Open Access

Quantitative Simultaneous Estimation of Water Soluble Vitamins, Riboflavin, Pyridoxine, Cyanocobalamin and Folic Acid in Neutraceutical Products by HPLC

Shahnaz Perveen[*,a], Arfa Yasmin[a] and Khalid Mohammed Khan[b]

[a]*PCSIR Laboratories Complex, Karachi, Shahrah-e-Dr. Salimuzzaman Siddiqui, Karachi-75280, Pakistan*

[b]*H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*

Abstract: Water soluble vitamins *e.g.* riboflavin (B_2), pyridoxine (B_6), cyanocobalamin (B_{12}) and folic acid in neutraceutical product have been determined simultaneously by using a rapid, precise and time saving new high performance liquid chromatographic method and its validation. The method involves gradient elution of mobile phase through C_{18} discovery column (Supelco, Sigma-Aldrich) in a reverse phase chromatography with UV detection at 254 nm at ambient temperature. The ranges for quantification for B_2, B_6, B_{12} and folic acid were 0.13 mg g^{-1} (0.57-131 μg g^{-1}), 0.235 mg g^{-1} (3-235 μg g^{-1}), 7.94 x 10^{-2} mg g^{-1} (8-80 μg g^{-1}) and 9.66 x10^{-2} mg g^{-1} (10-97μg g^{-1}), respectively. For the validation of the method, linearity, precision, accuracy and robustness have been performed. The repeatability was measured in terms of RSD value. The RSD for all vitamins was below 1%. Recovery of vitamins ranges from 98.6 to 100.5%.

INTRODUCTION

In 1933, Kuln and co-workers first isolated riboflavin from eggs in a pure crystalline state and named it ovoflavin. Riboflavin in free form is found in the retina of the eye, in whey and in urine. Riboflavin is distributed in some degree in virtually all naturally occurring foods, *e.g.* liver, heart, kidney, milk, eggs, lean meats and fresh leafy vegetables are particularly good source of riboflavin [1]. Riboflavin can be assayed by chemical, microbiological, and biological methods. Both fluorometric [2] and microbiological [3] assays are official methods of the Association of Official Analytical Chemicals (AOAC). High performance liquid chromatography (HPLC) has been applied to the determination of riboflavin in a variety of foods by reverse-phase HPLC method [4]. Its use as a column additive is approved by the Food and Drug Administration 21 CFR (73:450).

Vitamin B_6 is identified and named pyridoxine in 1934. The analysis of vitamin B_6 in food is complicated by the fact that six forms (vitamers) are found in nature, therefore microbiological, colorimetric and HPLC methods are currently used [5-7]. Rich sources of vitamin B_6 are chicken, pork, fish, organ meats, and eggs.

Vitamin B_6 deficiency symptonology includes the following chemical signs: eczema and seborrheic dermatosis, in the ears, nose, and mouth; cheilosis, glossitis and angular stomatitis and hypochromic and microcytic anemia.

Vitamin B_{12} deficiency includes paresthesias of the hands and feet, decreased deep-tendon reflexes, unsteadiness and potential psychiatric problems. Such as moodiness, hallucinations, delusions and psychosis. Vitamin B_{12} is not present in plants, and therefore dietary deficiencies can occur in strict vegetarians. Cyanocobalamin is the commercial form of vitamin B_{12} and specifications are found in the codex for use as food [8], and in the USP for pharmaceutical use [9].

Vitamin B_{12} can be determined by microbiological, radioisotope dilution, spectrophotometric, chemical or biological methods employing animals [10-12]. Spectrophotometric determination at 550 nm is relatively insensitive and is useful for the determination of vitamin B_{12} in high potency products such as premixes. Thin layer chromatography and open column chromatography have been applied to both direct assays of cyanocobalamins. An indirect method is atomic absorption spectrophotometric analysis of cobalt in dry feeds. Recently a high performance liquid chromatographic (HPLC) method is reported which is suitable for premixes, raw materials and pharmaceutical products containing 20-100 μg vitamin B_{12} [13].

Folic acid deficiency is the result of megaloblastic anemia. One of the chemical signs of acute folate deficiency includes a red, painful tongue. Folic acid as pteroylglutamic acid is not found naturally in foods. Methods for determining folic acid in food include biological, microbiological, chemical, chromatographic and radiometric assays [14-17].

