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Simultaneous measurement of whole blood vitamin B1 and vitamin B6 using LC-ESI–MS/MS

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ABSTRACT

Vitamin B1 and B6 have recently been included in the Dutch clinical guidelines for the general practitioner in the differential diagnosis of dementia. To keep up with the sharp rise in the number of requests, an LC–MS/MS method using stable isotopes as internal standards was developed. The active vitamers thiamine pyrophosphate (TPP) and pyridoxal-5′-phosphate (PLP) in whole blood are simultaneously measured with a short run time of 2 min. Whole blood is mixed with internal standard solution containing both TPP-d3 and PLP-d3, followed by deproteinization with a trichloroacetic acid (TCA) solution. A UPLC–MS/MS system from Waters™ was used for chromatographic separation and subsequent detection by electrospray ionization in the positive mode with mass transitions of 425.1 > 121.85 for TPP and 247.9 > 149.9 for PLP. The method is linear across the range of 12–4870 nmol/L for TPP and 6–4850 nmol/L for PLP. The mean intra-assay and inter-assay precision are 3.5% and 7.6% respectively for TPP and 3.4% and 6.1% for PLP. The relative matrix effect (TPP 97%, PLP 93%), recovery (TPP 99%, PLP 94%) and lower limit of quantification (TPP 12 nmol/L, PLP 6 nmol/L) meet the applied acceptance criteria. The comparison of the new LC-ESI–MS/MS method for TPP with our current HPLC-Fluorescence method for total thiamine yields the following equation: TPP LC–MS/MS = 0.97 × total thiamine HPLC – 10.61 (r^2 = 0.94). The comparison of the new LC-ESI–MS/MS method for PLP with our current LC-ESI–MS/MS method results in PLP LC–MS/MS new = 1.01 × PLP LC–MS/MS old – 1.58 (r^2 = 0.99). In conclusion, this LC–MS/MS based assay is characterized by simple sample processing with a short run time and comparison with the current methods is excellent. The new LC–MS/MS method is a convenient method to determine TPP and PLP in whole blood for both clinical routine and research applications.

1. Introduction

Both vitamin B1 and B6 are present in the human body as several vitamers. Thiamine and its mono, di and triphosphate esters are collectively called vitamin B1, with thiamine pyrophosphate (TPP, the diphosphate) being the biologically active form [1]. Vitamin B6 consists of six interconvertible forms: the alcohol pyridoxine (PN), the aldehyde pyridoxal (PL), the amine pyridoxamine (PM), their corresponding 5′-phosphate derivatives and the degradation product pyridoxic acid (PA). Pyridoxal-5′-phosphate (PLP) is the main metabolically active vitamer [2]. TPP and PLP act as cofactors in numerous enzymatic reactions, mainly in carbohydrate and amino acid metabolism. Vitamin B1 deficiency causes the clinical phenotypes of beriberi and Wernicke-Korsakoff syndrome. Vitamin B6 deficiency as well as an excess of this vitamin can cause neurological symptoms [3,4].

It is a subject of discussion how vitamin status in the human body is best assessed with respect to vitamer, method and matrix. Roughly 80% of vitamin B1 is present in red cells, predominately in the form of TPP. The plasma content consists mostly of unphosphorylated thiamine in low concentrations, making this less precise to measure [5,6]. The concentration of TPP in erythrocytes has been shown to be a good indicator of body stores, because it depletes at a rate similar to those of other major organs [7]. Talwar et al. demonstrated that the TPP concentration in whole blood correlates well with that in washed erythrocytes, and thus is a suitable matrix for vitamin B1 assessment [8].

