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Rapid method for the determination of total 5-methyltetrahydrofolate in blood by liquid chromatography with fluorescence detection

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Abstract

A liquid chromatographic method is described for the determination of total 5-methyltetrahydrofolate (5-MTHF) in whole-blood samples. The method was applied to a survey of whole-blood total 5-MTHF levels of women at child-bearing age. To determine whole-blood total 5-MTHF content, a whole-blood sample was frozen and thawed to break red blood cells and the 5-MTHF polyglutamates were released and hydrolyzed into 5-MTHF monoglutamate by endogenous polyglutamates hydrolase in the plasma. In brief, an aliquot of 0.1 ml whole-blood sample was mixed with 0.3 ml 57 mmol/l ascorbic acid and incubated at 37°C for 60 min, then diluted with 0.6 ml buffer solution (0.2 mol/l potassium phosphate dibasic and 30 mmol/l mercaptoethanol, pH 8.5). After the sample was heated at 100°C for 10 min and centrifuged, the supernatant was analyzed by reversed-phase liquid chromatography with fluorescence detection. The recoveries from spiked samples were from 95 to 105% with within-day and day-to-day relative standard deviations less than 6.5%. The detection limit was estimated to be 30 nmol/l based on three times the noise level (peak to peak). Application of the method to a survey of whole-blood total 5-MTHF levels of women at child-bearing age showed that the method was reliable and suitable for the determination of blood total 5-MTHF.

Keywords: 5-Methyltetrahydrofolate

1. Introduction

Folate is a water-soluble vitamin and acts in the body as an one-carbon unit carrier. It is generally considered a serum folate at a level greater than 6.0 ng/ml, or a red cell folate at a level greater than 160 ng/ml, indicates normal folate status, while a serum folate level less than 3.0 ng/ml, or a red cell folate level less than 140 ng/ml indicates a deficiency [1]. Folate deficiency can cause megaloblastic anemia and is related to neural tube defects of newborn infants. Epidemiological research in recent years have indicated that even mild folate deficiency is correlated to high blood levels of homocysteine, while a high blood level of homocysteine is a risk factor of cardiovascular diseases. Decreased folate

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levels have also been considered as a risk factor for neoplastic transformation [2,3].

Serum folate content is relatively low and is not constantly correlated to the status of folate store in the body; red blood cell folate content is more than 10 times of that in blood serum and is generally correlated to the folate nutritional status of the body [4,5], although not without exceptions [6,7]. Determination of folate content in whole blood has been used for the evaluation of folate nutritional status [8], but there are still few data reported concerning whole-blood folate level. Different methods have been used in the analyses of folates in blood samples. These include microbiological assay, competitive protein binding assay, chemiluminescence methods, ion-capture, and chromatographic methods [9–14]. Microbiological assay and competitive protein binding assay have been widely accepted and used for folate analysis in clinical laboratories. Rapid and fully automatic competitive protein binding assay kits for folate analysis are now available commercially. Some other methods have not been widely accepted due to the troublesome sample preparation or non-popularity of the instruments used. The results of an international round robin for serum and whole-blood folate levels indicated that the variations between different methods and different laboratories were relatively high [15].

High-performance liquid chromatography (HPLC) methods are more specific but usually require tedious sample pre-clean up procedures [8,16]. Folates in red blood cells occur mainly as 5-methyltetrahydrofolate (5-MTHF) polyglutamates that can be hydrolyzed into 5-MTHF monoglutamate by endogenous polyglutamates hydrolase in the plasma. The enzymatic deconjugation of erythrocyte polyglutamyl folates to monoglutamate had been studied thoroughly by Pfeiffer and Gregory III [5]. Based on the work by Pfeiffer and Gregory III [5], a relatively simple and rapid liquid chromatographic method for the determination of total 5-MTHF in whole-blood samples was developed and described in this report.

2. Experimental

2.1. Chemicals

5-MTHF monoglutamate disodium salt was purchased from Sigma (St. Louis, MO, USA). L-Ascorbic acid, 2-mercaptoethanol, and potassium phosphate monobasic and dibasic and other reagents were analytical grade. All solvents were HPLC grade (Tedia, Fairfield, OH, USA) and water was Milli-Q deionized water. Human whole-blood samples with heparin as anticoagulant were obtained from a local hospital in Shantou City, China and stored at −70°C.

