Journal of Chromatography B, 877 (2009) 1882–1886

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of thiamin diphosphate in whole blood samples by high-performance liquid chromatography—A method suitable for pediatric diagnostics

Robert Walter Körner[a], Anne Vierzig[b], Bernhard Roth[b], Carsten Müller[a,*]

[a] Department of Therapeutic Drug Monitoring, Institute of Pharmacology, University of Cologne, Gleuelerstrasse 24, D-50931 Cologne, Germany
[b] Department of Neonatology and Pediatric Intensive Care, Childrens's Hospital, University of Cologne, Kerpenerstrasse 62, D-50937 Cologne, Germany

ARTICLE INFO

Article history:
Received 12 February 2009
Accepted 9 May 2009
Available online 15 May 2009

Keywords:
Thiamin
Thiamin diphosphate
Determination
HPLC
Preterm infants
Pediatric diagnostics

ABSTRACT

An improved and easy to use method for the determination of thiamin diphosphate (TDP) in 100 μl of whole blood was developed. The small sample volume makes it possible to assess the nutritional status of vitamin B_1 in infants and even in preterm infants. Sample preparation comprises the extraction of TDP from whole blood by hemolysis, protein precipitation with trichloroacetic acid, and subsequent centrifugation. Potassium ferricyanide is used for pre-column derivatization of TDP to its fluorescent thiochrome derivative. Chromatographic separation was carried out using a reversed-phase column and an isocratic elution which consisted of a phosphate buffer and acetonitrile. TDP was detected fluorimetrically and quantified by external standardization. Method validation showed a high precision, almost complete recovery, and a high sensitivity. The lower limit of detection and the lower limit of quantification were 0.2 ng/ml and 4 ng/ml, respectively. Linearity was demonstrated over the expected concentration range of 4–400 ng/ml. In conclusion, we present a convenient HPLC method for the determination of TDP which is precise, sensitive and suitable for pediatric diagnostics.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Vitamin B_1 (thiamin) is a water-soluble vitamin which plays an essential role in carbohydrate metabolism. It is required by the pyruvate dehydrogenase complex for the transformation of pyruvate to acetyl-CoA. More enzyme complexes rely on vitamin B_1 as a coenzyme, such as the oxoglutarate dehydrogenase and the transketolase.

Thiamin exists in four different forms in the human body: thiamin, thiamin monophosphate (TMP), thiamin diphosphate (TDP), and thiamin triphosphate (TTP). TDP is the coenzyme form of thiamin and takes part in the metabolic pathways mentioned above. Its concentration in erythrocytes exceeds the concentration of thiamin, TMP, and TTP by far [1]. The physiological functions of TMP and TTP are unclear. Findings indicate that TTP might play a neurophysiological role in signal transduction [2]. Bettendorff et al. suggest that TTP is an activator of chloride channels [3]. Recently, it was observed that in *E. coli* TTP plays a role in the adaption to amino acid starvation [4].

Thiamin deficiency can be found in the form of the classical thiamin-deficiency disease Beriberi in developing countries in people whose diet does not meet the thiamin requirement. In developed countries thiamin deficiency can only rarely be encountered because the prevalent diet contains sufficient thiamin. It predominantly occurs in the form of Wernicke-Korsakoff syndrome in people suffering from alcoholism. However, in the year 2003 a life-threatening thiamin deficiency was reported in infants due to a thiamin-deficient formula nutrition [5]. Case reports as the latter one induce the need for a diagnostic tool that can be used to assess the thiamin status in neonates and young infants.

Various methods for the detection of thiamin deficiency, including microbiological assays, have been developed and are still in use nowadays. Before the introduction of HPLC the erythrocyte transketolase activity (ETKA) assay was a widely used method to assess the nutritional status of vitamin B_1 [6]. In thiamin deficiency transketolase activity decreases and when adding TDP to the sample it can be observed that enzyme activity increases abnormally. This is known as the TDP effect and reflects the undersaturation of the transketolase with TDP [7]. Obviously, the changes in enzyme activity are the consequence of a diminished coenzyme concentration and therefore describe the nutritional status of vitamin B_1 in an indirect way. That is why Warnock et al. suggested to directly measure the functional form of vitamin B_1 which is TDP [8].