Vitamin B6 is present in plasma mainly as PLP and PL, with no significant amounts of PN and PM present in peripheral plasma with normal vitamin B6 intake [9]. In red cells PLP and PMP predominate, of

Abbreviations: TPP, thiamine pyrophosphate; PLP, pyridoxal-5′-phosphate; PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine; PA, pyridoxic acid; FA, formic acid; TCA, trichloroacetic acid; HCl, hydrochloric acid; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry

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the total amount of PLP in whole blood, about 60% is present in erythrocytes [10]. There is debate whether measurement of a sole vitamer is sufficient to establish the nutritional status of a patient [11]. Primarily, PLP is determined, but complementary indices of vitamin B6 status may be PA in urine or plasma, PL and functional vitamin B6 biomarkers [12]. In healthy people, either plasma or red cell concentrations of PLP can be used as markers of vitamin B6 status, because they are highly correlated. However, in critically ill patients a shift of PLP from plasma to red cells has been observed, implying that whole blood may be a more reliable matrix [13].

Since the determination of vitamin B1 and B6 have been included in the Dutch clinical guidelines for the general practitioner in the differential diagnosis of dementia, the number of analyses has risen sharply and they are mainly requested in combination [14]. Consequently, it became desirable to measure both vitamins with a short run time in a combined analysis. Currently, this is mainly done using HPLC techniques with fluorescence detection, yet the first LC–MS/MS method to simultaneously quantify TPP and PLP has been described by, as it happens, a Dutch laboratory [15]. Commercial companies also anticipated on this demand, as Phenomenex recently published a comparable method on their website [16] and also Shimadzu has an LC–MS/MS based method available [17].

The biggest advantage of an LC–MS/MS method is the simple work up, only protein precipitation under acidic conditions suffices before the sample can be injected. This is in sharp contrast to HPLC methods, where derivatization of both molecules is required because of the lack of a fluorophore in their chemical structures (see Fig. 1) [8,13].

However, it is still challenging to transfer an existing LC–MS/MS method to another system without additional optimization. The choice of column, mobile phase, gradient and MS-settings are very delicate matters and often it comes down to redeveloping the method for the new setup [18,19]. Furthermore, every laboratory has its own routines and preferences, mostly dictated by other tests that are run on the same MS/MS system. It is therefore useful to demonstrate that the analysis of TPP and PLP is also possible under different conditions. The previously described LC–MS/MS methods for the combined determination of TPP and PLP were based on alkaline conditions using a high end mass spectrometer [15,16]. In this paper, we show that it is also possible to use acidic conditions and an entry level mass spectrometer to quantify these vitamers, making the implementation of this test in a routine clinical laboratory more versatile.

2. Material and methods

2.1. Materials

PLP and TPP were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). The internal standards, TPP-d3 and PLP-d3, were 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 obtained from Biosolve B.V. (Valkenswaard, The Netherlands).

The blood samples used for the development and validation of the method were residual specimens from daily routine analyses (stored at −20 °C) in our laboratory, which were suitably anonymized. In agreement with the Erasmus MC Code of Conduct for responsible use, no permission from the Ethical Committee for the use of anonymous leftover samples is required. Patients do have the option to declare a no-cooperation statement for this procedure.

Quality control blood was obtained from a healthy donor from the Bloodbank (Sanquin Blood Supply Foundation, Amsterdam, the Netherlands), which was aliquoted and stored at −80 °C.

2.2. Standard preparation

Stock standard solutions of TPP and PLP were each prepared at 750 µmol/L in 0.1 mol/L HCl. Stock standard solutions of the internal standards TPP-d3 and PLP-d3 were separately prepared at 30 µmol/L in 0.1 mol/L HCl. All stock standards were aliquoted and stored at −80 °C. A working standard of 7.50 µmol/L TPP and PLP was obtained by further diluting the respective stock standards with water. It was made freshly for each new series of measurements and was used immediately after preparation. An internal standard working solution of 600 nmol/L was obtained by further diluting TPP-d3 and PLP-d3 with 0.1 mol/L HCl. After sample preparation, the remainder of the TPP-d3 and 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 TPP and PLP working solution was further diluted with 960 µL EDTA whole blood of patients with low vitamin B1 and B6 levels. Spiked calibration standards at six different levels of TPP and PLP standard were obtained by 1:1 serial dilution with the same EDTA whole blood, ranging from 0 to 300 nmol/L. Commonly, the samples with the lowest values in the previous run are kept to be used for the preparation of the calibration standards for the next run. After the measurements, adjustments were made for the endogenous amount of TPP and PLP, which was calculated as the ratio of intercept to slope from the calibration line $y = ax + b$.