2.2. Preparation of standard solutions and other reagents

An accurately weighed 5-MTHF monoglutamate disodium salt standard compound (about 2 mg) was dissolved in a 0.01 mol/l potassium phosphate dibasic buffer solution (pH 7.1) which contained 57 mmol/l ascorbic acid, calibrated to 10 ml and stored at 4°C as the stock standard solution. An intermediate standard was prepared by diluting the stock standard 100 times with the same buffer used for preparing stock standard. The stock standard solution was prepared monthly, and the intermediate standard solution was prepared weekly and stored at 4°C. Daily working standard was prepared by diluting the intermediate standard solution with the same buffer solution. The true content of the original 5-MTHF monoglutamate disodium salt standard was determined by measuring the absorbance of a standard solution with pure water as solvent at 290 nm (ϵ: 32 000 l mol^{-1} cm^{-1}) [17]. All operations with 5-MTHF standard were conducted in dim light. An ascorbic acid solution of 57 mmol/l was prepared daily. A 0.2 mol/l potassium phosphate dibasic buffer solution (pH 8.5) which contained 30 mmol/l mercaptoethanol was prepared weekly and stored at 4°C.

2.3. Sample preparation

Heparinized whole-blood samples stored at −70°C were thawed at room temperature and fresh heparinized whole-blood samples were frozen completely and thawed to break red blood cells. An aliquot of 0.1 ml thawed whole-blood sample was pipetted into a 1.5-ml plastic microcentrifuge tube and 0.3 ml 57 mmol/l ascorbic acid was added into the tube, which was capped tightly and vortex-mixed for 30 s and incubated for 60 min in a 37°C water bath. Then the sample was diluted to 1 ml by adding 0.6 ml buffer

solution (0.2 mol/l potassium phosphate dibasic and 30 mmol/l mercaptoethanol, pH 8.5) to the tube. The sample was mixed well and heated in a 100°C water bath for 10 min and then vortex-mixed for 30 s and centrifuged at 10 000 *g* for 15 min. The supernatant was collected into a sample vial and ready for HPLC analysis. All sample preparation steps were conducted under dim light.

2.4. Liquid chromatographic analysis

The HPLC system was a HP 1100 HPLC (Hewlett-Packard, USA), which consisted of a pump, an autosampler, a column chamber, a fluorescence detector, and a HP ChemStation for LC system. The column chamber temperature was set at 25°C and the sample injection volume was set at 10 μl. The excitation wavelength and the emission wavelength of the fluorescence detector were set at 295 nm and 360 nm, respectively. The mobile phase was methanol–0.6% acetic acid in water (14:86, v/v) with a flow-rate of 1 ml/min. The HPLC column was an HP Zorbax StableBondSB-C_{18}, 5 μm, 150 mm×4.6 mm column (Germany). The peak area was used for quantitative calculation.

2.5. Calibration

The total 5-MTHF content of a pooled whole-blood sample was determined by method of standard addition. A known amount of 5-MTHF monoglutamate standard was spiked into the pooled whole-blood sample and the spiked sample was then used as calibration standard. To establish a calibration curve, a series of spiked pooled-blood samples contained different levels of known amount of 5-MTHF monoglutamate standard were analyzed following the procedure described above. Blood samples with very low concentrations of folate were intentionally selected to make the pooled whole-blood sample. Then it was possible to construct with this pooled whole-blood sample a calibration curve which covered a concentration range started from very low level. The same pooled-blood sample was used as sample matrix for the preparation of calibration standards for routine analysis of surveyed blood samples. The pooled-blood sample used in this study was determined to contain a total 5-MTHF concentration of 43.3 nmol/l. The six-point calibration curve included 43.3, 133, 222, 401, 759, 1116 nmol/l that were prepared by spiked proper amounts of 5-MTHF standard solution to the pooled whole-blood samples.

2.6. Application of the method for survey of blood total 5-MTHF level

The established methodology was used to survey the blood total 5-MTHF levels of women at child-bearing age who lived in the local city (Shantou, China). Ethical approval to use the blood samples for folate determination was obtained from the hospital. Blood samples were collected from the women who came to the hospital for a physical examination that is required for obtaining a marriage certificate. Heparinized whole-blood samples from 98 healthy subjects (female, 87 subjects aged 20–29 years, nine subjects aged 30–39 years, and two subjects aged 40–43 years) were analyzed and one aliquot from each of the 98 samples was spiked with 5-MTHF monoglutamate standard at the level of 358 nmol/l in order to determine the recoveries of each spiked sample.

