Journal of Chromatography B, 903 (2012) 134–141

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

A stable isotope dilution LC–ESI-MS/MS method for the quantification of pyridoxal-5′-phosphate in whole blood

Bertrand D. van Zelst*, Robert de Jonge

Department of Clinical Chemistry, Erasmus MC, University Medical Center Rotterdam, PO Box 2040, 3000 CA Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 2 April 2012
Accepted 11 July 2012
Available online 20 July 2012

Keywords:
Method validation
Vitamin B6
Pyridoxal-5′-phosphate
LC–ESI-MS/MS
Whole blood

ABSTRACT

Vitamin B6 is a cofactor in numerous biologic processes that include gluconeogenesis, neurotransmitter synthesis and amino acid metabolism. The aim of this study was to develop a method to measure the concentration of the biologically active form of vitamin B6 (pyridoxal-5′-phosphate, PLP) in whole blood with stable isotope dilution LC–ESI-MS/MS and compare this new procedure with an established HPLC method based on derivatization of pyridoxal-5′-phosphate. 50 μl of stable isotope (PLP-d3) was added to 250 μl of sample, followed by deproteinization with 10% trichloroacetic acid. After centrifugation, 20 μl of the supernatant was injected into the LC–ESI-MS/MS. Reversed phase chromatography was performed on a UPLC system, using a Waters™ Symmetry C18 column, with a gradient of 0.1% formic acid in methanol. PLP was measured on a tandem MS with a mass transition of 247.8 > 149.8 in the positive ion mode with a collision energy of 14 eV. The chromatographic run lasted 4 min. The method was linear from 4 to 8000 nmol/l. The intra-day and inter-day precision ranged between 1.7–2.8% and 3.0–4.1%, respectively. The mean absolute matrix-effect was 99.3% [97–102%]. The relative matrix-effect was 98.8%. The mean recovery was 98% [89–103%]. The lower limit of quantification was 4 nmol/l. The comparison of the LC–ESI-MS/MS method with our current HPLC method yielded the following equation: LC–ESI-MS/MS = 1.11 [confidence interval, CI: 1.03–1.20] × HPLC + 4.6 [CI: −1.3 to 11.0] (r^2 = 0.94). This LC–ESI-MS/MS based method is characterized by simple sample processing and a short run time. The comparison with the current HPLC method is excellent although a significant proportional bias was detected. To conclude, the LC–ESI-MS/MS method is an appropriate method to determine PLP in whole blood.

1. Introduction

Vitamin B6 serves as a cofactor in several important biological processes, including heme-synthesis, amino acid metabolism, neurotransmitter synthesis and gluconeogenesis [1]. The biologically active form of vitamin B6 present in the human body is pyridoxal-5′-phosphate (PLP) and reflects long term body storage. Malnutrition, alcoholism or chronic renal insufficiency may lead to low vitamin B6 levels and result in anemia, pellagra, and changes in mental status. Furthermore, low levels of vitamin B6 may be associated with an increased risk for coronary artery disease and stroke [2,3].

Assessment of vitamin B6 status is predominantly done by measuring the concentration of PLP in plasma or whole blood. There is a strong correlation between plasma-PLP and whole blood-PLP in healthy individuals [4]. However, in patients who suffer from systemic inflammatory diseases, reduced plasma-PLP levels are found even after supplementation with B vitamins [5]. In these cases, whole blood PLP levels were found to be normal or, in the case of supplementation, elevated and hence, whole blood PLP is a better indicator of tissue vitamin B6 status [5–7]. The low plasma-PLP levels in these inflammatory disease patients might be caused by the low plasma albumin concentrations because PLP is predominantly bound to albumin. Alternatively, increased hydrolysis of PLP by high levels of alkaline phosphatase is another possible explanation for these low plasma PLP levels. On a more general note, whole blood vitamin levels are probably more sensitive markers of tissue vitamin status than plasma vitamin levels [8].

There are different analytical techniques to determine PLP levels. Enzymatic [9] and microbiological [10] methods have been described but HPLC-based methods with fluorescence detection are most frequently used [4,11–17]. These methods require a derivatization-step of PLP due to the lack of a fluorophore in the PLP molecule. Besides the need for derivatization, some of these methods have long chromatographic run times of about 10 min or more. Midttun et al. [18] and van der Ham et al. [19] reported methods in

Abbreviations: PLP, pyridoxal-5′-phosphate; PMP, pyridoxamine-5′-phosphate; PNP, pyridoxine-5′-phosphate; CSF, cerebrospinal fluid.

* Corresponding author. Tel.: +31 107033587; fax: +31 104367894.
E-mail address: b.vanzelst@erasmusmc.nl (B.D. van Zelst).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.07.007

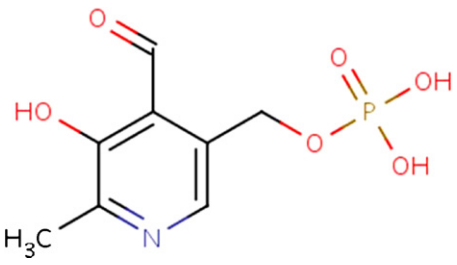

Fig. 1. Chemical structure of pyridoxal-5′-phosphate.

which all vitamin B6 vitamers were measured in plasma or CSF with liquid chromatography–tandem mass spectrometry (LC–MS/MS). Mass spectrometric methods are commonly more sensitive and more selective than HPLC methods, no derivatization is needed and shorter run times are more easily achieved. However, a drawback in mass spectrometry is the possible occurrence of matrix effects that get more pronounced with more complex matrices such as whole blood. To the best of our knowledge, no MS-based methods to quantify PLP in whole blood have been described in the literature. The objective of this study was to develop a method to measure the concentration of PLP in heparin whole blood for clinical practice, using stable isotope dilution LC–ESI-MS/MS, validate the method, and compare it with an established HPLC method based on derivatization of PLP.

