Journal of Chromatography B, 877 (2009) 3151–3158

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Application of the high-performance liquid chromatography method with coulometric detection for determination of vitamin B_6 in human plasma and serum

Marcin L. Marszałł[a,*], Anna Lebiedzińska[b], Wojciech Czarnowski[a], Ryszard Makarowski[c], Mateusz Kłos[a], Piotr Szefer[b]

[a] Department of Toxicology, Medical University of Gdańsk, Al. Gen. J. Hallera 107, Gdańsk 80-416, Poland
[b] Department of Food Sciences, Medical University of Gdańsk, Al. Gen. J. Hallera 107, Gdańsk 80-416, Poland
[c] Department of Psychology, University of Gdańsk, Pomorska 68, Gdańsk 40-343, Poland

ARTICLE INFO

Article history:
Received 7 May 2009
Accepted 4 August 2009
Available online 20 August 2009

Keywords:
Vitamin B_6
Pyridoxic acid
HPLC
Coulometric detection
Plasma
Serum

ABSTRACT

A reversed-phase high-performance liquid chromatography method (HPLC) with coulometric electrochemical detection has been developed and validated for the simultaneous analysis of pyridoxamine-5′-phosphate (PMP), pyridoxamine (PM), pyridoxal 5′-phosphate (PLP), pyridoxal (PL), pyridoxine (PN) and 4-pyridoxic acid (4-PA) in human plasma and serum. The isocratic separation was achieved on C_{18} column (250 mm × 4.6 mm, I.D., 5 μm) with a mobile phase consisted of 35 mM sodium phosphate containing 2.5 mM heptanesulfonic acid, adjusted to pH 3.2 with 85% orthophosphoric acid and 12% methanol (v/v). Within-run and between-run precisions expressed by the relative standard deviations were less than 2.7% and 7.7% for all the analysed vitamins and 4-PA, respectively. The limits of detection (LOD) and quantification (LOQ) were: 0.8 and 2.6 nM, 1.1 and 3.8 nM, 1.5 and 4.5 nM, 1.3 and 4.2 nM, 1.1 and 3.7 nM, 2.1 and 6.3 nM for PMP, PM, PLP, PL, PN and 4-PA, respectively. The recoveries ranged from 90.4% to 98.4%. Stability of vitamins was checked under a variety of storage conditions. The developed application demonstrated acceptable sensitivity, precision, accuracy, stability, and linearity over the physiological concentration range. The major advantage of the proposed method is its great simplicity.

1. Introduction

Vitamin B_6 can be found in three natural forms: pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL). These chemically similar molecules differ within the functional group branching from the fourth position, inside 2-methyl-3-hydroxyl-5-hydroxymethyl pyridines. PM, PL, and PN contain an aminomethyl ($-CH_2-NH_2$), an aldehyde (–CH–O) and a hydroxymethyl ($-CH_2-OH$) groups, respectively (Fig. 1). Each form is phosphorylated in 5-hydroxymethyl position during metabolic conversion. However, both pyridoxamine-5′-phosphate (PMP) and pyridoxal-5′-phosphate (PLP) interconvert as coenzyme which is formed during aminotransferase catalysed reactions [1].

Vitamin B_6 is involved in the metabolism of macronutrients participating in more than 100 enzymatic reactions. Vitamin B_6 is essential for the synthesis of both serotonin and GABA-neurotransmitters that control depression, pain perception, and anxiety, as well as the activation of glycine in the initial stages of heme production. Most of the vitamin B_6-dependent enzymes participate in amino acid reactions including transamination, decarboxylation, dehydratation, desulfhydration, racemization, cleavage, and synthesis [1–4].

Clinical manifestation of vitamin B_6 deficiency is reported to be rare, since the vitamin is widely present in food. However, several groups within the general population have been described as having low biochemical indices of vitamin B_6 status [5]. Most of the studies included women during pregnancy and lactation [6,7], alcoholics [8,9], patients with diabetes mellitus [10], adolescents [11] and hiperhomocysteinemia [12] as well as some genetic disorders, such as cystathioninuria and homocystinuria [3].

High variability of vitamin B_6 forms and their low concentration in human matrices cause difficulties in analysis. The biochemical assessment is usually conducted by means of chemical analysis of the vitamers, their metabolites or functional tests. Analytical techniques include determination of PLP in plasma and red cells. PLP metabolite, e.g. 4-pyridoxic acid (4-PA) can be measured in urine or plasma, as well as the activity and activation coefficient with the red cell aminotransferases, and the tryptophan load metabo-

* Corresponding author. Tel.: +48 583493175; fax: +48 583493175.
E-mail address: marmartox@amg.gda.pl (M.L. Marszałł).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.08.014

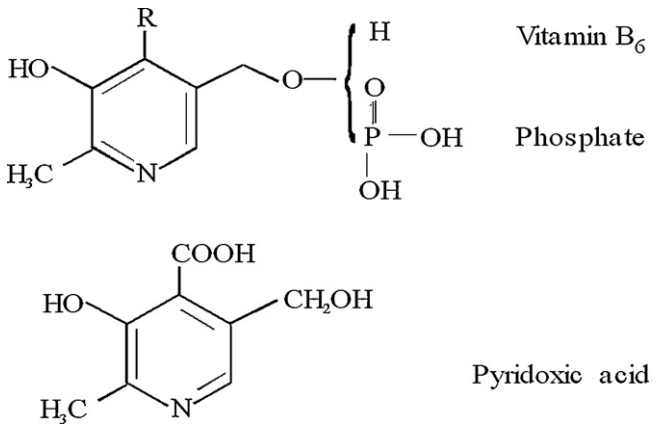

Fig. 1. Chemical structure of free and phosphorylated forms of vitamins B_6 and 4-pyridoxic acid. R = CH_2OH for pyridoxine, CH_2NH_2 for pyridoxamine, and CHO for pyridoxal.

lite test [13,14]. Combination of these markers provides a reliable approach for practical laboratory methods. Consequently, the use of microbiological [15,16], enzymatic and radio-enzymatic [17–20], spectrofluorometric [21] and liquid chromatographic methods [13,14,22] has been reported for this purpose.

The majority of separation procedures is achieved by the use of reversed-phase high-performance liquid chromatography (HPLC) coupled with fluorescent [23–41] and UV [42] detections. Corbun and Mahuren [27] reported a cation-exchange HPLC procedure, involving a gradient elution and a post-column bisulfide derivatization followed by fluorescence detection that was suitable for quantifying B_6 components in human plasma. Some other methods have been also used to enhance the fluorescence of PL and PLP by bisulfide adduct formation [26,28,31,33], formation of semicarbazones by reaction with semicarbazide [34–36], and oxidation by cyanide to carboxylic acid [37–39]. Modified fluorescent methods eliminated cyanide by post-column conversion of PLP to 4-PA and 5′-phosphate by chlorite [40,41]. Midttun et al. [43] reported the potential of liquid chromatography combined with tandem mass spectrometry (LC–MS) for the quantification of all known forms of vitamin B_6 and riboflavin in human plasma.