3. Results and discussion

In the method developed, a blood sample was simply incubated, diluted, heated and centrifuged. No complicated and costly sample preparation techniques were needed for this procedure. Since the sample preparation procedure was so simple, the sample throughput was very high. Red blood cells in heparinized whole-blood samples were broken by freeze–thaw cycle. In the preliminary study, the freeze–thaw cycle was compared with sonication. Both sonication and freeze–thaw cycle gave satisfactory results and there was no significant difference in the results of the total 5-MTHF content between two methods. In this study, all blood samples were stored frozen, and thawed before analysis. For fresh blood samples, either sonication or freeze–thaw cycle can be used to break down the red blood cells. An aliquot of 0.1 ml whole-blood sample was mixed with 0.3 ml 57 mmol/l ascorbic acid and incubated at 37°C for 60 min. The acidity created by ascorbic acid accelerated the conversion from 5-MTHF polyglutamates to 5-MTHF monoglutamate according to

the report by Pfeiffer and Gregory III [5]. In their report, whole-blood sample was diluted 10 times with 57 mmol/l ascorbic acid, and incubated at 37°C for 90 min for complete conversion of 5-MTHF polyglutamates to 5-MTHF monoglutamate. In our procedure, whole-blood sample was diluted with ascorbic acid only four times, and a different incubation time at 37°C was studied. Incubation at 37°C for 60 min was found to give satisfactory results (Fig. 1). Before heating at 100°C for 10 min, the sample was diluted with 0.6 ml of 0.2 mol/l potassium phosphate dibasic buffer solution that also neutralized the ascorbic acid. No significant difference in blood total 5-MTHF contents was found when comparing heating at 90°C with 100°C. 5-MTHF was stable under the conditions of the sample preparation procedure. The purpose of heating the samples at 100°C for 10 min was to denature the proteins that were then removed by centrifugation.

A pooled whole-blood sample was used as matrix to prepare calibration standards. The total 5-MTHF content of a pooled whole-blood sample was determined by the method of standard addition. The pooled-blood sample used in this study was determined to contain a total 5-MTHF concentration of 43.3 nmol/l with a standard deviation of 3.2 nmol/l ($n=5$). In the preliminary study, when the standard of 5-MTHF monoglutamate was diluted with phosphate buffer (0.01 mol/l potassium phosphate dibasic buffer solution which contained 57 mmol/l ascorbic acid, pH 7.1) to low concentration (e.g., 400 nmol/l), and injected into the HPLC system repeatedly, the peak response of the compound was not consistent. We suspected that the compound was not stable or may be adsorbed to the glass surface of the sample vial and could not be used as a calibration standard. Standards of 5-MTHF monoglutamate spiked into blood which was treated following the sample preparation procedure, however, were very stable (at least for 2 days) in the vial of the HPLC autosampler at room temperature. A six-point calibration curve was therefore prepared by using a pooled whole-blood sample. The regression equation of the calibration curve was $y=a_1x+a_0$ with a linear regression coefficient (γ) of 0.9997, where y is the peak area count of the chromatographic peak of total 5-MTHF, x is the concentration (nmol/l) of total 5-MTHF in blood sample. The coefficients a_1 and a_0 are 0.0082 and 0.039, respectively. The confidence interval ($\alpha=0.05$) of the intercept (a_0) of the regression equation was calculated to be (0.039 ± 0.095), which included the point of 0. Therefore, in routine analysis, a one-point calibration in duplicate could be used instead of a calibration curve. In this study, the concentration of the one-point calibration standard was 401 nmol/l (43.3+358 nmol/l). But a new calibration curve should be prepared to check the instrumental system in case of any deviation.

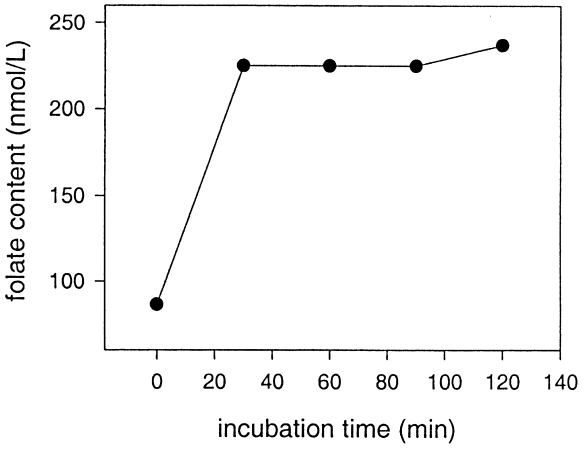

Fig. 1. The effect of incubation time on the blood total 5-MTHF content determined by the HPLC method described.