2. Materials and methods

2.1. Materials

PLP and PMP were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). The structure of PLP is shown in Fig. 1. The internal standard, PLP-d3, was purchased from Buchem B.V. (Apeldoorn, The Netherlands). Formic acid (FA), trichloroacetic acid (TCA) and hydrochloric acid (HCl) were purchased from Merck (Darmstad, Germany). LC–MS grade water and LC–MS grade methanol were from Biosolve B.V. (Valkenswaard, The Netherlands).

The blood samples used for the development and validation of the method were leftover specimens from daily routine analyses in our laboratory and thus, no informed consent was necessary.

2.2. Standard preparation

Stock standard solution of PLP was prepared at 750 μmol/l in 0.1 mol/l HCl. Stock standard solution of the internal standard PLP-d3 was prepared at 30 μmol/l in 0.1 mol/l HCl. Both stock standards were aliquoted and stored at −80 °C. The stock standard of PLP was further diluted in water to obtain a working standard of 7.50 μmol/l, which was made freshly for each new series of measurements and which was used immediately after preparation. The stock standard of PLP-d3 was further diluted with 0.1 mol/l HCl to obtain a working solution of 600 nmol/l. After sample preparation, the remainder of the PLP-d3 working solution was kept at room temperature under yellow light until the next series of measurements.

2.3. Calibrator preparation

40 μl of the PLP working solution was further diluted with 960 μl lithium heparin whole blood to obtain spiked calibration standards at six different levels of PLP, ranging from 0 to 300 nmol/l. After the measurements, correction took place for the endogenous amount of PLP, which was calculated as the ratio of intercept to slope from the calibration line $y = ax + b$.

2.4. Sample preparation

PLP was measured in lithium heparin whole blood. 250 μl of whole blood, calibrator or QC was mixed with 50 μl of the internal standard working solution. Protein precipitation took place by dropwise adding 1750 μl of a 10% TCA solution whilst mixing. Thereafter, the samples were kept at room temperature for 1 h under yellow light. At 30 min, the samples were mixed one more time. After 1 h, the samples were centrifuged for 7 min at 21,380 × *g*. The supernatants were transferred into black vials and placed in the autosampler at 15 °C.

2.5. LC–ESI-MS/MS

Chromatography was performed using a UPLC (Waters Corporation, Etten-Leur, The Netherlands). Separation was achieved by full loop injection of 20 μl prepared sample on a Symmetry C18 column (2.1 mm × 100 mm, 3.5 μm) with a column temperature of 35 °C. Gradient elution utilized 0.1% FA in water as solvent A and methanol as solvent B at a flowrate of 0.25 ml/min. The gradient was as follows: 0–0.1 min (85%A and 15%B), 2 min (45%A and 55%B), 2.1 min (85%A and 15%B), 4 min (85%A and 15%B). Both gradient steps were linear with a total run time of 4 min for each sample.

Mass spectrometry was performed using a Quattro Premier XE (Waters Corporation, Etten-Leur, The Netherlands) tandem mass spectrometer. PLP was measured by electrospray ionization (ESI) in the positive mode with the following selected reaction monitoring mass transitions: m/z 247.8 > 149.8 and 247.8 > 93.9 for PLP and m/z 250.9 > 152.9 and 250.9 > 96.9 for PLP-d3 (Table 1). Other mass spectrometer settings were: capillary voltage 4.00 kV, cone voltage 30 V, desolvation temperature 350 °C at a gasflow of 1000 l/h and cone gasflow 50 l/h. Argon was used as collision gas at a flowrate of 0.20 ml/min.

2.6. Method validation

The validation of the assay was performed according the FDA guidelines [20], CLSI guidelines [21] and the recommendations as described in the publications of Matuszewski et al. [22,23] for determining matrix effects and extraction recovery.

2.6.1. Linearity

The analytical linearity of the method was determined by spiking whole blood with 10 different concentrations of PLP ranging from 0 to 8000 nmol/l. A second linearity experiment was performed covering the clinical range of PLP at six different concentrations, ranging from 0 to 300 nmol/l. All measurements were done 5-fold. Linearity was assessed by application of the

Table 1
MS–MS conditions for the single reaction monitoring of PLP and PLP-d3.

Molecule	Parent ion (m/z)	Daughter ion (m/z)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
PLP	247.8	93.9	0.1	30	28
	247.8	149.8	0.1	30	14
PLP-d3	250.9	96.9	0.1	30	28
	250.9	152.9	0.1	30	14

'lack-of-fit' model as described in the CLSI EP-6 guidelines. The method was accepted as being linear within the 95% confidence interval, when the 'lack-of-fit' criterium was <3.29.

2.6.2. Lower limit of quantification

As clinical samples with extremely low PLP concentrations were not available, PLP-d3, which is absent in patient samples, was used to determine the lower limit of quantification (LLOQ). Whole blood was spiked with different concentrations of PLP-d3, ranging from 0 to 60 nmol/l and measured 10-fold. The lowest concentration of PLP-d3 where the imprecision is less than 20% and where the S/N ratio is at least 10 is regarded as the LLOQ. Noise is defined as the mean blank PLP-d3 response of twenty different whole blood samples.