There were also successfully validated other methods of vitamin B_6 detection based on a redox reaction with electrochemical detectors, i.e. amperometic or coulometric detectors. The oxidation of PN at a carbon paste electrode has been firstly examined. Consequently, modification of the glassy carbon electrodes, flow and sequential injection techniques improved speed and selectivity as compared with electrochemical assays [14,44–46]. The chemical structure of all the particular vitamin B_6 forms enables their measurement by electrochemical detection at nanomolar concentrations in endogenous plasma and serum. It should be mentioned that use of electrochemical detection has been already reported in literature [44–48]. However, such application was limited to samples other than pharmaceuticals and foods.

The aim of this study was development and validation of the reversed-phase HPLC method for the simultaneous quantification of PMP, PM, PLP, PL, PN and 4-PA in human plasma and serum.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of pump P 580, an automated sample injector ASI-100, column thermostate STH 585, all from Dionex Corporation (Sunnyvale, CA, USA) and Coulochem II electrochemical detector-equipped with a 5020 model guard and 5010 model analytical cell (ESA, Chelmsford, MA, USA) operated by the Chromeleon chromatography-management system, version 6.8 (Dionex). Compounds were separated on a 250 mm × 4.6 mm I.D., 5 μm particle, Hypersil Gold C_{18} column with 10 mm × 4.6 mm I.D., 5 μm particle, Hypersil Gold guard column, both from Thermo Electron Corporation (Dreieich, Germany).

2.2. Reagents and chemicals

All reagents were of analytical grade. Pyridoxamine dihydrochloride (PM, purity ≥99.0%), pyridoxamine-5′-phoshate (PMP, purity ≥98.0%), pyridoxal hydrochloride (PL, purity ≥99.5%), pyridoxal 5′-phosphate (PLP, purity ≥98.5%) and pyridoxine hydrochloride (PN, purity ≥99.0%) were obtained from Fluka (Biochemica, Switzerland, Italy). 4-Pyridoxic acid (4-PA, purity ≥98.0%), 1-heptanesulfonic acid, sodium salt, sodium phosphate dibasic heptahydrate, sodium EDTA were from Sigma–Aldrich (St. Louis, MO, USA). Orthophosphoric acid 85% (w/w) (Riedel de Haën, Seelze–Hannover, Germany), perchloric acid 60% (w/w) (Merck, Darmstadt, Germany), acetonitrile and methanol HPLC grade were from Baker (Deventer, Holland). Purified water was obtained from Compact Milli-Q system (Millipore, Milford, MA, USA).

Standard stock solutions (10 mM) were prepared by dissolving solid PM (25.9 mg), PMP (24.8 mg), PL (20.4 mg), PLP (26.5 mg), PN (20.6 mg) and 4-PA (18.4 mg) in 10.0 ml pure water. Stock solutions were kept separately in polypropylene tubes protected from light and frozen in liquid nitrogen and then stored at −80 °C. These stock solutions were then diluted with water to yield a series of spiking standard solutions.

Serum control lyophilised material, based on human serum (QC) containing PLP, PL and other vitamins (A, E, B_1, B_2 and B_{12}) was obtained from ClinCheck Recipe (Chemicals & Instruments GmbH, München, Germany) and reconstituted with water according to manufacturer instruction.

2.3. Sample preparation

Blood samples were taken from the ulnar vein after fasting for at least 12 h. Blood samples were collected in tubes with lithium heparine and in tubes without additive (Vacuette, Linz, Austria). After centrifugation at 1500 × *g* for 20 min at 4 °C, plasma and serum fractions were separated, frozen in liquid nitrogen and stored at −80 °C for batch analysis.

All samples were prepared by simple protein precipitation. Plasma and serum samples (250 μl) were vigorously shaken with the same volume of 0.8 M perchloric acid included 2.5 mM EDTA, and the mixture was allowed to equilibrate for 20 min in ice. Perchloric acid solution with EDTA was freshly prepared and stored at 4 °C for 12 h. Samples were centrifuged at 34,000 × *g* for 10 min, supernatant was transferred to a 1.5 ml capped polypropylene tube and neutralized with 0.05 ml of 5 M sodium hydroxide (final pH between 3.0 and 4.0). Samples were then frozen in liquid nitrogen and stored at −80 °C until HPLC analysis.

For QC assurance ClinCheck Control level 1, 2 and 3 were prepared in the same manner as plasma and serum samples.

2.4. Chromatographic conditions

The isocratic mobile phase for chromatography was 35 mM sodium phosphate containing 2.5 mM heptanesulfonic acid, adjusted to pH 3.2 with 85% orthophosphoric acid and 12% methanol (v/v). The flow rate was 1.0 ml min^{-1}. The mobile phase was filtered through a 0.22 μm membrane filter and vacuum degassed before use. The injection volume was 20 μl. The column and automated sample injector thermostats were set at 20 and 8 °C, respectively. The column eluate was monitored by coulometric

detection, in which both selectivity and sensitivity were adjusted by varying the potential between the working and reference electrodes. Analysed compound flowed across the working porous graphite coulometric electrode and chemical reactions occurred whereby the analysed vitamins B_6 and 4-PA lose one or more electrons. These electrons were detected as a current flux thorough the working electrode. Any substance in the column effluent, not oxidized below the designated cell potential, was invisible to the coulometric detector [49,50]. A guard cell connected in line before the automated sample injector was used to remove oxidizable impurities from the mobile phase, to eliminate interference with baseline stability. The electrochemical behaviour of vitamins B_6 was studied by repeated injection of working standard solutions (0.01 mM) and by detection at potentials from −0.5 to +1.0 V. Information from hydrodynamic voltammograms was used to establish the optimum operating potential of the detector for quantification of PMP, PM, PLP, PL, PN and 4-PA. The cell cleaning procedure after analysis minimized a degradation of cell performance and solubility of particulate matter from the coulometric cell, i.e. phosphate, heptanesulfonic acid as well as contaminants from plasma and serum samples. For this purpose, acetonitrile and pure water were used after HPLC analysis in the following sequence: 90% acetonitrile and 10% pure water (v/v) for 30 min at flow rate 1.5 ml^{-1} and next 60% acetonitrile and 40% pure water (v/v) for 120 min, at flow rate 1.0 ml^{-1}.

