before carrying out the three extraction procedures mentioned above.

In multivitamin preparations, recovery studies were carried out using only thiamine and riboflavin standards. For B1, about 30 to 100% of the amount present in the pharmaceutical products, that is, 1 to 20 mg were added from a stock solution of 5 mg/ml in 0.1N HCl. Because of the difficulty in dissolving vitamin B2 to prepare a standard solution which is sufficiently concentrated for addition in recovery studies, the crystalline standard was weighed and added directly to the test samples. About 50 to 100% of the amount present in pharmaceutical products, usually 2 to 4 mg, were added directly to the recovery sample during extraction. These two standards were added to 1 g of the test sample contained in a 125 ml amber erlenmeyer flask before the addition of reagents for extraction using the acid hydrolysis method.

**Table 1.** Recovery of individual vitamin\* standards extracted using different procedures

Vitamin standards	Amount of standard added (mg)	Extraction Methods					
		Acid hydrolysis		Acid plus enzyme hydrolysis		Alkaline hydrolysis	
		mg recovered	% recovery	mg recovered	% recovery	mg recovered	% recovery
Thiamine	5	4.74	94.7	4.61	92.3	1.55	31.0
Riboflavin	2	2.31	115.7	2.20	110.1	0.53	26.3
Pyridoxine	10	11.77	117.7	11.13	111.3	7.45	74.5
Cyanocobalamin	3	2.16	71.9	1.17	60.1	ND	ND
Niacin	10	8.98	89.8	9.10	91.0	8.52	85.2
Niacinamide	10	9.90	90.0	10.12	101.2	8.24	82.4
Folic acid	1	0.93	92.7	1.19	119.2	1.05	104.5

\* Each vitamin was analysed in duplicate  
ND = not detected

**Table 2.** Recovery of vitamin\* in standards mixtures extracted using different procedures

Vitamin standards mixture with the following vitamins	Amount of standard added (mg)	Extraction Methods					
		Acid hydrolysis		Acid plus enzyme hydrolysis		Alkaline hydrolysis	
		mg recovered	% recovery	mg recovered	% recovery	mg recovered	% recovery
Thiamine	5	4.73	94.6	4.91	98.2	na	na
Riboflavin	2	2.10	105.0	1.94	96.9	na	na
Pyridoxine	10	10.23	102.3	10.09	100.8	na	na
Cyanocobalamin	1	0.44	44.4	0.42	42.1	na	na
Ascorbic acid	10	7.13	71.3	5.60	56.0	na	na
Niacin	10	9.88	98.8	9.88	98.8	na	na
Niacinamide	10	10.33	103.2	10.08	100.8	na	na
Folic acid	1	0.75	74.7	1.01	101.2	0.96	95.1

\* Each vitamin was analysed four times  
na = unable to analyse because of merging of peaks due to instability of vitamins