Studies which compare the ETKA assay to the measurement of TDP by HPLC found several advantages of the HPLC method. It is easier to standardize, offers improved robustness, and improved

* Corresponding author. Tel.: +49 0 221 478 5066; fax: +49 0 221 478 7184.
E-mail address: c.mueller@uni-koeln.de (C. Müller).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.05.013

analyte stability [9]. Herve et al. and Talwar et al. observed a high correlation between red cell TDP concentration and TDP effect [10,11]. Both describe the measurement of TDP as a more sensitive and specific index of thiamin status. The assessment of TDP is more specific since the ETKA assay may be influenced by factors other than thiamin deficiency [12–16].

Moreover, Talwar et al. found a strong correlation between the concentration of TDP in erythrocytes and in whole blood. They recommended the assessment of TDP in whole blood in conjunction with hemoglobin determination as it is less time-consuming.

Several HPLC methods for the determination of TDP in whole blood have been published before [11,17–19]. However, the majority requires a sample volume in the range of 500–1000 μl. The aim of this work was to establish a method which is as reliable as the existing HPLC methods and which requires a smaller sample volume making it applicable also in preterm infants.

2. Experimental

2.1. Chemicals and reagents

HPLC gradient grade acetonitrile (ACN), sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, hydrochloric acid (HCl), and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). Potassium ferricyanide (purity ≥99%), HPLC grade thiamin diphosphate (purity ≥95%), thiamin monophosphate, and thiamin were purchased from Sigma–Aldrich (Steinheim, Germany). Throughout all steps of analysis deionized water purified by an Ultra Clear System (SG Water conditioning and Regeneration, Barsbüttel, Germany) was used.

2.2. Materials and equipment

The method was developed using a Waters HPLC system (Waters Chromatography Division, Milford, MA, USA) which consisted of a 717 plus autosampler and a 510 solvent delivery system. The system was connected to an HP 1046A fluorescence detector (HP, Palo Alto, CA, USA). Column temperature was maintained by a CH-500 column oven (Eppendorf, Hamburg, Germany). Instrument control and data acquisition were performed with a personal computer running the software Waters Millennium 32 (Waters Chromatography Division, Milford, MA, USA). Samples were centrifuged by a Centrifuge 5402 (Eppendorf, Hamburg, Germany) and vortexed by a Vortex-Genie (Scientific Industries, Inc., Bohemia, NY, USA).

2.3. Chromatographic conditions and detection

Separation was achieved using a Hypersil GOLD column (4.6 mm × 250 mm, 5 μm, 175 Å; Thermo Fisher Scientific Inc., Waltham, MA, USA). This column is packed with a silica-based material with C18 selectivity for which the manufacturer reports stability within the pH range of 1–11. It was protected by a corresponding guard column of the same material (4.6 mm × 10 mm, 5 μm, 175 Å; Thermo Fisher Scientific Inc., Waltham, MA, USA). The column was maintained at 30 °C and was equilibrated for 30 min with mobile phase before the injection of samples.

The isocratic mobile phase consisted of a 50 mM phosphate buffer and ACN (buffer/ACN, 95:5, v/v). The buffer's pH was 8.2 since the fluorescence of TDP reaches a maximum above pH 8 [20]. The mobile phase was delivered at a flow rate of 1.0 ml/min. It was prepared freshly prior to each run and degassed for 10 min in an ultrasound bath. Samples stored in the carousel of the autosampler were protected from light and kept at room temperature. The injection volume was 45 μl and detector settings were: excitation, 375 nm; emission, 430 nm; gain, 15; response time, 6; and lamp, 3.

2.4. Derivatization

To be able to detect TDP fluorimetrically it has to be oxidised to a thiochrome derivative first. This derivatization can be achieved in alkaline solution in the presence of potassium ferricyanide [21]. It is also possible using cyanogen bromide [22] which yields a stronger fluorescence [23]. However, we preferred potassium ferricyanide due to its lower grade of toxicity.