2.6.3. Precision

The precision of the method was determined using whole blood sample pools at three different PLP concentrations, 39 nmol/l, 59 nmol/l and 103 nmol/l, corresponding to a PLP deficient concentration, a PLP concentration at the lower end of the normal range and a PLP concentration at the higher end of the normal range. Sample pools with a PLP concentration closer to the LLOQ could not be obtained due to the lack of patients that were severely PLP-deficient. The samples were measured 5-fold on twenty consecutive working days to assess interday precision. The intraday precision was determined by measuring the samples 20-fold in one run. Interday and intraday precisions were expressed as coefficients of variation (CV%). The FDA's acceptance criteria for precision (CV < 15%) were applied.

2.6.4. Recovery

The sample extraction recovery was determined by spiking two aliquots of twenty different blood samples with 100 nmol/l PLP. Aliquot one of each blood sample was spiked before sample preparation, aliquot two of each blood sample was spiked after sample preparation. A third aliquot was used to determine endogenous PLP. The recovery was calculated as the difference in PLP concentration between pre-sample preparation spiking and post-sample preparation spiking after correcting for endogenous PLP [23]:

$$\text{Recovery (\%)} = \frac{\text{PLP spiked before sample prep} - \text{endogenous PLP}}{\text{PLP spiked after sample prep} - \text{endogenous PLP}} \times 100$$

Recoveries of 100 ± 15% were regarded as being admissible in this method validation.

2.6.5. Matrix effect

Twenty calibration lines were obtained by spiking twenty different whole blood samples with four different concentrations (0 nmol/l, 50 nmol/l, 100 nmol/l, and 200 nmol/l) of PLP and subsequent calculation of slope and intercept. The slopes of the whole blood calibration lines were compared with the slope of a calibration line in water as a measure of an absolute matrix-effect [23]:

$$\text{Absolute matrix effect}: \frac{\text{Slope whole blood sample}}{\text{Slope water}} \times 100\%$$

The relative matrix effect was calculated as 100% – CV% of the slopes of the twenty whole blood calibration lines. Furthermore, the maximum slope difference was calculated as the difference in percentage between the lowest slope and highest slope of the 20 calibration lines. All matrix effects were calculated with and without correction by the internal standard.

When using the internal standard as correction, a relative matrix effect of 100 ± 5% was used as a cut-off value for the method to be acceptable [23].

Matrix effects were also studied by the direct infusion method. A standard solution of the stable isotope was directly infused into the LC–MS/MS while at the same time a regular sample was injected into the system. A blank injection was performed and subtracted from the mass spectrum of the regular sample.

2.6.6. Interferences

There are two known vitamin B6 vitamers that could interfere with the detection of PLP or PLP-d3 because of similar structure and similar molecular mass. Pyridoxamine-5′-phosphate (M = 248.2) and PLP (M = 247.2) differ only in one mass unit causing a potential interference in the measurement of PLP by PMP. Pyridoxine-5′-phosphate (M = 249.2) and PLP-d3 (M = 250.2) also differ in one mass unit where the detection of PLP-d3 might become compromised when significant concentrations of PNP are present in whole blood.

To check for interference, 20 μl of a neat 1000 nM PMP-solution was introduced as sample and the chromatographic window for both PLP mass transitions was monitored for interfering peaks caused by PMP. PNP is commercially not available, therefore possible interferences with PLP-d3 peak detection was performed by injecting 20 PLP-d3 blank samples into the LC–ESI-MS/MS and checking the chromatograms for unknown peaks.

2.6.7. Stability

The stability of the PLP stock standard was checked by storage at −80 °C for 1 year. The stability of PLP in whole blood was studied by storing five whole blood samples under the following conditions: 4 h in ambient light, 1 week at −20 °C, 6 weeks at −80 °C and 6 months at −80 °C. Freeze/thaw stability of whole blood PLP was investigated by up to three successive cycles of freezing at −80 °C and unassisted thawing. Stability of PLP after sample preparation was evaluated by keeping the freshly prepared and measured samples in the autosampler at 15 °C for 96 h. All results obtained from these stability experiments were compared with the results of the same samples freshly measured.

Quantitative release of PLP from the matrix was investigated by performing the sample extraction procedure with different strengths of acid (TCA 10% and TCA 20%).

2.6.8. Carry-over

Carry-over was determined by triplicate measurement of a sample with a high concentration of PLP (3400 nM) followed by triplicate measurement of a sample with a low (39 nM) concentration of PLP and calculated as $((L1 - L3)/(H3 - L3)) \times 100\%$ [24].

2.6.9. Method comparison and accuracy

A method comparison study between the described LC–ESI-MS/MS method and the currently used HPLC method was carried out. This HPLC-method is based on extraction by TCA and derivatization of PLP with semicarbazide followed by a fluorometric detection [25].

120 whole blood samples for routine analysis of PLP were aliquoted 2-fold and stored at −20 °C until analyzed. Measurements were done in duplicates in 4 independent runs with both methods and the mean of the duplicates were plotted against each other. The new method was considered significantly different when both the slope and/or the intercept did not encompass 1 or 0, respectively and the observed difference was more than 5%.