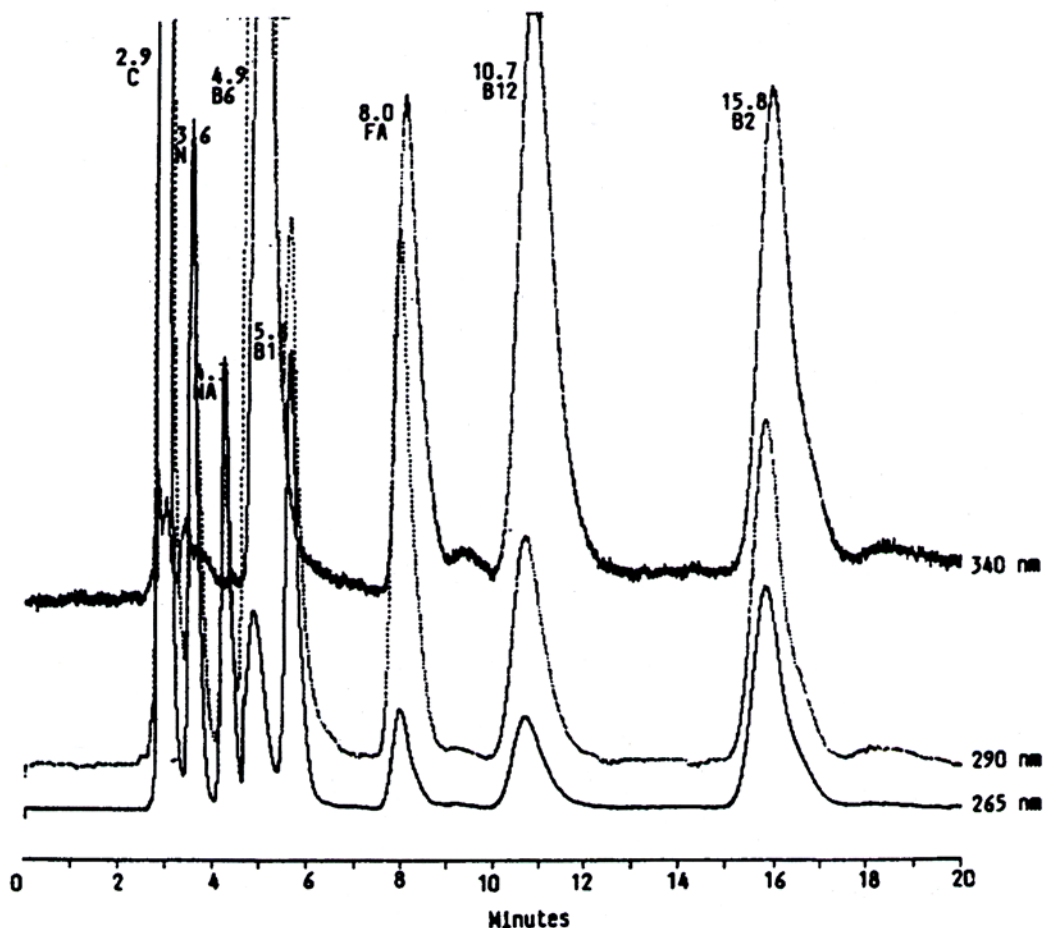


Figure 1. Chromatogram showing the separation of vitamin mixture using mobile phase containing methanol-glacial acetic acid-water, 26.5: 0.5: 73 (V/V/V) with 10 mM sodium pentanesulfonic acid and 0.09% triethylamine (TEA).

B<sub>1</sub> - thiamine, B<sub>2</sub> - riboflavin, B<sub>6</sub> - pyridoxine, B<sub>12</sub> - cyanocobalamin, C - ascorbin acid, N - niacin, NA - niacinamide, FA - folic acid.

Other chromatography conditions are as given in the text.

## RESULTS AND DISCUSSION

### Chromatography of vitamin mixture

Figure 1 shows the HPLC chromatogram of a mixture of eight vitamins, separated using the mobile phase of methanol:glacial acetic acid:water 26.5:0.5:73 with 10 mM sodium pentane sulfonic acid and 0.09% TEA. All eight vitamins gave quite good separation at the retention time (in minutes) of 2.9 for ascorbic acid, 3.6 for niacin, 4.3 for niacinamide, 4.9 for pyridoxine, 5.6 for thiamine, 8.0 for folic acid, 10.7 for cyanocobalamin and 15.8 for riboflavin.

### Studies on extraction procedures on vitamin standards

The three methods of extraction, namely acid, acid plus enzyme and alkaline hydrolysis, were

applied to solutions of individual vitamin standards. It was found that acid hydrolysis gave good recoveries of 90 to 118% for all the vitamins studied, except for vitamin B<sub>12</sub> whose recovery was only 72% (Table 1). The method of acid plus taka-diastrase enzyme gave an overestimation of 119% for folic acid and it was noted that there was an increase of retention time of the peak by 1.4 minutes. Recovery for B<sub>12</sub> was even worse, at about 60%, probably because the longer procedure required for the extraction resulted in losses of the vitamin. However recoveries for the other vitamins, that is, B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, niacin and niacinamide were still good, ranging from 91 to 111%. With alkaline hydrolysis, good recovery was obtained only for folic acid (105%), the retention time remaining the same as that of the standard before extraction. With regards to the other vitamins, B<sub>12</sub> was not detectable after hydrolysis, whereas recoveries for the other vitamins ranged from 26% for B<sub>2</sub> to 85% for niacin (Table 1).