2.5. Preparation of calibrators

A standard solution was prepared freshly before each calibration by dissolving 20 mg of TDP in 100 ml of 0.1 M HCl. From this solution standard solutions of different concentrations were prepared.

Five calibrators were prepared by spiking samples of a pooled whole blood hemolysate. We added 100 μl of a TDP standard solution to 1500 μl of hemolysate. Standard solutions for spiking had concentrations of 0, 400, 800, 1600, and 3200 ng/ml to increase the hemolysate's concentration in steps of 0, 25, 50, 100, and 200 ng/ml, respectively. Calibrators were aliquoted and stored at −80 °C.

The hemolysate used for calibration can be checked anteriorly for the presence of thiamin. If thiamin is not detectable calibrators can be injected in intervals of 7 min.

2.6. Assay calibration and quantification

Calibration was carried out prior to each assay and after 30 samples to correct for drifts that might occur in chromatography. Each calibrator was analyzed three times. Peaks were integrated by the software Waters Millenium 32. After analysis the chromatograms were separately checked and in case of deviation from peak detection settings reintegrated manually.

The calibration curve was obtained by weighted linear regression of peak area and concentration. Weighted regression was chosen because standard deviations of the area showed a trend to increase with increasing concentration. It was carried out by using the software SPSS (SPSS Inc., version 15.0, Chicago, Illinois, USA). Although changes to slope and intercept were minimal compared to ordinary least square regression standard errors could be reduced.

2.7. Preparation of samples

All whole blood samples were collected using tubes which contained EDTA as anticoagulant (Becton, Dickinson and Company, Plymouth, UK; Part No. 368856). The samples were immediately transferred to screw cap micro tubes made of polypropylene (Sarstedt, Nümbrecht-Rommelsdorf, Germany; Part No. 72.730, 0.5 ml). These vials were frozen directly at −18 °C and then transferred to our laboratory using dry ice. Samples were stored for at least 24 h at −80 °C until analysis to achieve complete hemolysis.

At the day of analysis samples were thawed at room temperature. For sample preparation we used polypropylene micro tubes (Sarstedt, Nümbrecht-Rommelsdorf, Germany; Part No. 72.690, 1.5 ml). First of all, to 100 μl of whole blood 40 μl of 1.5 M TCA were added to precipitate the proteins. The sample was immediately vortexed at the highest velocity for 1 min. Thereafter, it was centrifuged at 14,000 × *g* for 10 min. The clear supernatant was transferred to a new vial and centrifuged in the same manner a second time to settle the remaining debris. Subsequently, 50 μl of the supernatant were transferred to a new vial and mixed with 10 μl of derivatization solution which was prepared freshly prior to each assay by adding 1.15 M sodium hydroxide to 30.4 mM potassium ferricyanide ($NaOH/K_3[Fe(CN)_6]$, 2:1, v/v). Finally, the

Table 1
Intra-assay precision of spiked blood samples.

TDP (ng/ml)	CV (%, $N = 10$)
111.6	1.5
147.2	3.0
197.6	2.8

resulting yellowish solution was transferred to an autosampler vial and stored light-protected in the carousel of the autosampler until analysis.

2.8. Internal quality control (QC)

QC1 samples were prepared in the same way as was the calibrator for the concentration of 50 ng/ml. One QC1 sample was run after each five patient samples to monitor for any drift in the chromatographic system. The whole assay was rejected if deviation was greater than 15% in two out of six QC1 samples.

QC2 samples were aliquots of a pooled whole blood sample. One QC2 sample was analyzed after each calibration to detect faults in calibration. If the TDP concentration deviated more than 10% from the concentration originally determined the assay was recalibrated. QC samples were stored at −80 °C.

2.9. Assay validation

2.9.1. Precision

To determine intra-assay precision we analyzed a whole blood sample from the same specimen ten times. Moreover, we measured intra-assay precision of whole blood samples spiked with low, intermediate, and high additions of TDP (Table 1). To determine inter-assay precision a whole blood sample was aliquoted, stored at −80 °C and analyzed on five different days.