The accuracy of the method was determined according the FDA guidelines [20]. Twenty different blood samples were spiked with three concentrations of PLP, 50 nmol/l, 100 nmol/l and 200 nmol/l and accuracy was expressed at each concentration as the

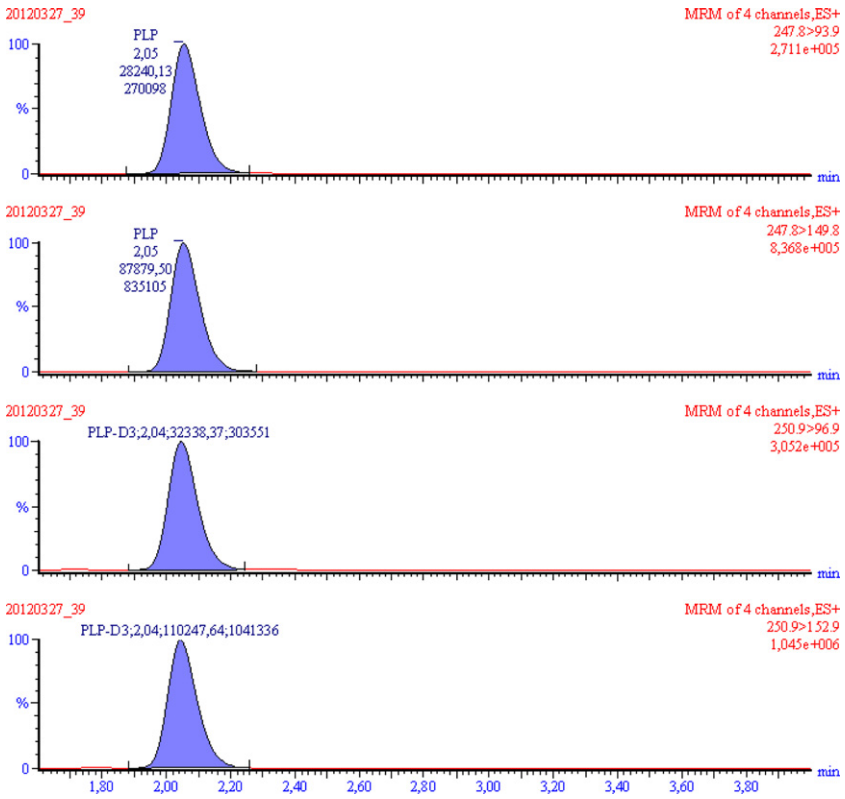

Fig. 2. Example of the chromatograms of a PLP measurement, showing the PLP-peaks (upper) and the PLP-d3 peaks (lower) for both mass transitions.

deviation between the mean measured PLP value and the true PLP value. The method was determined as being accurate when the difference between the measured value and true value was not larger than 15% at all tested concentrations.

2.7. Statistics

Quantification was performed using the peak area ratio of PLP to PLP-d3. Calibration lines were calculated using the Masslynx software. Linear extrapolation was used to quantify samples whose concentrations were below the lowest calibrator value. The LLOQ was calculated using the peak area ratio of PLP-d3 to PLP. Microsoft Excel® and Analyse-It were used to calculate linearity according the CLSI EP-6 criteria. Passing & Bablok method comparison with 95% confidence intervals and Bland & Altman method comparison with 95% limits of agreement were used for determining method agreement.

3. Results

To establish the appropriate mass spectrometric conditions, a standard solution of 5 μM PLP was directly infused into the MS at a flowrate of 10 μl/min. Collision induced dissociation (CID) of the protonated molecule was performed and the product ions giving the best signal to noise ratio were selected for SRM analyses. The mass transitions obtained in the positive ionization mode were m/z 247.8 > 149.8 and 247.8 > 93.9 for PLP, m/z 250.9 > 152.9 and 250.9 > 96.9 for PLP-d3. The first mass transition gave the better S/N ration for both PLP and PLP-d3 and was used as quantifier. The second transition was used as qualifier (Table 1). An example of an SRM-chromatogram is visualized in Fig. 2.

3.1. Linearity and LLOQ

The calibration curves were linear over the clinical range of 4–300 nmol/l (CLSI EP-6 lack-of-fit: 0.62) and over the analytical range of 4–8000 nmol/l (CLSI EP-6 lack-of-fit: 0.25). The linear correlation coefficients were $r^2 > 0.999$ for both calibration curves.

The lowest concentration where the CV% did not exceed 20% was at 2 nmol/l. However, the lowest concentration at which the response is at least ten times the blank response was 4 nmol/l (data not shown). Therefore, the LLOQ was specified at 4 nmol/l.

3.2. Precision and recovery

The inter- and intraday precisions are presented in Table 2. Interday variation expressed as CV% was determined at three different concentrations and ranged from 3.0% to 4.1%. Intraday variation of those samples ranged from 1.7% to 2.8%.

Recovery was calculated as the difference in PLP concentration between pre and post sample-preparation spiked samples. The mean recovery after correcting for endogenous PLP was 98% (range: 89–103%) and showed a CV of 3.6%.

Table 2
Summary of precision.