Analysis of water soluble vitamins B_2, B_{12}, folic acid, biotin and pantothenic acid based on biosensor-based vitamin analysis technology, this method is sensitive but did not analyze the vitamin B_6 simultaneously [18]. In another method simultaneous determination of seven water soluble vitamins nicotinamide, thiamin, riboflavin, pyridoxine, pyridoxal, pyridoxamine, cyanocobalamine and folic acid were carried out by using ion-pair chromatography [19]. Nevertheless, the detection time is very high and the UV detector has to set at different wavelengths. Literature showed simul-

*Address correspondence to this author at the PCSIR Laboratories Complex, Karachi, Shahrah-e-Dr. Salimuzzaman Siddiqui, Karachi-75280, Pakistan; Tel: +92-21-4655557/62; Fax: +92-21-4655557;
E-mail: dr_shahnaz_perveen@yahoo.com

1874-0650/09 2009 Bentham Open

taneous determination of four B-group vitamins, B_1, B_3, B_6 and B_{12} but B_{12} has been detected separately at 550 nm [20].

We want report herein a new method which simultaneously analyses four water soluble vitamins B_6, folic acid, B_{12}, and B_2 in a complex mixture (neutraceutical) by HPLC using UV detector at 254 nm. Details of the method and its validation were reported.

MATERIALS AND METHODS

Potassium dihydrogen phosphate (BDH, Anala R), formic acid (BDH, Anala R) and methanol (BDH, Anala R) were used. Standard solutions of riboflavin (B_2), pyridoxine (B_6), cyanocobalamin (B_{12}) and folic acid were freshly prepared. Folic acid, vitamins B_2, B_6 and B_{12} were purchased from Sigma-Aldrich. Acetic acid (BDH, Anal R), HCl (reagent grade and water (deionized) was used.

HPLC Method

The high performance liquid chromatographic system used was equipped with a solvent delivery 200 HPLC pump (Perkin Elmer Series) with online degasser, UV/VIS detector (Perkin Elmer Series 200), Perkin Elmer NCI 900 network chromatography interface, and a data processing unit compaq.

HPLC column discovery C_{18}, 25cm x 4.6mm, 5μm (Supelco, Sigma-Aldrich) was used for the separation of vitamins. A gradient of methanol and buffer (30:70, in eight minutes) of 50 mM (0.05M) potassium dihydrogen phosphate having pH 4.2 $\pm$ 0.1, adjusted with formic acid was used as mobile phase. The flow rate was maintained at 1 ml min^{-1}. Wave length of detection was 254 nm. An injection volume of 20μL was chromatographed, and the whole chromatography was performed at ambient temperature.

Preparation of Standard Solution

The standard samples B_2, B_6, B_{12} and folic acid 100 mg (each) were accurately weighed and transferred into three 100 mL volumetric flask separately and 100 mg of B_2 was transferred into 250 mL volumetric flask. Initially 7 mL of acetic acid and 50 mL of methanol were added to each flask; the contents were dissolved by sonication for 10 min and allowed to cool to ambient temperature. The contents were diluted to volume with water and thoroughly mixed. These solutions were used as reference working standard solution (Fig. **1**). Prior to injecting into the liquid chromatograph, the solution was filtered through 0.45 μm membrane filter. The samples were quite stable at room temperature. The stock solutions of standards were kept in a refrigerator for further use and remain unchanged for a period of a month.

Preparation of Sample Solution

15.07 g of neutraceutical enriched with vitamins was accurately weighed and transferred into a 250 mL round bottom flask. Initially about 10 mL of 0.1 N HCl and 80 mL water was added and then reflux on boiling water bath for 15 min. After completion of refluxing period the flask was cooled and volume made up to 100 mL in a volumetric flask. The content was centrifuged (1400 rpm) to remove suspended material. The supernatant solution was first filtered through a Whatman No. 1 filter paper and the resulting filtrate was again filtered through 0.45 μm membrane filter before injection into LC system (Fig. **2**). The stock solutions of sample were kept in a refrigerator for further use and remain unchanged for a period of a month.

RESULTS AND DISCUSSIONS

Standard solutions containing B_2, B_6, B_{12} and folic acid equal to 20, 50 for linearity, 80, 100, 120, 150 and 180% were prepared and examined by the assay procedure. The peak area responses measured for B_2, B_6, B_{12} and folic acid were plotted versus concentration and a linear response was obtained over the range of concentrated studied for all four ingredients. The slope of calibration curve and proximity of all points to the calibration curve demonstrates that the method has adequate sensitivity to the concentrated of vitamins B_2, B_6, B_{12} and folic acid.