2.9.2. Recovery

Recovery was measured by comparing spiked whole blood samples to standard solutions of TDP. Standard solutions were processed in the same manner as were whole blood samples except for the addition of TCA. Instead of 40 μl TCA we added 40 μl of H_2O. Recovery experiments were carried out at three different concentrations (Table 2). At a certain concentration recovery is expressed as the percentage of the area of the spiked blood sample, minus the area of the blank blood sample, compared to the area of the standard solution. The area of the unextracted standard solution represents 100%. Each sample was analyzed three times and mean values were determined.

2.9.3. Sensitivity and linearity

For sensitivity and linearity studies standard solutions of TDP were used. They were processed like whole blood samples but replacing TCA by H_2O to achieve an alkaline pH when adding the derivatization solution. To determine the lower limit of detection (LOD) and the lower limit of quantification (LLOQ) standard solutions were gradually diluted and injected.

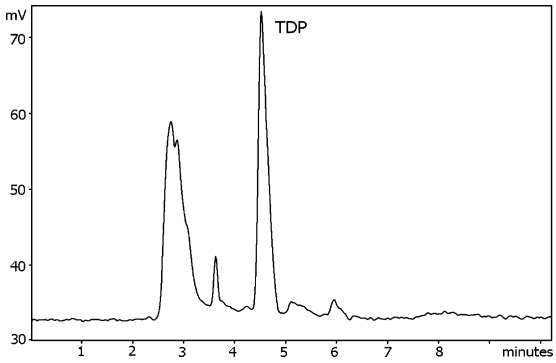

Fig. 1. Whole blood sample; c(TDP) = 88.6 ng/ml; RT(TDP) = 4.6 min.

2.9.4. Interferences

To check for interferences aqueous solutions of drugs were derivatized, analyzed and checked for interfering peaks at the retention time of TDP. The following drugs were analyzed: caffeine (27 mg/l), theophylline (16 mg/l), vancomycin (32 mg/l), meropenem (50 mg/l), cefotaxime (100 mg/l), ceftazidime (100 mg/l), gentamicin (10 mg/l), ampicillin (100 mg/l), sulbactam (50 mg/l), fluconazole (18 mg/l), furosemide (4 mg/l), hydrochlorothiazide (0,5 mg/l), and indometacin (2 mg/l).

2.9.5. Analyte stability

To check analyte stability at room temperature aliquots of a whole blood specimen obtained by venous puncture were frozen at −80 °C in intervals of 20 min after taking the blood sample. The samples were protected from light. To check sample stability at −18 °C aliquots of a whole blood specimen were stored at −18 °C and analyzed in intervals of three days. The stability of freshly prepared standard solutions of TDP was checked exclusively at room temperature because they were always prepared freshly and not stored.

While processing the samples it was not always feasible to maintain the same time from the addition of TCA to the addition of the derivatization solution. Therefore, we studied whether a decrease of TDP can be observed in samples which remained acid over a longer time.

We checked analyte stability at three freeze–thaw cycles. A whole blood samples was aliquoted and frozen at −80 °C for 24 h. With each cycle it was thawed completely and refrozen at −80 °C for 24 h. Finally, we studied the stability of processed samples which were stored in the autosampler.

2.10. Reference values of TDP in an adult population

We analyzed the blood of 30 healthy adults (14 males, 16 females, age range 18–65, mean age 40.5). The persons were residents of the state North Rhine-Westphalia, Germany.

Table 2
Recovery of TDP at three different concentrations; mean recovery = 98.1% ± 0.3(S.D.).

TDP (ng/ml)	Area (standard) ± S.D.	Area (whole blood) ± S.D.	Area (whole blood) − Area (whole blood)$_0$	Recovery (%)
0	–	412476 (±12374)	–	–
50	299602 (±4494)	705428 (±20457)	292952	97.8
100	596023 (±10132)	999016 (±36964)	586540	98.4
200	1195013 (±14340)	1583469 (±55421)	1170993	98.0