In order to avoid any complicated sample prepurification step, efforts were focused on looking for an optimum combination of mobile phase and column able to separate 5-MTHF monoglutamate from the interfering substances in the blood samples. Different solvents, ion paired reagents, pH values, columns and their combinations were screened. Finally, the chromatographic parameters described in the Experimental section gave satisfactory results. It is shown in Fig. 2 that the chromatographic peak of 5-MTHF monoglutamate was well separated from the interfering substances. Although there was no complicated procedure used for sample pre-clean up, the HPLC column was still in good condition after more than 1000 injections. Considering that the injection volume was only 10 μl for each injection, and the whole-blood sample was diluted 10 times in the procedure, and the final sample was an aqueous buffer extract, there should not be too much contamination of the column by the samples. But as a good

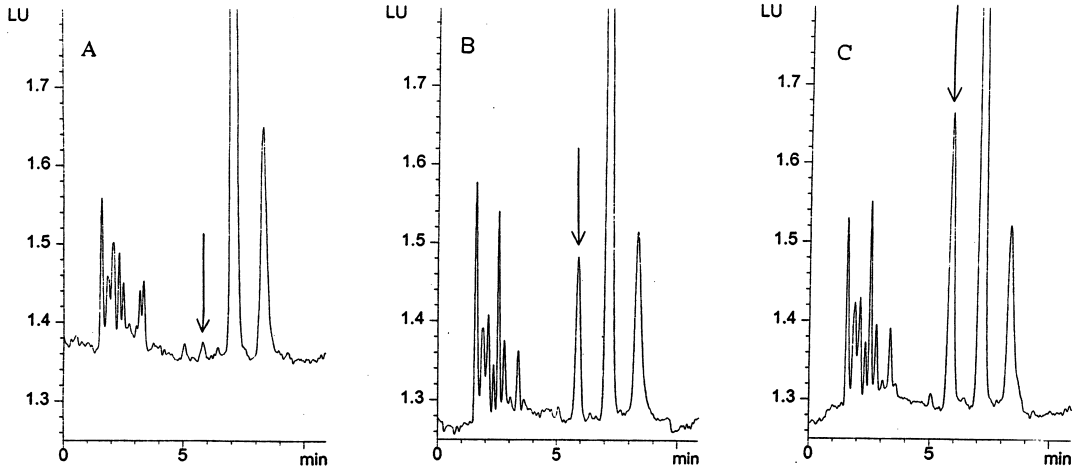

Fig. 2. Chromatograms of whole-blood samples with injection volume of 10 μl. (A) A whole-blood sample with total 5-MTHF level of 41 nmol/l; (B) a whole-blood sample with total 5-MTHF level of 334 nmol/l; (C) the same sample of chromatogram B spiked with 5-MTHF monoglutamate standard at 358 nmol/l.

practice, the HPLC system was washed with methanol or acetonitrile for 60 min every day after all samples were run.

In order to evaluate the accuracy and precision of the methodology, aliquots of a pooled whole-blood sample were spiked with 5-MTHF monoglutamate standard compound at different levels and analyzed in different days. Spike recoveries with within-day variations and day-to-day variations at different levels were therefore calculated based on the analytical results and summarized in Table 1 and Table 2. The average recoveries were within the range of 95~105% with within-day and day-to-day variations less than 6.5%. Low-level spike recoveries of samples with low concentration of blood total 5-MTHF are summarized in Table 3. The recoveries at low level of 5-MTHF were satisfactory as well. The injection volume was 10 μl for samples with blood total 5-MTHF level higher than 100 nmol/l, and the detection limit of the method for total 5-MTHF in whole-blood samples was estimated to be 30 nmol/l (15 pg of 5-MTHF disodium salt on column) based on three times the noise level (peak to peak). Injection volume was increased to 30 μl for samples with blood total 5-MTHF levels lower than 100 nmol/l, and the detection limit was estimated to be 12 nmol/l (18 pg of 5-MTHF disodium salt on column) based on three times the noise level (peak to peak). A whole-blood sample with 5-MTHF at 68.9 nmol/l was analyzed with an injection volume of 30 μl and its chromatogram is depicted in Fig. 3.