The effects of different methods of extraction on recoveries of a mixture of standard solutions were next investigated. Using the acid hydrolysis and acid plus enzyme hydrolysis methods, recoveries for thiamine, riboflavin, pyridoxine, niacin and niacinamide were good for both methods, ranging from 95 to 105% (Table 2). However recovery for B<sub>12</sub> in a mixture of standards was poor, with values as low as 44% in acid hydrolysis and 42% in the acid plus enzyme method. Ascorbic acid gave variable results with a mean of 71% in acid hydrolysis and even poorer results in the acid plus enzyme method. The poor recovery values of some vitamins could be due to their instability in the presence of other vitamins (Sober, 1970). For folic acid, the average recovery with acid hydrolysis was 75%, but the acid plus enzyme extraction method gave a good recovery of about 100%. Using the alkaline hydrolysis method, only folic acid gave a satisfactory recovery of about 95%. It was unable to analyse the other vitamins because the peaks were merged together with several other peaks, presumably arising from the degradation of the vitamins. As can be expected, most of these B-vitamins are unstable in alkaline medium (Sober, 1970).

### **Studies on extraction methods on pharmaceutical preparations**

Studies were carried out to determine the effects of utilising the acid hydrolysis and acid plus enzyme hydrolysis methods for the extraction of several tablets of individual vitamins from the General Hospital, Kuala Lumpur and other pharmaceutical outlets. Both methods gave good recovery values of 89 to 110% for thiamine, riboflavin, pyridoxine, B<sub>12</sub>, ascorbic acid and niacinamide tablets (Table 3). The recovery obtained for folic acid was poor, that is, < 14%, for both the acid and acid plus enzyme methods of hydrolysis. However when the alkaline hydrolysis procedure was used, the recovery value improved to 87.3%, confirming that the acid and acid plus enzyme methods were not suitable for use with folic acid.

The next step was the application of the acid and acid plus enzyme extraction methods on multivitamin tablets or capsules to determine if the presence of other vitamins affect the analysis of a particular vitamin. The amount of niacinamide, pyridoxine, thiamine and riboflavin in four test samples obtained by the two methods are as tabulated in Table 4. For all the four vitamins, the difference in the percentage of vitamins obtained compared with declared values for the two methods was less than 10% for most of the samples. It was therefore decided to omit the enzyme hydrolysis step, thereby shortening the time of extraction and permitting chromatographic runs to be carried out within the same day as the extraction. This would be advantageous, especially for

ascorbic acid which was noted to be unstable on standing.

Table 3. Recovery of vitamins\* in individual vitamin tablets extracted using different procedures

Individual vitamin tablets	Amount declared (per tablet)	Extraction methods			
		Acid hydrolysis		Acid plus enzyme	
		mg recovered	% recovery	mg recovered	% recovery
Thiamine	10 mg	9.81 mg	98.1	10.43 mg	104.3
Riboflavin	3 mg	3.28 mg	109.4	3.07 mg	102.3
Pyridoxine	10 mg	11.06 mg	110.6	9.97 mg	99.73
Vitamin B <sub>12</sub>	100 mg	102.6 mg	102.6	93.06 mg	93.1
Ascorbic acid	50 mg	44.34 mg	88.7	45.48 mg	91.0
Niacinamide	50 mg	45.19 mg	90.7	44.76 mg	89.5
Folic acid	5 mg	0.68 mg	13.6	1.95 mg	7.8

\* Each vitamin was analysed in duplicate

### **Determination of multivitamin tablets or capsules**

Tables 5(a), 5(b) and 5(c) present the results obtained for the analysis of 10 different proprietary brands of multivitamin tablets or capsule obtained from various pharmaceutical outlets. All the preparations were determined using the acid hydrolysis extraction method. The tables present the levels obtained for the eight vitamins determined, compared with the amounts declared on the label. Recovery values obtained are tabulated in Table 5(b). The main findings obtained are highlighted in the following paragraphs.

#### *Niacinamide*

In almost all the products determined, the amount of the vitamin obtained was close to the amount declared, ranging from 95% to 125%. Product 9, however, gave a poor ratio of 78%.

#### *Pyridoxine*

Out of the 10 multivitamin preparations examined, the amount obtained over the amount declared for 8 of them ranges from 83 to 122%. However 2 of the products had a ratio of 130 to 140%.