2.4. Sample preparation

250 µL EDTA whole blood, calibrator or QC was mixed with 250 µL of the internal standard solution containing both TPP-d3 and PLP-d3. Protein precipitation took place by the dropwise addition of 2000 µL of a 10% (w/v) TCA solution, while mixing on a vortex. The sample was left standing at room temperature, and again mixed after 30 and 60 min. Following this, the sample was centrifuged at 21250 RCF/21,380*g* for 7 min at RT. The supernatant was transferred into a clean vial and placed in the autosampler at 15 °C.

2.5. LC-ESI–MS/MS

Chromatography was performed using an ACQUITY UPLC® system (Waters Corporation, Etten-Leur, The Netherlands). Separation was achieved by a 20 µL full loop injection of the prepared sample on a Symmetry C18 column (2.1 mm × 100 mm, 3.5 µm) with a column temperature of 30 °C. A gradient elution utilizing 0.1% FA in water as solvent A and 0.1% FA in methanol as solvent B was performed, having a varying flow rate, non-linear gradient steps and a total run time of

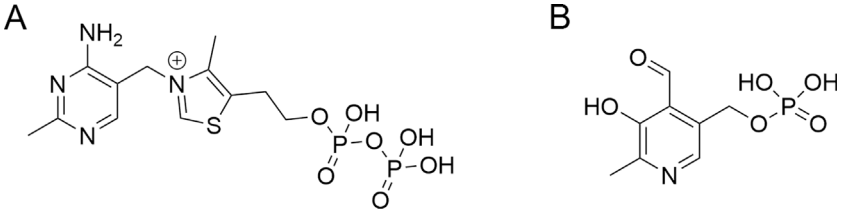

Fig. 1. Molecular structures of (A) TPP and (B) PLP.

Table 1
MS/MS conditions for the single reaction monitoring of TPP, TPP-d3, PLP and PLP-d3.

Molecule	Parent ion (*m/z*)	Daughter ion (*m/z*)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
TPP	425.1	121.85	0.025	28	25
	425.1	303.9	0.025	28	19
TPP-d3	428.1	124.85	0.025	28	25
PLP	247.9	149.9	0.025	20	18
	247.9	93.8	0.025	20	28
PLP-d3	250.9	152.9	0.025	20	18

1.90 min. The gradient was as follows: 0 min (97%A and 3%B, 0.50 mL/min, curve initial), 0.96 min (70%A and 30%B, 0.50 mL/min, curve 6), 1.44 min (3%A and 97%B, 0.80 mL/min, curve 11), 1.70 min (97%A and 3%B, 0.80 mL/min, curve 11) and 1.90 min (97%A and 3% B, 0.50 mL/min, curve 11).

Mass spectrometry was performed using a Quattro Premier XE (Waters Corporation, Etten-Leur, The Netherlands) tandem mass spectrometer. TPP and PLP were measured by electrospray ionization (ESI) in positive ionization mode with the following selected reaction monitoring mass transitions: *m/z* 425.1 > 121.85 and 425.1 > 303.9 for TPP, *m/z* 428.1 > 124.85 for TPP-d3, *m/z* 247.9 > 149.9 and 247.9 > 93.8 for PLP and *m/z* 250.9 > 152.9 for PLP-d3 (Table 1). Other mass spectrometer settings were: capillary voltage 3.0 kV, cone voltage 28 V for TPP and 20 V for PLP, desolvation temperature 350 °C at a gasflow of 1000 L/h and cone gasflow 50 L/h. Argon was used as the collision gas at a flowrate of 0.20 mL/min. Detection took place between 0.90 and 1.44 min, but flow was otherwise set to waste.