	Intra-day ($n = 20$)		Inter-day ($n = 20$)	
	Mean (nmol/l)	CV (%)	Mean (nmol/l)	CV (%)
PLP low	38.6	2.8	39.3	4.1
PLP medium	58.0	2.3	58.9	3.5
PLP high	101.4	1.7	102.6	3.0

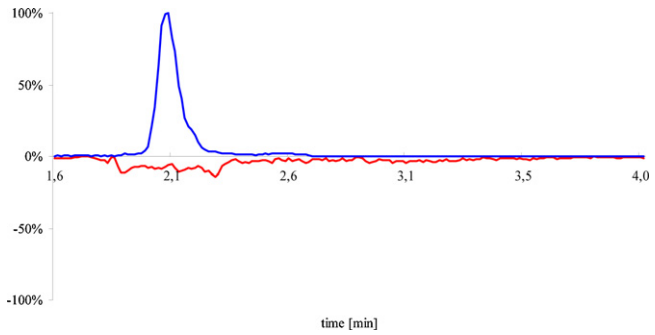

Fig. 3. Direct infusion chromatogram of PLP-d3 when a regular sample is injected (red chromatogram). A PLP peak is added to show the relevant retention time of the analyte (blue chromatogram). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Matrix effects

Two different types of matrix effects can occur. An absolute matrix effect can be defined as the difference in response between analyte spiked in sample matrix and the response of analyte spiked in water and can be quantified, in terms of percentage, as the agreement between the slopes of calibration lines made in matrix and water. A relative matrix-effect is described as the variation in response between different samples of the same matrix and can be expressed as the absolute matrix effect minus the CV% between the slopes of calibration lines made in different samples of the same matrix.

The mean absolute matrix effect of the PLP-assay was 103.6% (range: 93.4–114.8%) with a coefficient of variation of 5.3%, yielding a relative matrix effect of 100 − 5.3 = 94.7%. These calculations were done without internal standard correction. After recalculating the data with correction by the internal standard, the mean absolute matrix effect was 99.3% (range: 97.0–101.8%) with a coefficient of variation of 1.2% yielding a relative matrix effect of 100 − 1.2 = 98.8%. The maximum slope difference without internal standard correction was 22.9%. The maximum slope difference after internal standard correction was 4.9%.

The direct infusion method showed a loss of signal of approximately 10% of internal standard after a sample was injected (Fig. 3).

3.4. Interferences

Introduction of PMP as sample in the LC–ESI-MS/MS did not result in interfering peaks in the chromatogram of either PLP mass transition (Fig. 4). Also, after injection of 20 different PLP-d3 blank samples, no interfering peaks were observed that might influence PLP-d3 detection.

3.5. Stability and carry-over

We kept 5 different blood samples in daylight for 4 h directly after sample withdrawal and found no decline in PLP concentration when compared with the same samples that were stored in the dark (recovery: 100.2 ± 0.4%). The samples were also stored at different temperatures during different amounts of time whereby no loss of PLP was detected (Table 3). The stock standard of PLP was stable for at least 1 year at −80 °C. Prepared samples that were kept at 15 °C for 4 days also showed no decline in PLP concentration (recovery: 98.7 ± 1.9%). Freeze-thaw stability showed an increase in PLP concentration of 11.3% (±3.1%) after 3 unassisted cycles of freezing and thawing. This result was confirmed by a second independent experiment (increase 13.5 ± 6.2%). Two freeze/thaw cycles also showed an increase in PLP concentration (+8.1%), although this increase was not statistically significant. One freeze/thaw cycle showed PLP results that were comparable with freshly measured

Table 3
Whole blood PLP-stability after storage under various conditions.

Storage condition	Mean difference (%)	2SD (%)
4 h daylight	+0.2	0.4
1 week −20 °C	−1.7	5.0
6 weeks −80 °C	+0.0	6.2
6 months −80 °C	+2.5	5.6
1× freeze/thaw	+1.1	3.7
2× freeze/thaw	+8.1	10.6
3× freeze/thaw	+11.3	3.1
3× freeze/thaw repeat	+13.5	6.2
After sample prep.	−1.3	1.9

The mean difference: the difference of the PLP-results after storage under mentioned conditions compared with the PLP-results of the same samples freshly measured (n = 5).

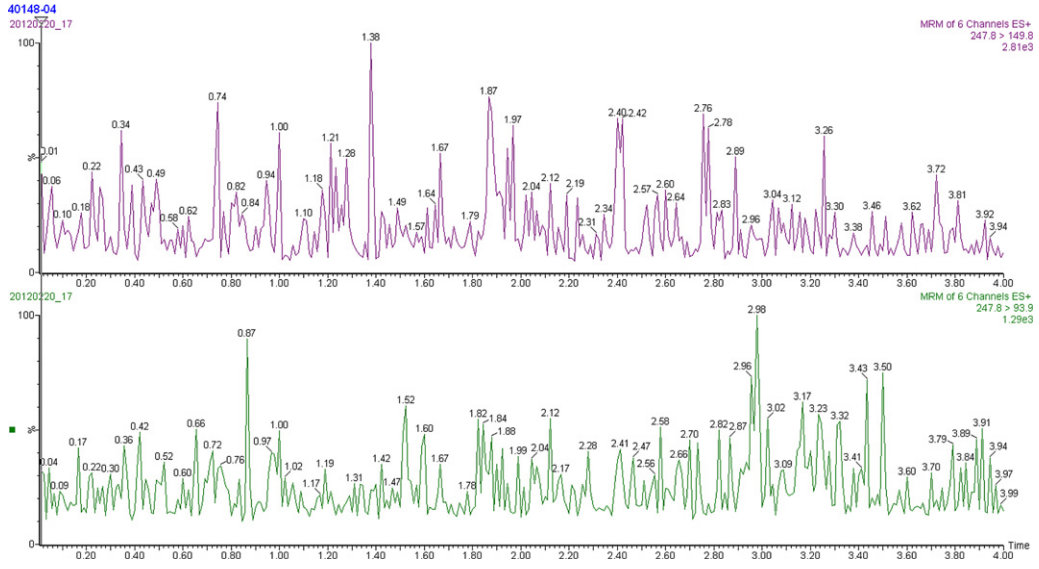

Fig. 4. Chromatogram monitoring PMP interference for both PLP mass transitions.