2.5. Calibration curve and validation procedure

2.5.1. Calibration, limit of detection and limit of quantification

Calibration curves were prepared using pooled blood samples from 11 volunteers, none of them was taking vitamin supplementation. The calibration curves for PLP, PL and 4-PA were determined after substraction of endogenous concentrations of these vitamins. To prepare calibration curves known amounts of working solutions, at six concentrations for PMP, PM, PLP, PL, PN and 4-PA (12.4, 24.5, 49.0, 96.2, 185.2, 227.3 nM, respectively) were added to plasma or serum samples (dilution factor was 1:10). The samples were prepared in the same manner as by the described above procedure (Section 2.3). Statistical analysis (determination of linear regression data, intercept, and slope) was performed by the use of Statistica for Windows, version 8.0 (Statsoft, Kraków, Poland).

The limit of detection (LOD) and limit of quantification (LOQ) of the method were defined as 3-times the chromatographic baseline-noise and 10-times the signal-to-noise ratio for PMP, PM and PN, respectively. To estimate LODs and LOQs, 10 independent determinations of PLP, PL and 4-PA in blood from 11 volunteers were taken. The LOD for these endogenous analytes was defined as three the standard deviations of 10 independent basal concentrations and LOQ, was calculated as 3 × LOD [51]. According to IUPAC recommendation LOQs are defined at the lowest concentration in the standard curve at which CV-defined precision is less than ≤20.0%, and calculated value, which is within 15% of target [52]. A good agreement was obtained between these LOQs approaches.

2.5.2. Recovery

Average recovery (as a measure of accuracy) was established by analysis of plasma samples spiked with four different concentrations (24.8, 60.9, 119.0, and 174.4 nM) of PMP, PM, PLP, PL, PN and 4-PA. Mean recovery from plasma samples was expressed as a percentage, calculated by comparing the analyte concentrations measured in plasma samples after substraction of the basal value with the concentration actually added. Recovery study for serum samples was determined by analysis of serum samples spiked with three different concentrations (24.8, 119.0 and 174.4 nM) of PMP, PM, PLP, PL and 4-PA. Mean recovery for serum samples was calculated and expressed as in the case of to plasma samples.

2.5.3. Precision

The within-run precision of the method was evaluated on one day by 10 replicates analysis of spiking samples (QC) containing PMP, PM, PLP, PL, PN and 4-PA at three different concentrations (24.5, 96.2, and 185.2 nM). The between-run precision was evaluated by six replicates analysis of QC samples every seven days for one month. The analyte concentrations were calculated using a calibration plot obtained in the same run from calibration standards prepared in plasma or serum samples. Within- and between-run precision were expressed as relative standard deviations (RSD% = 100 × SD/mean) for each calculated concentration.

2.5.4. Stability

The stability studies of PMP, PM, PLP, PL, PN and 4-PA were checked, under a variety of conditions, in plasma and serum samples spiked at concentrations of 24.5, 96.2 and 185.2 nM. The stability of analytes was evaluated after sample preparation (post-preparative stability, 24 h at 8 °C). The freeze–thaw stability of the analysed vitamins and 4-PA was tested following three cycles at −80 °C in spiked serum samples. The long-term freezer stability at −80 °C was tested with spiked plasma and serum samples analysed every seven days for two months. Furthermore, the stock solutions stability frozen at −80 °C for two months and stored at 4 °C for one month was assessed.

2.5.5. Interferences

To check for interferences, aqueous solutions of thiols (0.005 mM) were added to six QC serum samples which were checked for interfering peaks at the retention times of vitamins and 4-PA. The following thiols were analysed: reduced glutathione, glutathione disulfide, L-cysteine and L-methionine.

2.6. Study objects

The study group consisted of 40 volunteers (20 women and 20 men) aged 25–35 years, who were ethnically homogenous as inhabitants of the same urban area (Gdańsk, Poland). The subjects had neither history of cardiovascular disease and diabetes mellitus nor other metabolic diseases. Informed consent was obtained from each subject. This study was approved by the institutional review board of the Medical University of Gdańsk.

3. Results and discussion

3.1. Chromatography

Most widely acceptable technique for analysis of vitamin B_6 in biological and food matrices are reversed-phase systems using usually ODS-modified silica columns, as a stationary phase and gradient or isocratic elution [13,14]. These compounds can by analysed using low or medium pH eluent or by addition of ion-pairing agents, i.e. heptane- and octane-sulfonic acid, commonly employed for the resolution of basic compounds.

The isocratic elution using the mobile phase, 35 mM sodium phosphate containing 2.5 mM heptanesulfonic acid and adjusted to pH 3.2 with 85% orthophosphoric acid and 12% methanol (v/v) was selected in this study for separation of PMP, PM, PLP, PL, PN and 4-PA. This mobile phase was optimised with respect to signal of coulometric response, separation, column stability and analysis time by varying the pH (from 2.5 to 6.5), column temperature (from 20 to 40 °C) and concentration of heptanesulfonic acid (from 0.05 to 3.5 mM), methanol (from 4% to 20%) and phosphate buffer (from 15 to 75 mM).

The electroactivity of compounds monitored by coulometric detector was dependent on the presence of the functional group of molecules. The peak current of the PMP, PM, PLP, PL, PN and

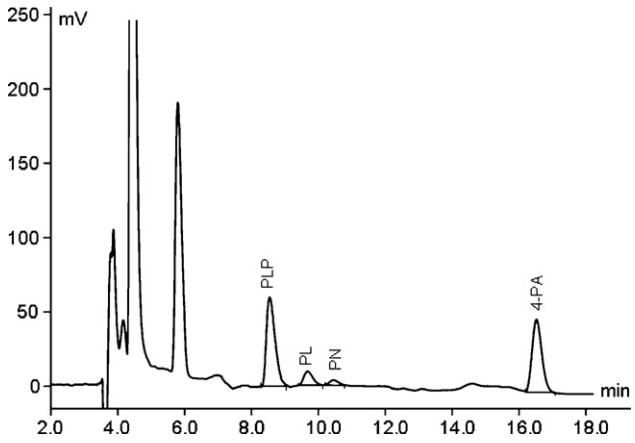

Fig. 2. Chromatogram of the studied vitamins in plasma samples obtained for single specimens: PLP (t_R 8.5 min, 63.4 nM), PL (t_R 9.8 min, 20.6 nM), PN (t_R 10.5 min, 8.2 nM), 4-PA (t_R 16.6 min, 47.2 nM).