#### *Thiamine*

For five of the products studied, the amount obtained was within 20% of the amount declared, with ratios ranging from 82 to 115%. The ratio was particularly low for product 9 that is, only 50% but very high for 4 of the products with ranges from 127 to 245%. Two of these products with high values obtained were reanalysed using the AOAC fluorometric assay (Deutsch, 1984). The results obtained were found to be even higher than those obtained by the HPLC method, that is, 20% higher for product 5 and 9% higher for product 1. However recoveries carried out for B<sub>1</sub> appears to be quite good ranging from 85 to 109% with only 2 cases giving slightly lower

recoveries of 67 and 78% (mean  $\pm$  SD,  $92.4 \pm 12.8$ ) (Table 5). The very results obtained could be due to some interfering compounds present in some samples, or it could be that the samples indeed had very high B<sub>1</sub> levels.

Table 4. Recovery of vitamins\* in multivitamin preparations extracted using different procedures

	Amount declared (tablet)	Amount obtained (mg/tablet)		% Obtained/declared		Difference (acid hydrolysis acid)
		Acid hydrolysis	Acid plus enzyme hydrolysis	Acid hydrolysis	Acid plus enzyme hydrolysis	
<b>Niacinamide</b>						
Sample 1	10	9.52	9.74	95.2	97.4	-2.2
2	50	48.76	51.3	97.5	102.6	-5.1
3	20	19.49	18.17	97.5	90.9	6.6
4	20	20.76	20.55	103.8	102.8	1.0
<b>Pyridoxine</b>						
Sample 1	1	0.89	0.91	89.0	91.0	-2.0
2	5	4.17	3.77	83.4	75.4	8.0
3	0.5	0.46	0.50	92.0	100.0	-8.0
4	15	14.43	13.18	96.2	87.9	8.3
<b>Thiamine</b>						
Sample 1	5	12.23	12.06	244.6	241.2	3.4
2	50	41.79	42.96	83.6	85.9	-2.3
3	3	2.45	2.03	81.7	67.7	14.0
4	10	20.83	20.07	208.3	200.7	7.6
<b>Riboflavin</b>						
Sample 1	1.5	1.42	1.49	94.7	99.3	-4.6
2	20	14.13	15.27	70.7	76.4	-5.7
3	2.5	2.18	1.62	87.2	64.8	22.4
4	2.5	2.27	2.03	90.8	81.2	9.6

### *Riboflavin*

In 5 out of the 10 products studied, the amount obtained over the amount declared were close, ranging from 87 to 109%. Exceptionally high value was obtained for one of the products, i.e. 155% for product 8. The other 4 products appeared slightly lower with values of 70 to 79%. Recoveries were carried out for 6 of the cases giving a range of 79 to 130% (mean  $\pm$  SD,  $98.4 \pm 18.4$ ). The larger variation in the recovery values compared to those obtained for thiamine might be attributed to the errors incurred in weighing small amounts (i.e. 2 to 4 mg) of B<sub>2</sub> standard to the extraction flask for recovery studies.



*B-vitamins & ascorbic acid in multivitamin preparations*

**Table 5(a).** Determination of several B-vitamins and ascorbic acid in 10 multivitamin preparations

		Amount obtained (mg/tablet or capsule)	Amount declared (mg*/table or capsule)	% Obtained/ declared	% Recovery
Niacinamide					
Product	1	9.52	10	85.2	-
	2	48.76	50	97.5	-
	3	19.49	20	97.5	-
	4	20.76	20	103.8	-
	5	61.04	50	122.1	-
	6	22.43	20	112.2	-
	7	27.49	25	110.0	-
	8	25.03	20	125.2	-
	9	7.00	9	77.8	-
	10	49.34	50	98.7	-
Pyridoxine					
Product	1	0.89	1	89.0	-
	2	4.17	5	83.4	-
	3	0.46	0.5	92.0	-
	4	14.43	15	96.2	-
	5	24.43	20	122.2	-
	6	2.83	2	141.5	-
	7	2.63	2	131.5	-
	8	2.91	3	97.0	-
	9	3.54	3.3	107.3	-
	10	9.94	10	99.4	-