The accuracy of the assay procedure was determined by carrying out recovery experiments by spiking the standard. Amounts of B_2, B_6, B_{12} and folic acid equivalent to 20, 50, 80, 100, 120 and 150% of the theoretical assay concentration were added to the formula amount of neutraceutical preparation and the mixtures were subjected to the assay procedure. The results so obtained are summarized in Table **1**. The recovery experiment shows that the method is sufficiently accurate and there is no significant interaction between the active components and excepients.

The precision of assay method was determined under repeatability condition by an experiment in which six preparations were made from the same batch of formulation and were analyzed by one operator on a single occasion. The results are presented in Table **2**. The intermediate precision was assessed by another experiment in which two analysts on two different instruments with six independent determinations assayed the same batch of formulation. The results are statistically valid as shown in Table **3**.

The robustness of the assay method was assessed with respect to alternations in flow rate, column (same column but of different batch) and change in wave length of UV of the standard and sample, as stability of working standard and test solutions stored in amber glass at ambient temperature. The results show that the new HPLC method is robust to small changes in flow rate, change in column, and the solutions exhibited a good degree of stability. The LOD for B_2, B_6, B_{12} and folic acid were found to 0.57, 3, 8 and 10 $\mu g\ g^{-1}$, respectively.

Neutraceutical preparation omitting B_2, B_6, B_{12} and folic acid was examined by the assay procedure. No peak due to excipients in the formulation was observed at the typical retention times for B_2, B_6, B_{12} and folic acid. Therefore, it is concluded that the assay method is specific for both active ingredient in the presence of excipients of formulated product. The suitability of the system was defined by determining the value of column efficiency, tailing factor and resolution factor using the method in VSP. Column efficiency was greater than 1000 per column, tailing factor was not more than 2 and resolution factor is greater than 3 for B_2, B_6, B_{12} and folic acid (Table **4**).

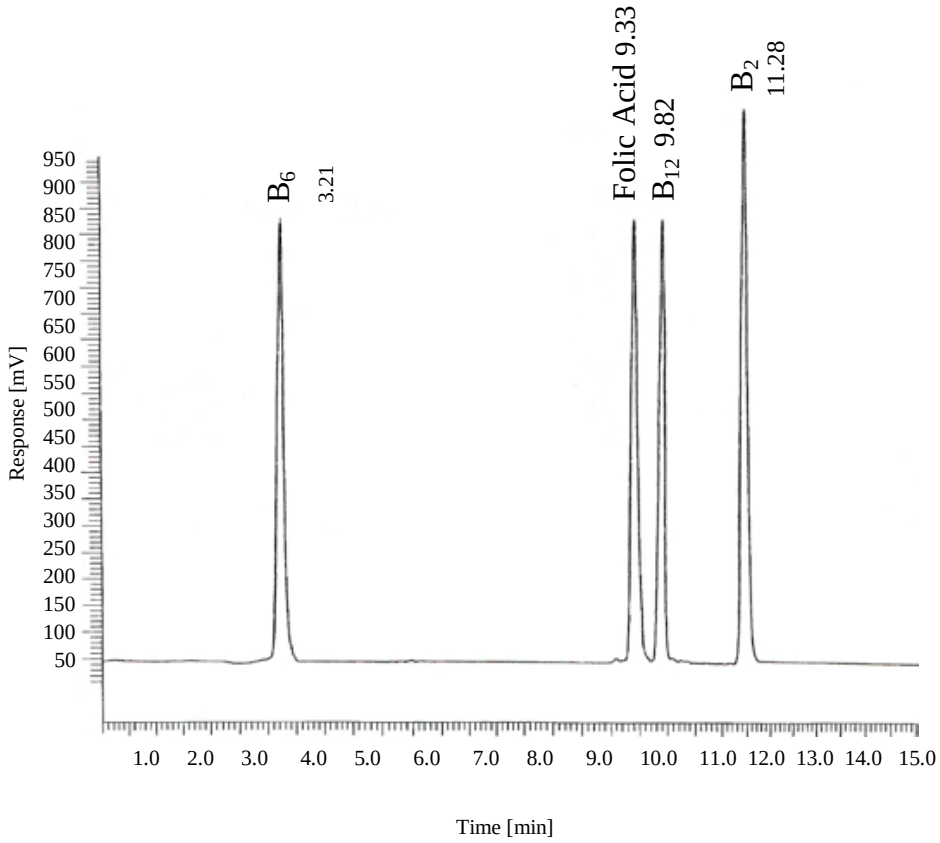

Fig. (1). Chromatogram of standards B_6, folic acid, B_{12} and B_2.