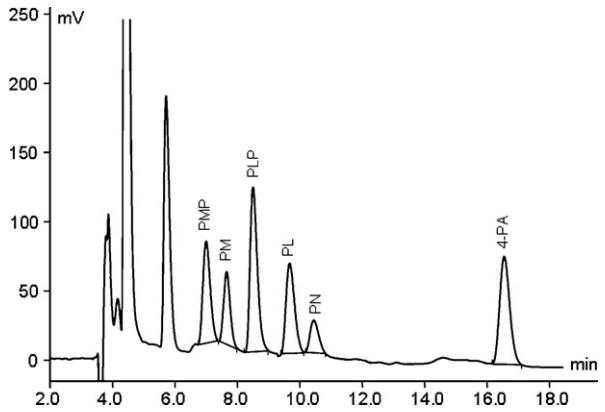

Fig. 3. Chromatogram of plasma sample obtained for pooled plasma samples spiked by 60.9 nM of the each analysed vitamin and 4-pyridoxic acid: PMP (t_R 7.0 min, 59.4 nM), PM (t_R 7.6 min, 54.7), PLP (t_R 8.5 min, 120.5 nM), PL (t_R 9.8 min, 80.7 nM), PN (t_R 10.5 min, 55.9 nM), 4-PA (t_R 16.6 min, 103.8 nM).

4-PA increased with the increase of positive potential applied to the working electrode showing that the response should be caused by these electroactive vitamins. Hydrodynamic voltammograms of the vitamins and 4-PA exhibited good responses in the ranges from +0.35 to +0.8 V. The potentials applied were: guard cell +0.90 V, first working electrode (E1) +0.35 V, and second working electrode (E2) +0.80 V.

In optimised procedure four phosphate buffers at different concentrations were tested. The experimental results showed that the phosphate buffer concentration of 35 mM determined stability of column and was good for peak broader. The concentration of methanol as the organic modifier, was selected to be 12% resulting in good retention and separation of the analysed vitamins. Addition of heptanesulfonic acid to the mobile phase caused the increase in retention of compounds due to the interactions between negative ion-pair heptanesulfonic acid and the basic vitamins. Optimum concentration of the ion-pair reagent was found to be 2.5 mM. At pH from 3.0 to 3.2 of the mobile phase, good resolution and retention time were produced. The column temperature of 20 °C was selected as the optional temperature for the effective separation of vitamins, and hence used in all experiments.

The isocratic mobile phase used enabled satisfactory separation of the vitamins within 18 min. Chromatograms of the studied vitamins in plasma samples obtained from single specimens are presented at Fig. 2. In plasma samples from volunteers, who were not taking vitamins supplementation, vitamins B_6 were found as PLP, PL and 4-PA and sometimes PN was detected while no PMP and PM were identified. Similar results for detection of various forms of vitamins B_6 in human matrices were published elsewhere [28,33,36,43,53]. Fig. 3 presents chromatogram for plasma sample obtained from pooled plasma samples spiked by 60.9 nM for each of the analysed vitamins and 4-PA. The retention times for PMP, PM, PLP, PL, PN and 4-PA were 7.0, 7.6, 8.5, 9.8, 10.5 and 16.6 min, respectively. Within-run retention time repeatability, expressed as the standard deviation, obtained for total of 36 injections over 12 h was 0.3% for PMP, 0.2% for PM, 0.5% for PLP, 0.6% for PL, 0.9% for PN and 0.6% for 4-PA. No noticeable change in retention time reproducibility or chromatographic separation was observed for the analytical column over the course of 40 volunteer sampler runs.

The parameters such as minimal potential of electrode oxidation, longer electrode half-life and good baseline stability are advantages of the coulometric detector. The porous graphite electrodes in coulometric detector are characterised by sufficient surface area. Thus, they do not require frequent electrode maintenance like noble metal electrodes (gold or platinum). From the practical point of view, fast and simple cell cleaning procedure after HPLC analysis is highly recommended [49]. This optimised procedure using acetonitrile and pure water was usually carried out after five days of detector continuous run. It gives good stability of the coulometric detector work, excellent day-to-day reproducibility and long period of operation without a reduction in signal.

3.2. Validation

3.2.1. Linearity, limit of detection and limit of quantification

Response to the analysed vitamins B_6 and 4-PA in plasma and serum samples was a linear function of concentrations between 12.4 and 227.3 nM, with a regression coefficient $r \geq 0.999$. The regression parameters of calibration curves and the limits of detection (LOD) and quantification (LOQ) of the method are summarized in Table 1.

The LODs were 0.8 nM for PMP, 1.1 nM for PM and 1.1 nM for PN. The LOQs were 2.6 nM for PMP, 3.8 nM for PM and 3.7 nM for PN. This calculation of LOD can be applied only when it is possible to record the baseline noises, obtained when a blank sample is subjected to final determination. There is no blank for plasma

Table 1
Validation of the method concerning regression parameters of calibration curves, the limits of detection (LOD) and quantification (LOQ).

Analyte	Regression parameters			LOD (nM)	LOQ (nM)
	Slope ± SD[a]	Intercept ± SD[a]	Coefficient of correlation (r)		
PMP	0.1122 ± 0.0005	−0.0295 ± 0.039	0.9994	0.8	2.6
PM	0.1032 ± 0.0007	0.0538 ± 0.041	0.9992	1.1	3.8
PLP	0.1048 ± 0.0003	0.0243 ± 0.023	0.9997	1.5	4.5
PL	0.0982 ± 0.0004	−0.0347 ± 0.044	0.9996	1.3	4.2
PN	0.0925 ± 0.0005	0.0342 ± 0.049	0.9994	1.1	3.7
4-PA	0.1017 ± 0.0009	0.0902 ± 0.061	0.9992	2.1	6.3

[a] Standard deviation of the slope and the intercept of calibration curves obtained by plotting mean coulometric response from six replicate analyses of six different concentrations of B_6 vitamers and 4-PA.

Table 2
Results of the recovery study of B_6 vitamers and 4-PA for plasma and serum.