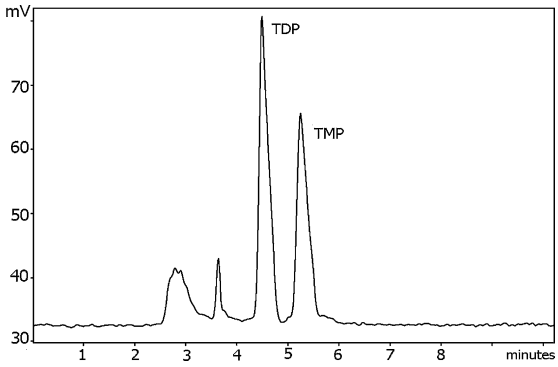

Fig. 2. Spiked whole blood sample; c(TDP) = 108.5 ng/ml; RT(TDP) = 4.6 min; RT(TMP) = 5.3 min.

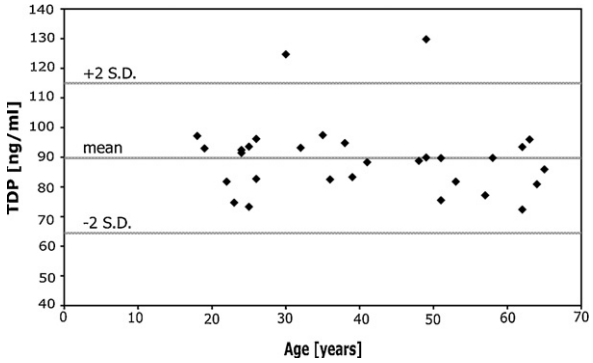

Fig. 3. Reference values of TDP in an adult population.

3. Results

3.1. Chromatographic separation

A chromatogram typically obtained of a whole blood sample is shown in Fig. 1. Prior to the elution of TDP unknown substances are eluted. These peaks might include a signal of TTP which usually is eluted prior to TDP when applying reversed-phase mode and might be present in very low concentrations in whole blood [24].

Fig. 2 shows a chromatogram of a whole blood sample spiked with TDP and TMP. The vitamers are separated clearly. TDP, TMP and thiamin elute approximately after 4.6, 5.3, and 41.8 min, respectively. Due to the late eluting thiamin injections were made in intervals of 50 min.

Throughout all conducted assays the baseline was stable and did not show any drifts. Retention times were stable, as well.

3.2. Assay validation

3.2.1. Precision

The intra-assay coefficient of variation (CV) was 1.1% for whole blood samples processed from the same specimen ($N = 10$, TDP concentration = 87.2 ng/ml) and smaller than 5% for *spiked* whole blood samples (Table 1). Inter-assay CV was 4.2% ($N = 5$, TDP concentration = 91.5 ng/ml).

3.2.2. Recovery

Recovery of the spiked blood samples was almost 100% throughout all concentrations tested (Table 2). Thus, the extraction process does not cause a significant loss of TDP.

3.2.3. Sensitivity and linearity

Based on a signal-to-noise ratio of 3 the LOD was 0.2 ng/ml. The LLOQ was 4 ng/ml considering a signal-to-noise ratio of 10. Samples of 4 ng/ml could be assessed with a CV of 3.7% ($N = 8$). The detector response was linear from 4 to 400 ng/ml.

A calibration done with whole blood calibrators ($N = 6$ per concentration) and five calibration points showed that the CV was smaller than 5% and accuracy was smaller than 7% throughout the whole concentration range. In all linearity experiments and all calibrations which were conducted the coefficient of determination was greater than 0.99.

3.2.4. Interferences

No interferences could be observed in the tested drugs.

3.2.5. Analyte stability

At room temperature whole blood samples were stable for at least 3 h after blood taking when protected from light. At −18 °C samples proved to be stable for at least 90 days. When stored at −70 °C whole blood samples are reported to be stable for at least 7 months [11]. Freshly prepared standard solutions of TDP were stable for 3 h at room temperature.

No change of the TDP level was noticed if extracted samples remain underivatized for up to 2 h. The analyte remained stable throughout three freeze–thaw cycles. Processed samples were stable for 16 h at room temperature when stored light-protected in the autosampler.