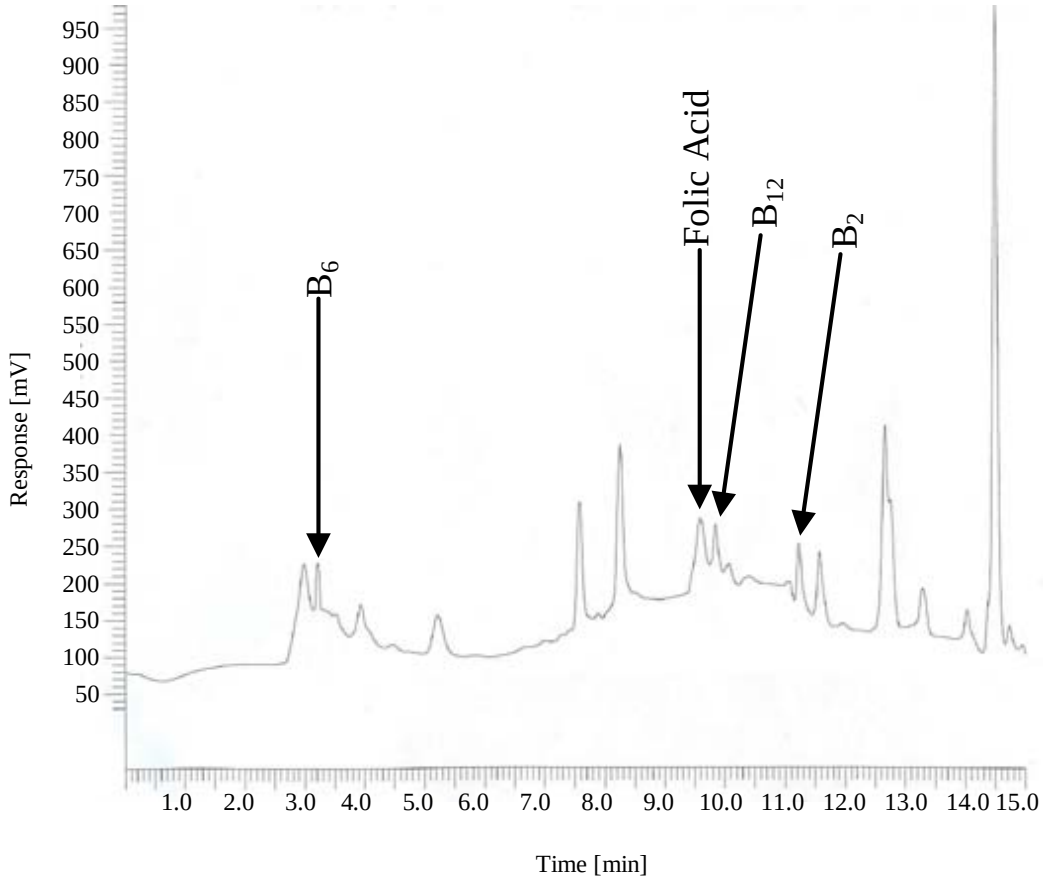

Fig. (2). Chromatogram of sample.

Table 1. Recovery Experiment (Reproducibility) for B_2, B_6, B_{12} and Folic Acid by Proposed HPLC Method (n = 6)