Matrix/Analyte	Initial concentration (nM ± SD)[a]	Concentration added (nM)[a]	Concentration found (nM ± SD)[a]	Percentage of recovery (±SD)[a,b]
Plasma				
PMP	ND[c]	24.8	23.2 ± 0.1	93.5 ± 0.4
	ND[c]	60.9	59.4 ± 0.2	97.5 ± 0.4
	ND[c]	119.0	115.8 ± 0.4	97.2 ± 0.5
	ND[c]	174.4	171.4 ± 0.3	98.3 ± 0.2
PM	ND[c]	24.8	22.8 ± 0.4	91.2 ± 1.4
	ND[c]	60.9	55.5 ± 0.9	91.1 ± 0.5
	ND[c]	119.0	114.8 ± 0.6	96.4 ± 0.5
	ND[c]	174.4	168.6 ± 0.6	96.7 ± 0.3
PLP	63.4 ± 0.2	24.8	86.8 ± 0.7	94.3 ± 2.6
	63.4 ± 0.2	60.9	120.5 ± 0.4	93.9 ± 0.7
	27.5 ± 0.1	119.0	143.3 ± 0.5	97.3 ± 0.5
	27.5 ± 0.1	174.4	198.3 ± 1.0	97.9 ± 0.6
PL	22.6 ± 0.2	24.8	45.1 ± 0.4	92.8 ± 1.2
	22.6 ± 0.2	60.9	80.7 ± 0.3	95.5 ± 0.5
	15.5 ± 0.1	119.0	131.7 ± 0.5	97.7 ± 0.4
	15.5 ± 0.1	174.4	185.7 ± 1.3	97.6 ± 0.7
PN	ND[c]	24.8	22.4 ± 0.5	90.4 ± 1.2
	ND[c]	60.9	55.9 ± 0.3	91.8 ± 0.4
	ND[c]	119.0	113.9 ± 0.6	95.7 ± 0.5
	ND[c]	174.4	164.2 ± 1.3	94.1 ± 0.7
4-PA	47.2 ± 0.3	24.8	69.8 ± 0.4	91.0 ± 1.7
	47.2 ± 0.3	60.9	103.8 ± 0.8	93.0 ± 1.4
	32.4 ± 0.1	119.0	148.9 ± 0.5	97.8 ± 0.4
	32.4 ± 0.1	174.4	203.5 ± 1.4	98.1 ± 0.8
Serum				
PMP	ND[c]	24.8	23.6 ± 0.4	95.0 ± 1.7
	ND[c]	119.0	115.8 ± 0.5	97.3 ± 0.5
	ND[c]	174.4	171.0 ± 1.2	98.0 ± 0.7
PM	ND[c]	24.8	23.0 ± 0.2	92.7 ± 0.8
	ND[c]	119.0	116.0 ± 0.3	97.5 ± 0.3
	ND[c]	174.4	168.5 ± 2.7	96.6 ± 1.6
PLP	62.8 ± 0.3	24.8	85.6 ± 0.4	97.7 ± 0.5
	62.8 ± 0.3	119.0	178.9 ± 0.8	98.4 ± 0.5
	26.4 ± 0.4	174.4	196.1 ± 0.7	97.7 ± 0.4
PL	19.6 ± 0.2	24.8	41.9 ± 0.2	94.0 ± 0.8
	19.6 ± 0.2	119.0	135.7 ± 0.6	97.9 ± 0.4
	13.8 ± 0.3	174.4	183.8 ± 1.0	97.6 ± 0.5
4-PA	35.7 ± 0.2	24.8	58.5 ± 0.9	91.8 ± 3.4
	43.3 ± 0.3	119.0	159.2 ± 0.3	97.4 ± 0.2
	24.2 ± 0.1	174.4	191.3 ± 3.3	95.5 ± 2.2

[a] $n = 3$.
[b] Sample dilution was taken into consideration.
[c] Not detected.

and serum samples for PLP, PL and 4-PA, thus LOD can be assessed with a pooled matrix consisted of the endogenous analyte. With this approach, the method had the LODs of 1.5, 1.4 and 2.1 nM and LOQs of 4.5, 4.2 and 6.3 nM for PLP, PL and 4-PA, respectively. The LOQ was similar to that established according to mentioned previously IUPAC recommendation (Section 2.5.1).

Another procedure to estimate a LOD and a LOQ, based on repetitive analysis of the low serum control samples and four dilutions was described by Rybak and Pfeiffer [40]. Although the LODs and LOQs obtained in our study are higher, excluding need for dilution of matrix compounds, however, is enough sensitive to quantify PLP, PL and 4-PA in nanomolar concentrations.

Table 3
Results of analysis of ClinCheck serum control samples.

Analyte/Level	Mean value (nM)	Control range (nM)	Mean concentration found (nM ± SD)[a]	Percentage of recovery (±SD)[a]
PLP				
Level I	72.0	57.5–86.6	68.6 ± 2.0	95.3 ± 2.8
Level II	113.0	90.2–136.0	111.1 ± 1.1	98.3 ± 0.8
Level III	158.0	127.0–190.0	153.3 ± 2.3	96.8 ± 1.6
PL				
Level I	29.4	23.6–35.3	27.1 ± 0.7	93.6 ± 2.5
Level II	64.0	51.2–76.6	62.1 ± 1.2	95.8 ± 3.1
Level III	98.1	78.4–118.0	95.8 ± 1.9	97.8 ± 1.9

[a] $n = 6$.

Table 4
Results for determination of precision.

Analyte	Plasma QC pool	Concentration found (nM ± SD)			
		Within-run (n = 10)	RSD (%)	Between-run (n = 24)	RSD (%)
PMP	Low	23.6 ± 0.4	1.7	23.1 ± 1.2	5.2
	Medium	95.8 ± 0.7	0.7	95.7 ± 2.6	2.7
	High	184.7 ± 0.9	0.5	183.8 ± 2.9	1.6
PM	Low	22.6 ± 0.6	2.6	22.9 ± 1.4	6.1
	Medium	95.4 ± 0.9	0.9	95.8 ± 2.8	2.9
	High	183.9 ± 1.4	0.8	184.7 ± 3.2	1.7
PLP	Low	23.7 ± 0.7	2.9	23.5 ± 1.2	5.1
	Medium	96.1 ± 1.3	1.4	95.8 ± 1.9	2.0
	High	184.9 ± 0.9	0.5	184.1 ± 4.8	2.6
PL	Low	23.3 ± 0.5	2.1	22.9 ± 1.8	7.7
	Medium	95.2 ± 0.8	0.8	95.1 ± 3.1	3.3
	High	184.1 ± 0.7	0.4	183.9 ± 3.6	2.0
PN	Low	22.4 ± 0.6	2.7	22.9 ± 1.3	5.7
	Medium	95.3 ± 1.0	1.1	95.7 ± 2.7	2.8
	High	183.7 ± 1.2	0.7	183.1 ± 3.9	2.1
4-Pa	Low	23.7 ± 0.6	2.5	23.5 ± 1.1	4.7
	Medium	95.9 ± 0.9	0.9	95.2 ± 2.9	3.0
	High	184.2 ± 1.4	0.8	184.1 ± 4.3	2.3

3.2.2. Recovery

Analytical recoveries, determined by analyses of spiking plasma and serum samples (Table 2) ranged from 93.5–98.0% for PMP, 91.1–97.5% for PM, 93.9–97.9% for PLP, 92.8–97.8% for PL, 90.4–95.7% for PN and 91.8–98.1% for 4-PA. Results of analysis of ClinCheck serum control samples are presented in Table 3. Chromatogram of serum control ClinCheck sample (level 2) is illustrated at Fig. 4.

3.2.3. Precision

The within-run precision of the method was evaluated on one day by 10 replicates analysis of spiking samples (QC) containing low, medium and high concentrations of the analysed vitamins and 4-PA, and the between-run precision was evaluated by six replicates analysis of QC samples every seven days during one month. Results of these assays are presented in Table 4. Other published HPLC methods [32,36,39–41] reported similar within-run (0.7–6.1%) and between-run (3.7–8.8%) RSD ranges for the analysed compounds in plasma and serum samples.