The methodology was used to survey the blood total 5-MTHF levels of the women at child-bearing age (20 to 43 years with an average age of 27.4

Table 1
Within-day recoveries and variations of total 5-MTHF from spiked whole-blood samples

Spike level (nmol/l)	Concentration (nmol/l)		Recovery[b] (%)	*n*	RSD (%)
	Calculated[a]	Determined			
179	222	220	98.7	5	6.1
358	401	391	97.1	5	4.3
715	758	740	97.4	5	1.8

Aliquots of pooled whole-blood sample that contained 43.3 nmol/l of 5-MTHF were spiked with the standard compound at different levels and analyzed to determine the recoveries and variations.

[a] Concentration calculated=spike level+43.3 (nmol/l).

[b] Recovery=[(concentration determined−43.3)/spike level]×100%.

Table 2
Between-day recoveries and variations of total 5-MTHF from spiked whole-blood samples

Spike level (nmol/l)	Concentration (nmol/l)		Recovery[b] (%)	*n*	RSD (%)
	Calculated[a]	Determined			
179	222	217	97.0	5	5.6
358	401	412	103.0	5	4.9
715	758	742	97.7	5	3.2

Aliquots of pooled whole-blood sample that contained 43.3 nmol/l of 5-MTHF were spiked with the standard compound at different levels and analyzed to determine the recoveries and variations.

[a] Concentration calculated=spike level+43.3 (nmol/l).

[b] Recovery=[(concentration determined−43.3)/spike level]×100%.

Table 3
Low-level spike recoveries of 5-MTHF with two pooled-blood samples that contain low concentration levels of blood total 5-MTHF

Spike level (nmol/l)	Concentration (nmol/l)		Recovery (%)	*n*	RSD (%)
	Calculated	Determined			
0	–	34.5	–	5	1.4
38.2	72.7	74.6	105	5	2.6
76.4	111	115	105	5	2.1
0	–	142	–	5	2.7
38.2	180	177	91.6	5	1.1
76.4	218	216	96.8	5	1.4

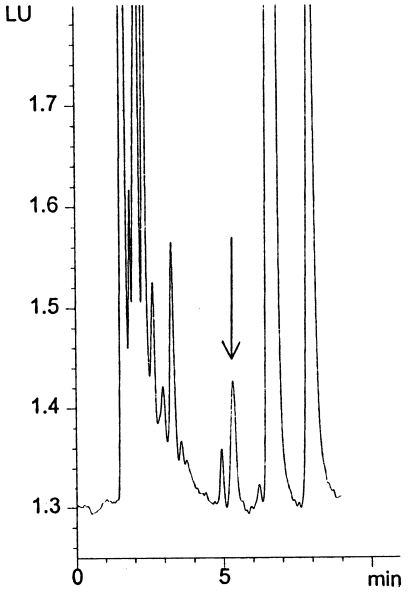

Fig. 3. Chromatogram of a whole-blood sample with injection volume of 30 μl. The blood total 5-MTHF level of the sample was determined to be 68.9 nmol/l.

years) who lived in the local city (Shantou). Whole-blood samples from 98 healthy subjects were collected and analyzed following the procedure developed in this research. In order to further evaluate the reliability and applicability of the methodology, one aliquot of each blood sample was spiked with 5-MTHF monoglutamate standard at the level of 358 nmol/l and also analyzed. Spike recoveries were calculated based on the analytical results of each blood sample and each spiked blood sample. The survey results are summarized in Fig. 4. The average recovery of the spiked blood samples was 97.3% with a relative standard deviation of 6.4% (n=98).