[a]B_2			[b]B_6			[c]B_{12}			[d]Folic Acid		
Amount Added mg g^{-1}	Amount Found mg g^{-1}	Recovery %	Amount Added mg g^{-1}	Amount Found mg g^{-1}	Recovery %	Amount Added mg g^{-1}	Amount Found mg g^{-1}	Recovery %	Amount Added mg g^{-1}	Amount Found mg g^{-1}	Recovery %
5.0265	5.0184	99.84	5.0418	5.0327	99.82	5.1347	5.1302	99.91	5.1385	5.0652	98.57
10.0147	10.0113	99.97	10.1357	10.1462	100.10	8.0364	8.0154	99.74	9.5417	9.5524	100.11
13.5461	13.5326	99.90	15.2481	15.2346	99.91	10.2207	10.2115	99.91	10.0132	10.0087	99.96
15.1135	15.0478	99.57	20.3108	20.3216	100.05	15.1184	15.1063	99.92	15.1167	15.0314	99.44
20.0028	20.0417	100.19	23.5107	23.5016	99.96	18.5174	18.5416	100.13	18.2145	18.2257	100.06
25.2314	25.3153	100.33	25.0159	25.1327	100.47	20.2168	20.2511	100.17	20.1548	20.1734	100.09

[a]Mean = 99.97, standard deviation ± 0.268; % RSD = ± 0.268
[b]Mean = 100.05, standard deviation ± 0.228; % RSD = ± 0.228
[c]Mean = 99.96, standard deviation ± 0.16; % RSD = ± 0.16
[d]Mean = 99.71; standard deviation ± 0.61; % RSD = ± 0.612

The dependence of retention time on flow rate has been observed for both standard and sample and it was found that a increase in flow rate decrease the retention time of B_2, B_6, B_{12} and folic acid in a regular manner. The HPLC method has been found to be time saving with a high degree of precision and accuracy.

Table 2. Precision Under Repeatability Conditions (n = 6)

Determination	Riboflavin (% L.S)	Pyridoxine (%L.S)	Cyanocobalamine (% L.S)	Folic Acid (% L.S)
01	98.24	99.46	97.57	100.24
02	98.76	99.35	97.87	100.51
03	98.57	98.98	97.48	99.85
04	99.01	99.32	98.09	99.89
05	99.56	98.84	98.04	99.25
06	99.36	99.02	98.24	100.15
Mean	98.75	99.16	97.90	99.98
SD	± 0.48	± 0.22	± 0.31	± 0.46
% RSD	±0.49	±0.22	± 0.32	± 0.46

Table 3. Intermediate Precision (n = 4)

Instrument	Analyst	B_2 (% L.S)	B_6 (% L.S)	B_{12} (% L.S)	Folic Acid (% L.S)
1	A	99.01	99.32	97.49	100.15
	B	98.79	98.89	98.09	99.25
2	A	98.81	99.01	98.11	100.74
	B	99.59	98.30	98.54	100.04
Mean		99.05	98.88	98.06	100.05
SD		± 0.373	± 0.427	± 0.432	± 0.612
% RSD		± 0.377	± 0.432	± 0.441	± 0.612

Table 4. Stability Indicating Results and Instrument Repeatability (n = 4)

Time Period (Hour)	Peak Area of Standard Solution (a. u)			
	B_2	B_6	B_{12}	Folic Acid
0	7515091	3151394	7028085	9831283
8	7501432	3152461	7014893	9824361
16	7500981	3150873	7019476	9826147
24	7501076	3145524	7011875	9819875
Time Period (Hour)	**Peak Area of Sample (a. u)**			
	B_2	B_6	B_{12}	Folic Acid
0	353222	107092	813628	298368
8	350146	109241	821476	297475
16	351485	119976	814385	289956
24	352673	118795	812469	287973

CONCLUSIONS

This paper describes a simple, rapid, economic and accurate quantitative simultaneous estimation of water soluble vitamins, riboflavin, pyridoxine, cyanocobalamin and folic acid in neutraceutical products by HPLC. A validation of this method was carried out and showed that specificity, robustness and precision are guaranteed.

REFERENCES

[1] Hui, YH. *Encyclopedia of Food Science and Technology*, Vol. 4 (Q-Z). Wiley Interscience Publication: New York, **1991**.

[2] Helrich, K. *Official Methods of Analysis of the Association of Official Analytical Chemicals*, 13th ed.; Association of the Official Analytical Chemists, Arlington Inc: VA, **1990**, pp. 65:970, 15:981.

[3] Helrich, K. *Official Methods of Analysis of the Association of Official Analytical Chemicals*, 13th ed.; Association of the Official Analytical Chemists, Arlington Inc: VA, **1990**, Vol. *33*, p. 940.

[4] Brubacher, G.; Müller-Mulot, W. *Vitamin B_2 (Riboflavin) in Foodstuffs: HPLC Method, in Methods for the determination of Vitamins in Food-Recommended by COST 91*, Elsevier Applied Science: New York, **1985**.