3.2.4. Stability study

The results of postpreparative stability study of spiked plasma samples at three different concentrations of each analysed vitamins and 4-PA, stored in dark at 8 °C are summarized in Table 5. At time points of 0, 6, 12, 18 and 24 h, three aliquots of each of the QC were analysed. After HPLC analysis, the mean concentrations of the QC were calculated and compared to these at time point of 0 h. The concentrations in the samples stored for 12 h were, on average, 4.0% lower than these in samples processed immediately. Therefore PMP, PM, PLP, PL, PN and 4-PA in plasma were stable for the time necessary for processing of batches of approximately 36 samples. Similar results referring to PLP in plasma samples, have been reported by Talwar et al. [36]. Samples prepared and stored at 8 °C for 24 h showed significant losses of each vitamins and 4-PA, on average 14.2%.

Table 5
Results of postpreparative stability study of spiked plasma stored in dark at 8 °C.

Analyte	Concentration added (nM)	Assayed concentration (nM ± SD)[a]					Ratio (%)			
		0 h	6 h	12 h	18 h	24 h	6 h/0 h	12 h/0 h	18 h/0 h	24 h/0 h
PMP	24.5	23.8 ± 0.1	23.9 ± 0.6	22.4 ± 0.2	21.3 ± 0.7	18.4 ± 0.6	99.8	94.1	88.6	77.3
	96.2	94.5 ± 0.9	94.2 ± 0.8	92.2 ± 0.4	89.7 ± 1.1	84.0 ± 1.4	99.8	97.8	94,8	88.9
	185.2	184.3 ± 1.1	183.8 ± 1.0	180.9 ± 0.4	177.8 ± 0.5	170.1 ± 0.8	99.9	98.2	94.7	92.3
PM	24.5	23.7 ± 0.4	23.6 ± 0.2	22.8 ± 0.6	20.7 ± 0.4	18.1 ± 0.8	99.6	96.2	87.3	76.3
	96.2	95.8 ± 0.2	95.2 ± 0.6	93.7 ± 0.4	91.6 ± 0.3	89.7 ± 0.7	99.4	97.8	95.6	93.6
	185.2	184.1 ± 0.6	182.3 ± 0.5	180.4 ± 0.6	176.3 ± 0.5	170.7 ± 0.4	99.0	97.9	95.8	92.7
PLP	24.5	23.8 ± 0.4	23.4 ± 0.2	22.5 ± 0.5	20.7 ± 0.6	18.5 ± 0.6	98.3	94.5	86.9	77.7
	96.2	95.8 ± 0.5	94.2 ± 0.3	92.6 ± 0.4	89.9 ± 0.6	85.3 ± 0.3	98.3	96.6	93.8	89.0
	185.2	184.5 ± 0.4	183.2 ± 0.5	180.6 ± 0.8	176.6 ± 0.4	169.3 ± 0.5	99.3	97.8	95.7	91.2
PL	24.5	23.9 ± 0.3	23.3 ± 0.4	22.3 ± 0.3	20.2 ± 0.6	17.8 ± 0.6	97.5	93.3	84.5	74.5
	96.2	95.6 ± 0.4	95.1 ± 0.6	93.2 ± 0.6	90.1 ± 0.5	87.1 ± 0.3	99.5	97.5	94.2	91.1
	185.2	184.4 ± 0.4	181.8 ± 0.6	177.3 ± 0.5	172.8 ± 0.6	167.3 ± 0.6	98.6	96.1	93.7	90.7
PN	24.5	23.7 ± 0.5	23.1 ± 0.3	21.9 ± 0.4	19.8 ± 0.6	17.3 ± 0.4	97.5	92.0	83.5	73.0
	96.2	95.6 ± 0.3	94.2 ± 0.4	91.1 ± 0.6	87.3 ± 0.5	83.2 ± 0.7	98.5	95.3	91.3	87.0
	185.2	184.9 ± 0.2	183.3 ± 0.4	180.0 ± 0.4	176.9 ± 0.5	172.7 ± 0.4	99.1	97.4	95.7	93.7
4-PA	24.5	23.8 ± 0.3	23.1 ± 0.5	22.2 ± 0.4	20.4 ± 0.4	18.3 ± 0.6	97.1	93.3	85.7	76.9
	96.2	95.9 ± 0.3	94.6 ± 0.2	90.6 ± 0.4	87.3 ± 0.6	82.8 ± 0.6	98.6	94.5	91.0	86.3
	185.2	184.7 ± 0.3	183.4 ± 0.5	179.2 ± 0.5	172.6 ± 0.5	169.1 ± 0.4	99.3	97.0	93.4	91.5

[a] n = 3.

Table 6
Results of stability study of spiked serum after repeated thawing and freezing.

Analyte	Concentration added (nM)	Assayed concentration (nM)				Mean concentration (nM)	RSD (%)
		Number of freeze-thaw cycles					
		0	1	2	3		
PMP	24.5	23.7	23.1	23.9	23.4	23.5	1.5
	96.2	95.9	95.0	94.9	95.6	95.3	0.5
	185.2	184.7	183.9	184.1	183.6	184.0	0.4
PM	24.5	23.7	23.4	24.0	23.1	23.6	1.7
	96.2	94.5	95.3	94.8	95.7	95.1	0.6
	185.2	184.7	184.2	183.6	183.1	183.9	0.4
PLP	24.5	23.2	23.6	23.1	23.7	23.4	1.3
	96.2	95.3	96.1	95.7	94.8	95.5	0.6
	185.2	183.5	184.2	183.7	182.3	183.4	0.4
PL	24.5	23.9	24.1	23.2	23.8	23.8	1.6
	96.2	95.7	94.9	94.0	95.1	94.9	0.7
	185.2	184.4	183.7	183.5	182.9	183.6	0.3
PN	24.5	24.1	23.6	23.1	23.3	23.6	2.0
	96.2	94.7	95.3	95.9	94.9	95.2	0.6
	185.2	184.8	184.1	183.2	183.7	183.9	0.4
4-PA	24.5	23.8	24.2	23.1	23.5	23.7	2.0
	96.2	95.8	95.1	94.3	94.8	95.0	0.7
	185.2	184.4	183.5	184.0	184.9	184.2	0.3

The freeze–thaw stability of PMP, PM, PLP, PL, PN and 4-PA in serum was studied by subjecting samples from the three serum QC pools to repeated freeze–thaw cycles (Table 6). Each cycle first involved thawing serum samples at room temperature for 30 min. Samples were then opened and allowed to remain at 8 °C for an addition 30 min before being analysed and returned to storage at −80 °C for the next analysis. The concentration measured up to three freeze–thaw cycles indicated no significant losses of each analysed vitamin and 4-PA. Similar results, but only for the determination of PLP and 4-PA in serum samples were obtained by previously reported HPLC method with fluorescent detection [40].