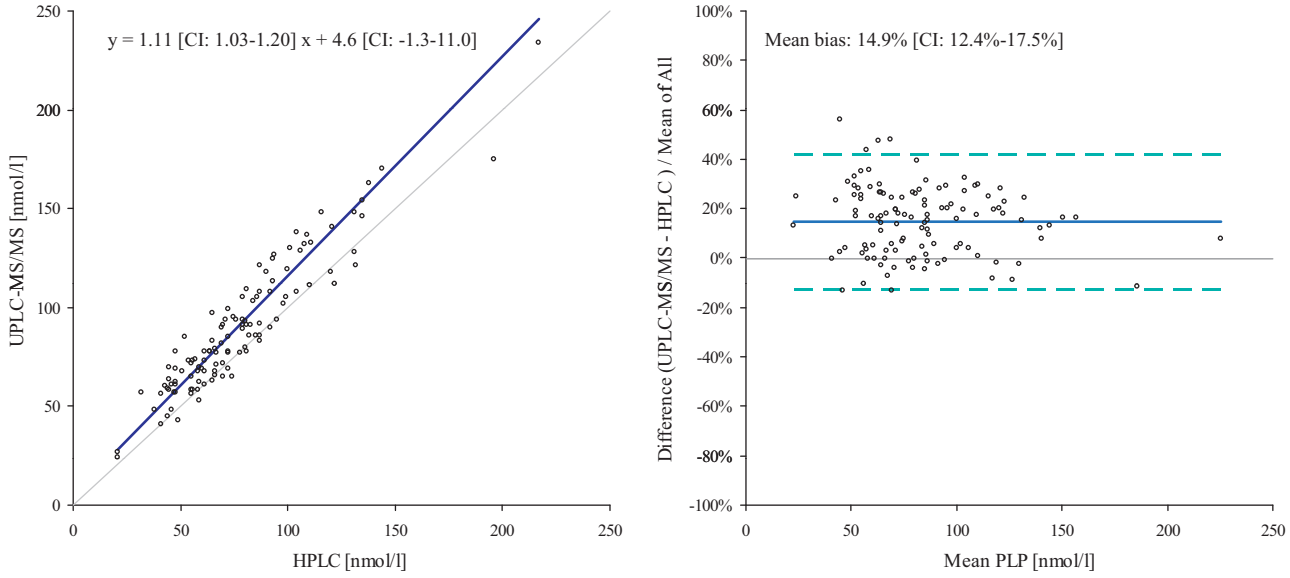

Fig. 5. Method comparison of the current HPLC-method with the newly developed LC–ESI-MS/MS-method. Left panel: method comparison by Passing & Bablok regression analysis. Dotted lines represent the 95% confidence interval. Right panel: Bland–Altman plot for agreement between both methods. The solid line represents bias (14.9%). The dotted lines represent the 95% limits of agreement (−12.4% to 42.3%).

Table 4
Summary of accuracy.

	True value (nmol/l)	Mean measured value (nmol/l)	Accuracy (%)	CV (%)
PLP low	50	49.5	98.9	1.7
PLP medium	100	98.7	98.7	1.9
PLP high	200	199.5	99.7	1.3

samples. Monitoring the release of PLP from its protein carriers was done by comparing the use of two different concentrations (10% and 20%) of TCA during sample pretreatment. The obtained results were comparable, yielding a small non-significant decline in result of 1.4% using 20% TCA.

The measured carry-over was 0.128%.

3.6. Method comparison and accuracy

The LC–ESI-MS/MS method was developed as potential replacement for the currently used HPLC-method [25]. The Passing & Bablok method comparison using patient samples yielded the following equation: LC–ESI-MS/MS = 1.11 [confidence interval, CI: 1.03–1.20] × HPLC + 4.6 [CI: −1.3 to 11.0]; $r^2 = 0.94$ (Fig. 5). The Bland–Altman agreement-plot showed a bias for the LC–ESI-MS/MS method of 14.9% [CI: 12.4–17.5%] with 95% limits of agreement between −12.4% and 42.3%.

The accuracy was expressed as the deviation between the mean measured value and the true value at three different concentrations and ranged from 98.7% to 99.7% (Table 4).

4. Discussion

We developed and validated the first stable isotope dilution LC–ESI-MS/MS method to measure vitamin B6 in whole blood. This method is characterized by higher sample throughput than traditional HPLC-based methods because of faster run times and simple sample preparation. Although the method is extensively validated for the use of heparin whole blood, separate matrix effect experiments showed that EDTA whole blood and plasma can also be used as long as patient samples and calibration curves have the same sample-matrix (data not shown). With this method, sample volumes as low as 50 μl can be used, which makes it suitable for pediatric use.

The newly developed method is linear from 4 to 8000 nmol/l. The clinical range of 4–300 nmol/l is applicable for most patients. However, in patients treated with pyridoxine or patients with a disturbed vitamin B6 metabolism like in hypophosphatasia [26], levels of PLP as high as 4000 nmol/l can be found. In the case of supplementation, strongly elevated concentrations of PLP are found within hours after pyridoxine intake in both red blood cells and plasma. When measuring PLP in these types of patients, an extended calibration range is needed. The choice of calibrator values can be easily determined if the patient population is known. During the last 2 years, no patients were found with PLP concentrations above the upper limit (8000 nmol/l) of the method.