\* cyanocobalamin concentration in µg per tablet or capsule

**Table 5(b).** Determination of several B-vitamins and ascorbic acid in 10 multivitamin preparations (cont)

		Amount obtained (mg/tablet or capsule)	Amount declared (mg*/table or capsule)	% Obtained/ declared	% Recovery
Thiamine					
Product	1	12.23	5	244.6	85.9
	2	41.79	50	83.6	95.7
	3	2.45	3	81.7	67.4
	4	20.83	10	208.3	91.1
	5	50.74	40	126.9	91.1
	6	2.59	2	129.5	102.5
	7	8.78	10	87.8	78.4
	8	2.46	3	82.0	85.6
	9	3.35	6.7	50.0	107.2
	10	23.06	20	115.3	109.0
Riboflavin					
Product	1	1.42	1.5	94.7	-
	2	14.13	20	70.7	-
	3	2.18	2.5	87.2	-
	4	2.27	2.5	90.8	-

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	5	14.81	20	74.1	78.8
	6	2.18	2	109.0	100.2
	7	9.38	10	93.8	87.7
	8	4.64	3	154.7	107.4
	9	5.28	6.7	78.8	129.5
	10	3.63	5	72.6	86.7
<b>Ascorbic acid</b>					
Product	2	444.57	500	88.9	-
	4	107.49	100	107.5	-
	5	150.88	150	100.4	-
	6	51.11	50	102.2	-
	7	62.51	75	83.4	-
	8	126.38	100	126.4	-
	9	49.81	66.6	74.8	-
	10	161.74	150	107.8	-
<b>Folic acid</b>					
Product	4	ND	0.25	-	-
	5	ND	0.025	-	-
	6	0.14	0.1	140.0	-
	7	0.14	0.1	140.0	-
	8	0.10	0.2	50.0	-
	9	0.13	0.125	104.0	-
	10	0.34	1	34.0	-
<b>Cyanocobalamin*</b>					
Product	2	ND	5	-	-
	3	ND	2	-	-
	4	ND	4	-	-
	5	ND	25	-	-
	6	ND	3	-	-
	7	ND	5	-	-
	8	ND	25	-	-
	9	ND	6.7	-	-
	10	ND	5	-	-

\* cyanocobalamin concentration in  $\mu\text{g}$  per tablet or capsule  
 ND = not detected

*Ascorbic acid*

For most of the products studied, the amount obtained agreed within 20% of the amount declared. It must be pointed out that these estimations were carried out almost immediately after processing the samples since vitamin C has been known to be unstable on standing and on exposure to light.

*Folic acid*

Unlike for the other B-vitamins so far discussed, results obtained for folic acid were not satisfactory. Out of the 7 products studied, folic acid was detected in only 5 of them, and the amounts obtained compared to the declared values were highly variable, ranging from 34 to 140%. It can be noted that the level of folic acid in the preparations studied were much lower

than for the other vitamins. Except for one product, all the samples had declared values of less than 1 mg per tablet. Chromatography peaks were thus very small and difficult to integrate accurately. Even for product 10, which had the highest declared content of folic acid of 1 mg, the amount obtained was only 34% of the declared value. It could be that the method used was not suitable for the determination of folic acid because of the low levels present or that the amounts declared were not correct. Further studies were carried out to investigate this further.

#### *Cyanocobalamin*

Cyanocobalamin was not detected in all the 9 multivitamin preparations examined, all of which had declared values of below 25 mg whereas the amount of other B-vitamins were in the region of several milligrams. Amin & Reusch (1987a) had reported that the determination of 1-5 mg of vitamin B<sub>12</sub> in the presence of 4-15 mg of B<sub>1</sub>, B<sub>2</sub> and B<sub>6</sub> was a major problem because of the large difference in the concentration of the B<sub>12</sub> compared with the other vitamins. Using the Water's 440 fixed-wavelength detector at 340 nm at 0.005 a.u. appeared to be not sensitive enough for B<sub>12</sub> detection in these multivitamin preparations. Detection of B<sub>12</sub> peak at a sensitivity higher than that of the other vitamins did not appear to help as B<sub>12</sub> still did not separate completely from the earlier eluting peak. Spiking samples with B<sub>12</sub> standard solution also did not resolve the problem as the amount required for spiking was 10 times greater than the amount present in the sample resulting in inaccurate values.

#### **Further trials on folic acid determination in vitamin preparations**

As has been discussed above, the method of acid hydrolysis appeared to be unsatisfactory for the analysis of folic acid. A number of trials were conducted on preparations containing folic acid by making modifications to the procedure.

Since folic acid was found to be insoluble in solutions with pH below 5, filtration of the final extract after acid hydrolysis could have resulted in losses in the vitamin. A series of experiments were thus conducted on individual folic acid tablets and multivitamin preparations to determine if omitting the filtration step could improve the results. The use of mobile phase was also examined to determine if it gives better results.