In the long-term temperature-stability study, spiking serum and plasma QC samples were frozen in liquid nitrogen and stored at −80 °C. Five samples at each analyte concentration were processed and analysed immediately after preparation and then every seven days for two months. There were no significant losses of PMP, PM, PLP, PL, PN and 4-PA during storage at −80 °C for two months. These results are similar to those reported elsewhere for PLP and 4-PA in plasma [39,54,55].

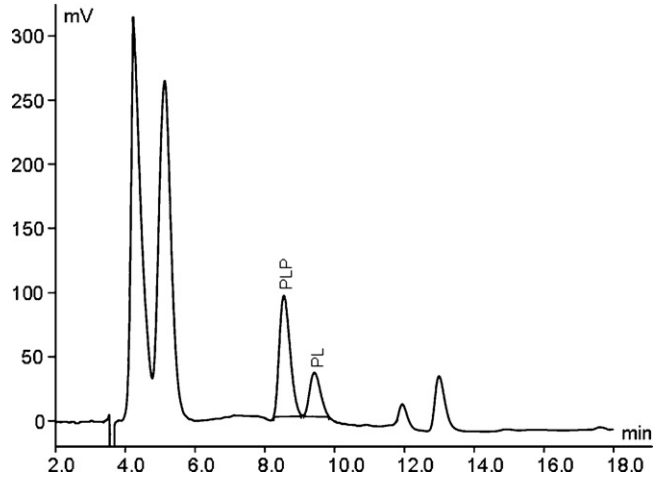

Fig. 4. Chromatogram of the analysed serum control sample, level 2: PLP (t_R 8.5 min, 111.2 nM), PL (t_R 9.8 min, 62.5 nM).

To assess the stability of stock solutions, two 10.0 mM solutions of each the analysed vitamin with 4-PA were prepared. One of these solutions was stored at 4.0 °C, and a second was frozen in liquid nitrogen and stored at −80 °C. After 0, 6, 12, 24 h and 2, 7, 14, 28, 45, and 60 days, these solutions were diluted to prepare working stock solutions (nominally 100.0 nM) and finally analysed. There were significant losses of each the analysed vitamin and 4-PA solutions stored at 4.0 °C after 24 h (approximately 12%). The frozen stock solutions of PMP, PM, PLP, PL, PN and 4-PA were stable throughout the study period. These results affirmed previously reported experiments [56,57].

3.2.5. Interferences

The endogenous matrix of thiols, such as glutathione, methionine and cysteine may cause some minimal interferences with the analysed vitamins B_6 [49]. These thiols may be detected in chromatographic conditions presented in our study. For this purpose, six replicates of the analysed plasma and serum QC samples, with added working solutions of the reduced glutathione, glutathione disulfide, L-cysteine and L-methionine, were analysed. No interferences were noticed in the retention time of vitamins B_6 and 4-PA. The deviation of the base line at the retention time of 6.5–7.0 min presented in all chromatograms of the plasma (Figs. 2 and 3) is probably related to the artefact which can be eliminated by performed routinely a baseline correcting procedure. It has been stated that all the vitamins analysed could be clearly separated from other non-specific eluted substances.

3.3. Assessment of vitamins B_6 and 4-pyridoxic acid concentrations in plasma and serum of study groups

Concentrations of vitamins B_6 in the analysed plasma and serum samples in the study groups (Table 7) were similar to that reported by several authors [30,33,40–43,53,58]. PN was identified in four samples collected from healthy women. PLP, PL and 4-PA were presented in amounts that can be measured with a relatively high precision. Bor et al. [53] reported that concentrations of vitamin B_6 from samples of healthy individuals are low unless the person takes extra supplements containing vitamins B_6. A guidance reference interval for PLP is 20 to 121 nM. Therefore, plasma concentrations

Table 7
The concentrations of vitamins B_6 and 4-pyridoxic acid concentrations in plasma and serum samples (means ± SD).

Subjects (*N*)	PLP (nM)	PL (nM)	PN (nM)	4-PA (nM)
Male (20)	51.4 ± 22.4	12.7 ± 6.7	ND	32.6 ± 17.8
Female (20)	62.7 ± 31.6	17.6 ± 8.2	6.9 ± 1.3	43.7 ± 21.2

N – number of subjects; ND – not detected.

less than 20 nM are considered as deficient [1]. The deficiency of PLP in plasma samples was not observed in the study groups.

4. Conclusions

The presented method displayed the satisfying sensitivity and selectivity of the electrochemical coulometric detection for the simultaneous determination of vitamins B_6 and 4-pyridoxic acid in human plasma and serum samples. According to our knowledge, this is the first report using coulometric detection in HPLC method for measurement of these vitamins in human plasma and serum. Simplicity of sample preparation, without derivatization and possibility of measurement at low sample volume makes this application an attractive alternative for the analysis of PMP, PM, PLP, PL, PN and 4-PA in human plasma and serum.