Since data of whole-blood total 5-MTHF level were rarely reported, and criteria using whole-blood total 5-MTHF level as indicator of folate nutrition status have not been well set up, more work might be needed before using whole-blood total 5-MTHF level as an indicator of folate nutrition status. Some other problems related to the use of whole-blood folate as an indicator of folate nutrition status include: healthy women and men have different hematocrit, lower

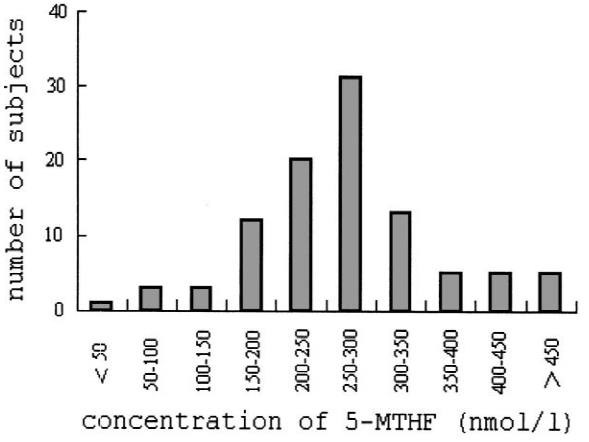

Fig. 4. The distribution the whole-blood total 5-MTHF among the 98 subjects (women at child-bearing age) surveyed. The horizontal axis is the concentration of 5-MTHF in whole-blood samples, and the vertical axis is the number of subjects.

whole-blood folate level in anemics might not always indicate folate deficiency, people with below-normal hematocrit because of mild thalassemial have a relatively low folate status. On the other hand, the combination of this method with the HPLC method for serum folate [16] will make possible the quantitative determination of 5-MTHF in whole-blood, red blood cells, and serum.

The method was tested on samples of serum and the chromatogram was similar to that of whole-blood samples. The chromatographic peak of 5-MTHF monoglutamate from serum was well separated from interfering substances but quite small, even with an injection volume as high as 100 μl due to the low concentration of the compound in serum.

In conclusion, a rapid and simple method for the determination of total 5-MTHF in whole-blood samples was developed. The sample preparation procedure and instrumental analysis were simple and straightforward, and no extensive or costly clean-up procedure was needed. The method described was precise and accurate.

References

[1] T. Brody, in: Nutritional Biochemistry, Academic Press, New York, 1993, p. 357.
[2] J. Sellhub, I.H. Rosenberg, in: E.E. Ziegler, L.J. Filer Jr. (Eds.), Present Knowledge in Nutrition, ILSI Press, Washington, DC, 1996, p. 200.
[3] Y.I. Kim, J.B. Mason, Nutr. Rev. 54 (1996) 259.
[4] S.M. Hunt, J.L. Groff, in: Advanced Nutrition and Human Metabolism, West Publishing, St. Paul, MN, 1990, p. 202.
[5] C.M. Pfeiffer, J.F. Gregory III, Clin. Chem. 42 (1996) 1847.
[6] M.D. Lucock, J. Wild, C.H. Lumb, M. Oliver, R. Kendall, I. Dasklakis, C.J. Schorah, M.I. Levene, Biochem. Mol. Med. 61 (1997) 28.
[7] F.A.J. Muskiet, F.P.L. van der Dijs, M.R. Fokkema, F.D. Muskiet, Clin. Chem. 46 (2000) 2015.
[8] S.R. Dueker, Y. Lin, A.D. Jones, R. Mercer, E. Fabbro, J.W. Miller, R. Green, A.J. Clifford, Anal. Biochem. 283 (2000) 266.
[9] C.R. Santhosh-Kumar, J.C. Deutsch, K.L. Hassell, Anal. Biochem. 225 (1995) 1.
[10] M. Kohashi, K. Inoue, H. Sotobayashi, J. Chromatogr. 382 (1986) 303.
[11] D.H. Wilson, R. Herrmann, S. Hsu, Clin. Chem. 41 (1995) 1780.
[12] J. van der Weide, H.C. Homan, E. Cozijnen-van-Rheenen, Clin. Chem. 38 (1992) 766.
[13] S.I. Hansem, J.A. Holm, Anal. Biochem. 172 (1988) 160.
[14] C. Klukas, C. Cimerci, J. Campbell, Clin. Chem. 35 (1989) 1194.
[15] E.G. Gunter, B.A. Bowman, S.P. Caudill, D.B. Twite, M.J. Adams, E.J. Sampson, Clin. Chem. 42 (1996) 1689.
[16] J. Chladek, L. Sispera, J. Martinkova, J. Chromatogr. 744 (2000) 307.
[17] H. van den Berg, P.M. Finglas, C. Bates, Int. J. Vitam. Nutr. Res. 64 (1994) 288.