3.3. Reference values of TDP in an adult population

The levels of TDP in whole blood ranged from 72.4 to 129.8 ng/ml with a mean value of 89.7 ng/ml (2S.D. = 25.3 ng/ml). This corresponds to a mean value of 194.7 nM (2S.D. = 54.9 nM) and is similar to the values found by Herve et al. [25] (mean = 176 nM, 2S.D. = 56 nM) and Lynch et al. [18] (mean = 174.2 nM, 2S.D. = 68.1 nM). Lower and higher mean values for TDP in whole blood have been reported in literature, and it was suggested that each laboratory sets up its own reference range [11]. The concentration of TDP is regionally different since it depends on dietary intake. Fig. 3 shows the obtained TDP values in relation to age. No decline with age could be observed. The two outliers were two women who have a vegetarian lifestyle and take vitamin supplements regularly.

4. Discussion

Several methods for the determination of thiamin and its phosphate esters were described in previous publications and different extraction and derivatization methods were used [17–20]. While other published methods focused on improvements in chromatography and time of analysis we are now proposing improvements which offer a convenient and fast sample extraction procedure and a small sample volume of 100 μl. In contrast, existing methods require from 500 μl [11,25] to 1000 μl [26] of whole blood. The use of small volumes of blood is consistent with the recommendations for clinical investigations of medicinal products in a pediatric population. Hence, this method enables investigation and routine assessment of the thiamin status in newborns and even in preterm infants. To our mind, this is an important step because young infants and preterm infants constitute a potential risk group for thiamin deficiency.

Developing this method efforts were made to use internal standardization to achieve a higher degree of precision and reliability. We tried to use the classical antimetabolites of thiamin: pyrithiamine and oxythiamine. Pyrithiamine could be detected, but its peak area showed a greater imprecision than did the peak area of TDP itself. Moreover, impurities contained in the pyrithiamine solu-

tion caused interferences with the TDP peak. Oxythiamine could not be detected fluorimetrically because it does not form a fluorescent product [27]. We also tried to use the anticoccidial drug amprolium as an internal standard. It could be detected and clearly separated from TDP, but proved to be unstable in the prepared samples. Its peak height and area began to decrease directly after derivatization whereas the TDP peak itself remained stable. We assume that derivatized amprolium is not stable in alkaline solution. Although, amprolium was used successfully as internal standard before by Vanderslice and Huang, they applied post-column derivatization which would balance the dissolution of amprolium [28]. Lynch et al. used acetylaneurine as an internal standard [18]. After all, this method yielded a high precision without the use of an internal standard. Establishing a calibration curve with five concentrations and analyzing each calibrator three times allowed an accurate determination of TDP.

Because most of the TDP found in whole blood is contributed by erythrocytes it was suggested to relate the level of TDP to hemoglobin [11] or to hematocrit [17]. However, we did not observe a relationship neither to hemoglobin, nor to hematocrit, nor to red blood cell count, nor to mean corpuscular volume. Higher levels of hemoglobin did not coincide with higher levels of TDP, and vice versa. Wyatt et al. who analyzed the blood of 323 pediatric patients claimed that adjustment to hematocrit was unnecessary since it contributed only to a small part of the variance of total or phosphorylated thiamin [29]. Whether TDP concentration in whole blood indeed needs to be corrected to red blood cell parameters remains to be evaluated.

The stability of TDP in whole blood is mainly influenced by temperature, light, and enzymatic activity. As anticoagulant we preferred EDTA over heparin since it inhibits alkaline phosphatase and the activation of thiamin diphosphatase [30]. However, only little is known about thiamin metabolism and it cannot be excluded that degradation and formation of TDP continues after collecting the sample in an EDTA-containing vial. Metabolic influence on the analyte is stopped when TCA is added to process the sample. To minimize the effects of the factors mentioned above we recommend freezing the sample directly after blood taking.

5. Conclusion

The HPLC method described here is easy to use and does not rely on sophisticated equipment. It does not require a separate pump for post-column derivatization. The method proved to be precise and reliable, and is the first method known to us which requires only 100 μl of whole blood making it suitable for pediatric patients.

Conflict of interest

The authors declare no conflict of interest.