References

[1] A. Shenkin, M. Baines, in: C.A. Burtis, E.R. Ashwood, D.E. Bruns (Eds.), Fundamentals of Clinical Chemistry, 6th ed., Elsevier Inc., St. Louis, 2008, p. 486.
[2] G.F.M. Ball, Vitamins in Foods, CRC Press, New York, 2006, p. 189.
[3] D. Bender, Br. J. Nutr. 81 (1999) 7.
[4] H.C. Lukaski, Nutrition 20 (2004) 632.
[5] A.L. Bailey, A.J.A. Wright, S. Southon, Eur. J. Clin. Nutr. 53 (1999) 448.
[6] H.W. Bruinse, H. van der Berg, Eur. J. Obstet. Gynecol. 61 (1995) 31.
[7] A. Leaf, S. Subramanian, S. Cherian, Curr. Pediatr. 14 (2004) 298.
[8] S.C. Larsson, E. Giovannucci, A. Wolk, Gastroenterology 128 (2005) 1830.
[9] M. Cravo, L. Glória, M.L. Camilo, M. Resende, J.N. Cardoso, C.N. Leitão, F.C. Mira, Clin. Nutr. 16 (1997) 29.
[10] S.K. Jain, Metabolism 56 (2007) 168.
[11] R. Monge-Rojas, Arch. Med. Res. 31 (2001) 70.
[12] F. Sofi, R. Marucci, P. Boli, B. Giambene, A. Sodi, S. Fedi, U. Menchini, G.F. Gensini, R. Abbate, D. Prisco, Artheriosclerosis 198 (2008) 223.
[13] R.R. Eitenmiller, L. Ye, W.O. Landen (Eds.), Vitamin Analysis for The Health and Food Sciences, 2nd ed., CRC Press, New York, 2008, p. 401.
[14] S.P. Coburn, in: W.O. Song, G.R. Beecher, R.R. Eitenmiller (Eds.), Modern Analytical Methodologies in Fat- and Water-Soluble Vitamins, John Willey & Sons, Toronto, 2000, p. 291.
[15] H. Baker, O. Frank, M. Ning, R. Gellene, S.H. Huntner, C.M. Levey, Am. J. Clin. Nutr. 18 (1966) 123.
[16] S.P. Coburn, J.D. Mahuren, R.R. Guilarte, J. Nutr. 114 (1984) 2269.
[17] B. Chaber, D. Livingston, Anal. Biochem. 34 (1970) 413.
[18] A. Hamfelt, Scand. J. Clin. Lab. Invest. 20 (1967) 1.
[19] V.M. Camp, J. Chippni, B.A. Faraj, Clin. Chem. 29 (1983) 642.
[20] Q. Han, M. Xu, L. Tang, X. Tan, X. Tan, Y. Tan, R.B. Hoffman, Clin. Chem. 48 (2002) 1560.
[21] J.J.B. Nevado, J.A.M. Pulgarín, M.A.G. Laguna, Talanta 42 (1995) 129.
[22] M.Á. Fermández-Muiño, M.T. Sancho-Ortiz, F. Valls-Garcia, in: W.J. Hurst (Ed.), Methods of Analysis for Functional Foods and Nutraceuticals, CRC Press, New York, 2008, p. 401.
[23] D.E. Burton, M.J. Sepaniak, J. Chromatogr. 24 (1986) 347.
[24] J.J.B. Nevado, J.A.M. Pulgarín, M.A.G. Laguna, J. Pharm. Biomed. Anal. 14 (1996) 1487.
[25] C.J. Argoudelis, J. Chromatogr. A 790 (1997) 83.
[26] J.T. Vanderslice, C.E. Marie, J. Chromatogr. 196 (1980) 176.
[27] S.P. Coburn, J.D. Mahuren, Anal. Biochem. 129 (1983) 310.
[28] P. Edwards, P.K.S. Liu, G.A. Rose, Clin. Chem. 35 (1989) 241.
[29] S.K. Sharma, K. Dakshinamurti, J. Chromatogr. 578 (1992) 45.
[30] M. Kimura, K. Kanehira, K. Yokoi, J. Chromatogr. A 722 (1996) 295.
[31] J.D. Mahuren, S.P. Coburn, Methods Enzymol. 280 (1997) 22.
[32] C.L. Deitrick, R.E. Katholi, D.J. Huddleston, K. Hardiek, L. Burrus, J. Chromatogr. B 751 (2001) 383.
[33] M.R. Bisp, M.V. Bor, E.-M. Heisving, M. Kall, E. Nexø, Anal. Biochem. 305 (2002) 82.
[34] J.F. Gregory 3rd, Anal. Biochem. 102 (1980) 374.
[35] J.B. Ubbink, W.J. Serfontein, L.S. DeVilliers, J. Chromatogr. 375 (1986) 399.
[36] D. Talwar, T. Quasim, D.C. McMillan, J. Kinsella, C. Williamson, D.St.J. O' Reilly, J. Chromatogr. B 792 (2003) 333.
[37] H. Millart, D. Lamiable, Analyst 114 (1989) 1225.
[38] H. Tsuge, Methods Enzymol. 280 (1997) 3.
[39] C.J. Bates, K.D. Pentieva, N. Matthews, A. Macdonald, Clin. Chim. Acta 280 (1999) 101.
[40] M.R. Rybak, C.M. Pfeiffer, Anal. Biochem. 333 (2004) 336.
[41] K.L. Ericson, J.D. Mahueren, Y.M. Zubovic, S.P. Coburn, J. Chromatogr. B 823 (2005) 218.
[42] A.E. Romero, M.E. Capella-Peiró, L. Monferrer-Pons, M.G. Augustí, Clin. Chim. Acta 348 (2004) 69.
[43] Ø. Midttun, S. Hustand, E. Solheim, J. Schneere, P.M. Ueland, Clin. Chem. 51 (2005) 1206.
[44] W. Qu, K. Wu, S. Hu, J. Pharm. Biomed. Anal. 36 (2004) 631.
[45] T. Pineda, M.I. Lopez-Cozar, J.M. Sevilla, M. Blazquez, J. Electroanal. Chem. 403 (1996) 101.
[46] M.F.S. Teixeira, G. Mariano, E.R. Dockar, É.T.G. Cavallheiro, Anal. Chim. Acta 508 (2004) 79.
[47] M.L. Marszałł, A. Lebiedzińska, W. Czarnowski, P. Szefer, J. Chromatogr. A 1094 (2005) 91.
[48] A. Lebiedzińska, M.L. Marszałł, J. Kuta, P. Szefer, J. Chromatogr. A 1173 (2007) 71.
[49] R.J. Flanagan, D. Perrett, R. Whelpton, Electrochemical Detection in HPLC. Analysis of Drugs and Poisons, The Royal Society of Chemistry, Cambridge, 2005, p. 56.
[50] K. Hensley, K.S. Williamson, M.L. Maidt, S.P. Gabitta, P. Grammas, R.A. Floyd, J. High Resolut. Chromatogr. 22 (1999) 429.
[51] P. Konieczka, J. Namieśnik, Quality Assurance and Quality Control in the Analytical Chemical Laboratory. A Practical Approach, CRC Press, 2009, p. 131.
[52] M.A. Currie, Pure Appl. Chem. 67 (1995) 1699.
[53] M.V. Bor, H. Refsum, M.R. Bisp, Ø. Bleie, J. Schneede, J.R. Nordrehaung, P.M. Ueland, O.K. Nygard, E. Nexø, Clin. Chem. 49 (2003) 155.
[54] W.M. Borschel, A. Kirksey, B.R. Hamaker, J. Pediatr. Gastroenterol. Nutr. 6 (1987) 409.
[55] M.C. Ocké, J. Schrijver, G.L. Obermenn-De Boer, B.P.M. Bloemberg, G.R.M.M. Haenen, D. Kromhout, J. Clin. Epidemiol. 48 (1995) 1077;
C.Y.W. Ang, J. Assoc. Off. Anal. Chem. 62 (1979) 1170.
[56] B. Saidi, J.J. Warthensen, J. Agric. Food Chem. 31 (1983) 876.
[57] C.J. Bates, K.D. Pentieva, A. Prentice, M.A. Mansoor, S. Finch, Br. J. Nutr. 81 (1999) 191.
[58] M.R. Rybak, R.B. Jain, C.M. Pfeiffer, Clin. Chem. 51 (2005) 1223.