The LLOQ was determined by use of whole blood spiked with different concentrations of PLP-d3. The FDA [20] prescribes that the experiment for determination of the LLOQ should be performed in the same biological matrix as the sample matrix. Under these recommendations, determination of the LLOQ for endogenous analytes like vitamin B6 poses the problem that the needed patient samples with a PLP-concentration near the LLOQ are often unavailable and as such an alternative strategy for determining the LLOQ is needed. We choose to use PLP-d3, which behaves identical to PLP in LC–MS/MS and is absent in patient samples, to determine the LLOQ with and use the whole blood endogenous PLP as internal standard. Comparing the found LLOQ (4 nmol/l) with those reported by others is difficult because (1) in most publications the needed information on how LLOQ was calculated is missing, (2) the LLOQ was calculated in various ways, and (3) different matrices were used to determine LLOQ. Comparison with the four other methods that determined LLOQ, similar values [4,27] or lower values [17,19] for LLOQ were found. Rybak et al. [17] used strongly diluted plasma as sample matrix and van der Ham et al. [19] used CSF as sample matrix for LLOQ determination. The cleanliness of those matrices, opposite to the whole blood matrix from our study, might explain their very low LLOQ.

The intra-day CV of <2.8% and inter-day CV of <4.1% for this method are equal to [11,15,17] or better than [4,9,10,12–14,18] other reported values for imprecision. The inter-day variance of the current HPLC method was 6.8% compared to 4.1% for the described LC–ESI-MS/MS method.

The recovery is a measure of the extraction efficiency and the most accurate way to determine recovery is by measuring the difference of concentration of analyte between pre sample preparation spiking and post sample preparation spiking of the same sample [23]. The mean recovery for our method was 98%. Most other published methods show similar recoveries [4,9–13,15–18,27].

The evaluation of matrix effects was performed according the recommendations of Matuszewski et al. [22,23]. Matrix effects can play a devastating role in LC–ESI-MS/MS analyses [28], and a thorough examination thereof is mandatory. Substances present in the sample matrix that co-elute with the analyte of interest are probably responsible for these matrix effects. The FDA prescribes in their guidance for industry [20] that during validation of LC–MS/MS methods the appropriate steps should be taken to minimize matrix effects. HPLC methods are also susceptible to matrix effects, although to a lesser extent than LC–MS/MS methods due to the traditional longer retention times and the more thorough sample preparation that are common with HPLC methods. To determine absolute and relative matrix effects, the slopes of 20 different matrix-matched calibration lines were compared with the slope of a calibration line in water. The observed absolute matrix effect of 103.6% and the relative matrix effect of 94.7%, both calculated without the use of an internal standard, indicate that matrix effects are hardly present. The introduction of a stable isotope as internal standard improved the absolute and relative matrix effects to 99.3% and 98.8%, respectively. In this case, matrix effects can be compensated for by the use of a stable isotope as internal standard and is highly recommended in general. The maximum slope difference is a different way of looking at a relative matrix effect. In this study, the maximum slope difference (without use of internal standard) was 22.9% indicating that when a QC sample with a concentration of 100 nmol/l would be analyzed, the result would range between 88.6 and 111.5 nmol/l. After correcting with the stable isotope, the maximum slope difference lowered to 4.9%, yielding concentrations between 97.5 and 102.5 nmol/l. This again proves the benefit of using a stable isotope. By only calculating the absolute and relative matrix effect, these differences are not directly evident and hence, determining the maximum slope difference proves another useful tool for measuring relative matrix effects. The direct infusion method is another useful manner of determining absolute matrix effects. The 10% loss of signal due to ion suppression with this method is comparable with the results of the absolute matrix effects using different calibration lines that showed a loss of signal of up to 7%.

PLP is known to be photosensitive and samples should be shielded from light when possible. The stability study showed that after exposure of the sample tube to natural light for 4 h (a time period during which blood tubes after sample withdrawal reach the laboratory from various outpatient departments), no loss of PLP could be detected in whole blood. This is in contrast to the results obtained by Talwar [4] who found a significant loss of 13% after keeping the samples in natural light for 2 h. An explanation for this discrepancy in PLP loss is hard to find. The only difference between our study and Talwar's study is that Talwar studied PLP stability in red blood cells, where in our study whole blood was investigated. To absolutely clarify this finding, further research is necessary. After keeping whole blood samples at −20 °C for 1 week or at −80 °C for up to 6 months, no decline in PLP concentration was observed. This is in concordance with reports by other authors [4,11,27]. Also, after keeping the prepared samples at 15 °C in the autosampler for 4 days, no change in PLP concentration was found. This finding makes it possible to re-measure a series of samples in cases of technical malfunction. The freeze/thaw stability experiment produced results that showed a gain in PLP concentration of 11% after 3 freeze/thaw cycles. Similar experiments done by others [17,27] showed stable PLP concentrations even after 5 freeze/thaw cycles, although those experiments were done in plasma and not in whole blood. Our finding was unexpected and for that reason replicated with the same result. Two freeze/thaw cycles showed a non significant gain in PLP concentration of 8%, but with a clear trend toward increasing levels. An explanation for the increase in PLP concentration is hard to find. A potential non quantitative release of PLP from its binders during sample pretreatment seems unlikely as it was demonstrated that higher concentrations of TCA as releasing agent during sample preparation did not result in higher PLP results. Further investigation is necessary to elucidate this finding. With this method it is advisable to measure only freshly prepared samples or samples that underwent only one freeze/thaw cycle.