[5] Polansky, M.M.; Klein, B.P.; Becker, D.A.; Venugopal, P.B. *Methods of Vitamin Assay*, 4th ed.; Association of Vitamin Chemists, Chapter 17, Wiley: New York, **1985**.

[6] Gregory, III J.F. *Methods for Determinations of Vitamins B_6 in Foods and other Biological Materials:* A Critical Review. *J. Food Compst. Anal.*, **1988**, *1*, 105-123.

[7] Vanderslice, J.T.; De Leenheer, A.P.; Lambert, W.E. Eds.; *Modern Chromatographic Analysis of the Vitamins*, Chapter 10, Marcel Dekker: New York, **1985**.

[8] Food and Nutrition Board, National Research Council, Food Chemicals Codex, 3rd ed.; National Academy Press: Washington DC, **1981**, p. 363.

[9] The United States Pharmacopeia XXII (USP XXII–NF XVII), United States Pharmacopeial Convention, Inc Rockville: MD, **1990**, p.343.

[10] Chin, H. B, Methods *of Vitamin Assay*, 4th ed.; Chapter 19, Wiley: New York, **1985**.

[11] Rosenthal, H.L.; Harris, R.S. *The Vitamins*, 2nd ed.; Academic Press, New York, **1968**, Vol. *II*, p. 145.

[12] a) Skeggs, H. R.; *The Vitamins*, 2nd ed.; Vol. VII, Academic Press, New York, **1968**, p. 277. b) Baker, H.; Frank, O. *The Vitamins*, 2nd ed.; Academic Press: New York, **1968**, Vol. *VII*, p. 293.

[13] Hudson, T.S.; Subramanian, S.; Allen, R.J. Determination of pantothenic acid, biotin and vitamin B_{12} in nutritional products. *J. Assoc. Off. Anal. Chemists*, **1984**, *67*, 994-698.

[14] a) Herbert, V.; Bertino, J.R. Folic acid, György P, Pearson W-N *The Vitamins*, 21st ed.; Academic Press: New York, **1967**, Vol. *VII*, p. 243. b) Baker, H.; Frank, O. *A Microbiological Assay for Folate Activity*, **1967**, ibid, p. 269.

[15] a) Keagy, P.M. *Folacin-Microbiological and Animal Assays*, 4th ed.; **1985**, pp 445. b) Augustin, J.; Klein B.P.; Becker, D.A.; Venugopal, P. B, *Methods of Vitamin Assay*, 4th ed.; Wiley, New York, **1985**, p. 445. c) Gregory III J.F. *Folacin,-Chromatographic and Radiometric Assays*, **1985**, ibid, p. 473.

[16] a) Mc Cormack, J.J.; Newman, R.A. *Chromatographic Studies of Folic Acid and Related Compounds*, Marcel Dekker: New York, **1985**. b) De Leenheer, A.P.; Lambert, W.E.; De Ruyter, M.G.M.; Eds.; *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker: New York, **1985**.

[17] Brubacher, G.; Muller, M.W.; Southgate D.A.T. *Folacin in Foodstuffs*, *in Methods for the Determination of Vitamin in Food-Recommended by COST 91*, Elsevier Applied Science, New York, **1985**, p. 158.

[18] Yanling, G.; Fei, G.; Sumangala, G.; Andrew, C.; Qinghai, S.; Mingruo, G. Quantification of water-soluble vitamins in milk-based infant formulae using biosensor-based assays. *Food Chem.*, **2008**, *110*, 769-76.

[19] Soledad, A-H.; Veciana-Nogue´s, T.M.; Izquierdo-Pulido, M.; Font, A. M. Determination of water-soluble vitamins in infant milk by high-performance liquid chromatography. *J. Chromatogr.*, **1997**, *778*, 247-53.

[20] Rada, A.; Jasmina, B.S.; Olivera, A.C.; Sote, M. V. RP-HPLC determination of vitamins, folic acid and B_{12} in multivitamin tablets. *J. Serb. Chem. Soc.,* **2005**, *70*, 1229-35 and references quoted there in.

Received: December 31, 2008 Revised: January 8, 2009 Accepted: February 12, 2009

© Perveen *et al.*; Licensee *Bentham Open*.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.