References

[1] M. Kimura, Y. Itokawa, J. Chromatogr. 332 (1985) 181.
[2] J.R. Cooper, J.H. Pincus, Neurochem. Res. 4 (1979) 223.
[3] L. Bettendorff, B. Hennuy, A. De Clerck, P. Wins, Brain Res. 652 (1994) 157.
[4] B. Lakaye, B. Wirtzfeld, P. Wins, T. Grisar, L. Bettendorff, J. Biol. Chem. 279 (2004) 17142.
[5] A. Fattal-Valevski, A. Kesler, B.A. Sela, D. Nitzan-Kaluski, M. Rotstein, R. Mesterman, H. Toledano-Alhadef, C. Stolovitch, C. Hoffmann, O. Globus, G. Eshel, Pediatrics 115 (2005) 233.
[6] M. Brin, M. Tai, A.S. Ostashever, H. Kalinsky, J. Nutr. 71 (1960) 273.
[7] T. Takeuchi, E.H. Jung, K. Nishino, Y. Itokawa, Int. J. Vitam. Nutr. Res. 60 (1990) 112.
[8] L.G. Warnock, C.R. Prudhomme, C. Wagner, J. Nutr. 108 (1978) 421.
[9] M. Baines, G. Davies, Ann. Clin. Biochem. 25 (Pt 6) (1988) 698.
[10] C. Herve, P. Beyne, P. Letteron, E. Delacoux, Clin. Chim. Acta 234 (1995) 91.
[11] D. Talwar, H. Davidson, J. Cooney, J.R.D. St, Clin. Chem. 46 (2000) 704.
[12] J. Fennelly, O. Frank, H. Baker, C.M. Leevy, Am. J. Clin. Nutr. 20 (1967) 946.
[13] M.J. Kaczmarek, P.F. Nixon, Clin. Chim. Acta 130 (1983) 349.
[14] F. Paoletti, A. Mocali, M. Marchi, F. Truschi, Biochem. Biophys. Res. Commun. 161 (1989) 150.
[15] O.E. Pratt, M. Jeyasingham, G.K. Shaw, A.D. Thomson, Alcohol Alcohol. 20 (1985) 223.
[16] J.A. Puxty, A.E. Haskew, J.G. Ratcliffe, J. McMurray, Ann. Clin. Biochem. 22 (Pt 4) (1985) 423.
[17] A. Floridi, M. Pupita, C.A. Palmerini, C. Fini, A. Alberti Fidanza, Int. J. Vitam. Nutr. Res. 54 (1984) 165.
[18] P.L. Lynch, E.R. Trimble, I.S. Young, J. Chromatogr. B Biomed. Sci. Appl. 701 (1997) 120.
[19] C.M. Tallaksen, T. Bohmer, J. Karlsen, H. Bell, Methods Enzymol. 279 (1997) 67.
[20] K. Ishii, K. Sarai, H. Sanemori, T. Kawasaki, Anal. Biochem. 97 (1979) 191.
[21] G. Berger, A. Bergel, A.R. Todd, Nature 136 (1935) 259.
[22] M. Fujiwara, K. Matsui, Anal. Chem. 25 (1953) 810.
[23] L.G. Warnock, Anal. Biochem. 126 (1982) 394.
[24] C.M. Tallaksen, T. Bohmer, H. Bell, J. Karlsen, J. Chromatogr. 564 (1991) 127.
[25] C. Herve, P. Beyne, E. Delacoux, J. Chromatogr. B Biomed. Appl. 653 (1994) 217.
[26] A. Losa, M.I. Sierra, A. Fernández, D. Blanco, J.M. Buesa, J. Pharm. Biomed. Anal. 37 (2005) 1025.
[27] C.J. Gubler, B.C. Hemming, Methods Enzymol. 62 (1979) 63.
[28] J.T. Vanderslice, M.H. Huang, J. Assoc. Off. Anal. Chem. 70 (1987) 920.
[29] D.T. Wyatt, D. Nelson, R.E. Hillman, Am. J. Clin. Nutr. 53 (1991) 530.
[30] J. Bettendorff, C. Michel-Cahay, C. Grandfils, C. De Rycker, E. Schoffeniels, J. Neurochem. 49 (1987) 495.