Table 6 shows that when samples were filtered and diluted in sodium hydroxide, the amounts obtained were low, with only one of the 7 samples studied giving 90% of the declared value. Without filtration, the samples diluted in sodium hydroxide resulted in much higher levels of folic acid obtained, with values obtained for 6 of the 7 samples studied within 10% of the declared values. The amount of folic acid obtained were clearly lower when the unfiltered samples were diluted in mobile phase; only one sample had an obtained value of over 90% of the declared value. It could thus be concluded that the acid hydrolysis procedure can still be applicable for folic acid estimation provided the filtration step was omitted and dilution made in 0.01N sodium hydroxide. The samples can then be filtered using membrane filters and chromatographed in the HPLC system described.

Table 6. Folic acid levels in samples with and without filtration after acid hydrolysis

Sample	Amount declared (mg per tablet)	Sample filtered		Samples not filtered			
		Dilution in 0.01N NaOH		Dilution in 0.01N NaOH		Dilution in mobile phase	
		Amount obtained (mg per tablet)	% Obtained / declared	Amount obtained (mg per tablet)	% Obtained / declared	Amount obtained (mg per tablet)	% Obtained / declared
Folic acid tablet 1	5	4.13	82.6	5.24	104.7	4.69	93.8
Folic acid tablet 2*	20	4.28	21.4	21.33	106.7	3.13	15.7
Multivitamin 1	1	0.61	61.0	0.96	96.1	0.56	55.6
Multivitamin 2	0.25	0.20	80.0	0.22	88.0	0.18	72.0
Multivitamin 3	0.20	0.13	65.0	0.21	105.0	0.17	35.0
Multivitamin 4	0.20	0.18	90.0	0.22	110.0	0.17	35.0
Multivitamin 5	0.05	0.02	40.0	0.03	60.0	0.02	40.0

\*four tablets were combined together

This laboratory conducted a longitudinal study on iron and folate supplementation to adolescent girls. The tablets used in the study were of two types, namely 60 mg iron and 120 mg iron, both of which contained 3.5 mg folate per tablet. The tablets were analysed for folic acid content prior to the supplementation study. It was found that the amount of folic acid obtained varied inversely with the amount of the tablet taken for analysis (Table 7). Comparing the results obtained for the two types of tablets, it would appear that the amount of folic acid obtained increased with lower amount of iron in the sample taken for analysis.

## CONCLUSIONS

The simultaneous determination of the eight water soluble vitamins that is, vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, B<sub>12</sub>, C, niacin, niacinamide and folic acid can be satisfactorily performed on a 10 mm mBondapak C18 column using mobile phase of 26.5% methanol, 0.5% glacial acetic acid and 73% water with 10 mM sodium pentanesulfonic acid and 0.09% TEA. Different methods for sample extraction were also evaluated and the method of acid hydrolysis with 0.1N sulphuric acid without the addition of enzyme taka-diastrase was sufficient for the extraction of pharmaceutical products. However for the determination of folic acid, the filtration of the final extract should be omitted and dilutions made with 0.01N sodium hydroxide before filtering with membrane filter for HPLC injection. The method developed was tried on tablets of individual vitamins and also on 10 different brands of multivitamins obtained from various sources. Vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, C, niacin, niacinamide and folic acid obtained showed good agreement with the levels stated on the labels. Vitamin B<sub>12</sub> was not detectable using the present chromatography system probably because of its low concentration of less than 25 mg.

Table 7. Determination of folic acid tablets\* containing iron

Amount of iron per tablet	Amount used in extraction (g)	Amount obtained (mg per tablet)	% Obtained / declared
60 mg	1	2.24	63.9
	0.5	3.41	97.4
120 mg	1	0.50	14.2
	0.5	1.62	46.3
	0.2	2.34	66.9
	0.05	3.86	110.3

\* each tablet had a declared value of 3.5 mg folic acid

In the next phase of the project, the developed chromatographic method will be applied to the determination of food samples. Considerable amount of sample cleaning procedures will have to be carried out first before successful separation by HPLC is possible. Detailed and systematic studies will be carried out to develop appropriate methods for various food groups. It remains to be seen how many of the water-soluble vitamins can be simultaneously determined in foodstuffs.

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