Carry-over was found to be 0.128%. The practical consequences are that a patient result gathered after measuring a sample with a very high concentration of PLP will be slightly elevated and as such the sample should be re-measured to correct the carry-over effect and obtain a reliable PLP-result.

To determine the accuracy of a method, a reference material or a reference technique or reference method is needed. None of those exist for the measurement of whole blood PLP. Therefore, we applied the method of accuracy determination as recommended by the FDA and found accuracy close to 100% for each concentration tested. Furthermore we compared our new LC–ESI-MS/MS method with the existing HPLC method that showed excellent correlation although a small but significant proportional bias of 11% was observed. During method validation no interferences in detecting PLP or PLP-d3 were witnessed that could account for this bias. The reference values were adapted according the Passing & Bablok method equation (35–110 nmol/l) and verified using Bhattacharya analysis on patient results measured with the LC–ESI-MS/MS method. Other researchers have also noticed differences between PLP-methods [29]. Future investigation will be conducted at our department in which the traceability of PLP-calibrators and PLP-methods will be explored.

5. Conclusions

This isotope dilution LC–ESI-MS/MS-method for determining the concentration of PLP in heparin whole blood is characterized by short run times and simple sample preparation. The method also showed excellent linearity, precision, accuracy, recovery and absence of significant matrix effects, where all these tested parameters met the applied acceptance criteria.

References

[1] J. Leklem, in: R. Rucker (Ed.), Handbook of Vitamins, 3rd ed., Marcel Dekker, New York, 2001, p. 339.
[2] S. Friso, D. Girelli, N. Martinelli, O. Olivieri, V. Lotto, C. Bozzini, F. Pizzolo, G. Faccini, F. Beltrame, R. Corrocher, Am. J. Clin. Nutr. 79 (2004) 992.
[3] P.J. Kelly, V.E. Shih, J.P. Kistler, M. Barron, H. Lee, R. Mandell, K.L. Furie, Stroke 34 (2003) e51.
[4] D. Talwar, T. Quasim, D.C. McMillan, J. Kinsella, C. Williamson, D.S. O'Reilly, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 792 (2003) 333.
[5] T. Quasim, D.C. McMillan, D. Talwar, A. Vasilaki, J.O.R.D. St, J. Kinsella, Clin. Nutr. 24 (2005) 956.
[6] D. Talwar, T. Quasim, D.C. McMillan, J. Kinsella, C. Williamson, D.S. O'Reilly, Clin. Chem. 49 (2003) 515.
[7] A.T. Vasilaki, D.C. McMillan, J. Kinsella, A. Duncan, D.S. O'Reilly, D. Talwar, Am. J. Clin. Nutr. 88 (2008) 140.
[8] A. Shenkin, Nutrition 13 (1997) 825.
[9] Q. Han, M. Xu, L. Tang, X. Tan, Y. Tan, R.M. Hoffman, Clin. Chem. 48 (2002) 1560.
[10] T.R. Guilarte, P.A. McIntyre, J. Nutr. 111 (1981) 1861.
[11] C.J. Bates, K.D. Pentieva, N. Matthews, A. Macdonald, Clin. Chim. Acta 280 (1999) 101.
[12] M.R. Bisp, M.V. Bor, E.M. Heinsvig, M.A. Kall, E. Nexo, Anal. Biochem. 305 (2002) 82.
[13] K.L. Ericson, J.D. Mahuren, Y.M. Zubovic, S.P. Coburn, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 823 (2005) 218.
[14] J.F. Gregory III, Anal. Biochem. 102 (1980) 374.
[15] K. Johansen, P.O. Edlund, J. Chromatogr. 506 (1990) 471.

[16] M. Kimura, K. Kanehira, K. Yokoi, J. Chromatogr. A 722 (1996) 295.
[17] M.E. Rybak, C.M. Pfeiffer, Anal. Biochem. 333 (2004) 336.
[18] O. Midttun, S. Hustad, E. Solheim, J. Schneede, P.M. Ueland, Clin. Chem. 51 (2005) 1206.
[19] M. van der Ham, M. Albersen, T.J. de Koning, G. Visser, A. Middendorp, M. Bosma, N.M. Verhoeven-Duif, M.G. de Sain-van der Velden, Anal. Chim. Acta 712 (2012) 108.
[20] FDA, Guidance for industry. Bioanalytical method validation, U.S. Department of Health and Human Services, 2001. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf.
[21] Evaluation of the Linearity of Quantitative Analytical Methods, NCCLS Document EP6-P, 1986, vol. 6, no. 18.
[22] B.K. Matuszewski, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 830 (2006) 293.
[23] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[24] P.M. Broughton, J. Autom. Chem. 6 (1984) 94.
[25] J. Schrijver, A.J. Speek, W.H. Schreurs, Int. J. Vitam. Nutr. Res. 51 (1981) 216.
[26] M.P. Whyte, J.D. Mahuren, L.A. Vrabel, S.P. Coburn, J. Clin. Invest. 76 (1985) 752.
[27] M.L. Marszall, A. Lebiedzinska, W. Czarnowski, R. Makarowski, M. Klos, P. Szefer, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 3151.
[28] M. Vogeser, C. Seger, Clin. Chem. 56 (2010) 1234.
[29] M.E. Rybak, R.B. Jain, C.M. Pfeiffer, Clin. Chem. 51 (2005) 1223.