2.6. Method validation

2.6.1. Linearity

The analytical linearity of the method was determined by spiking whole blood with 10 different concentrations of TPP and PLP ranging from 0 to 4800 nmol/L, and subsequent correction of the endogenous values. All samples were injected five times. Linearity was assessed by application of the 'lack-of-fit' model as described in the CLSI EP-6 guidelines [20]. 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 TPP and PLP concentrations were not available, TPP-d3 and PLP-d3, which are absent in patient samples, were used to determine the lower limit of quantification (LLOQ). The deuterated compounds have a similar analytical response as their undeuterated counterparts (data not shown). Whole blood was spiked with different concentrations of TPP-d3, ranging from 0 to 32 nmol/L and PLP-d3, ranging from 0 to 6 nmol/L and injected 10-fold. The lowest concentration of deuterated internal standard 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 of the respective deuterated vitamin response of twenty different whole blood samples.

2.6.3. Precision

The precision of the method was determined using whole blood sample pools (aliquoted and stored at −80 °C) at two different vitamer concentrations, 102 and 160 nmol/L for TPP and 71 and 181 nmol/L for PLP, corresponding to concentrations in the middle and just above the normal ranges. Unfortunately, sample pools with vitamer concentrations closer to the LLOQ could not be obtained due to the lack of patients that were severely deficient for both TPP and PLP. The samples were measured 5-fold on ten consecutive working days to assess inter-day precision. The intra-day precision was determined by measuring the samples 20-fold in one run. Inter-day and intra-day precisions were expressed as coefficients of variation (%CV). The FDA's acceptance criteria for precision (%CV < 15) were applied [21].

2.6.4. Recovery

The sample extraction recovery was determined by spiking two aliquots of twenty different blood samples with 100 nmol/L of both vitamers. 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 vitamin concentrations. The recovery was calculated as the difference in vitamin concentration between pre-sample preparation spiking and post-sample preparation spiking after correcting for endogenous vitamin concentration [22]:

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

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, 100 nmol/L, 200 nmol/L, and 400 nmol/L) of TPP and PLP and subsequent calculation of the slope and intercept values. 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 [22]:

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

The relative matrix effect was calculated as 100% – CV% of the slopes of the twenty whole blood calibration lines. When using the internal standard as correction, a relative matrix effect of 100 ± 10% was used as a cut-off value for the method to be acceptable [22].

2.6.6. Carry-over

Carry-over was determined by triplicate injections of a sample with a high concentration of TPP and PLP (~5000 nM) followed by triplicate measurements of a sample with a low (~100 nM) concentration of TPP and PLP and calculated as ((L1 − L3)/(H3 − L3)) × 100%.

2.6.7. Method comparison

A method comparison study between the LC–MS/MS method for the simultaneous quantification of TPP and PLP and the currently used methods for the separate determinations of vitamin B1 and B6 was carried out. The current vitamin B1 method comprises the measurement of total thiamine with HPLC after extraction by TCA, enzymatic hydrolysis by acid phosphatase, post-column derivatization and fluorometric detection of thiochrome [5,23]. PLP is already measured with an in-house developed LC–MS/MS method [24].

Whole blood samples for routine analysis (n = 30 for TPP and n = 48 for PLP) were aliquoted in duplicate and stored at −20 °C until analysis by both methods. Measurements were taken 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%.

2.7. Statistics

Quantification was performed using the peak area ratio of the respective vitamers to their internal standards. Calibration lines were calculated using MassLynx™ software version 4.1 (Waters Corporation, Etten-Leur, The Netherlands). 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 TPP-d3 to TPP and PLP-d3 to PLP. Microsoft Excel® and Analyse-It (software package 2.30) were used to calculate linearity according the CLSI EP-6 criteria. Passing & Bablok method comparison with 95% confidence intervals was used for determining method agreement and a% difference plot to determine the total bias.

3. Results and discussion

3.1. Calibrator preparation

Quantification of endogenous compounds in whole blood represents a challenge for the preparation of calibration standards. The main drawback of using whole blood is that linear extrapolation has to be used to quantify patient samples with TPP and PLP concentrations below the lowest calibration standard. On the other hand, use of water or a surrogate matrix instead of whole blood may result in a high matrix effect and inaccuracy in LC–MS/MS assay performance. Since whole blood with vitamin B1 and B6 levels around or below the lower limit of the respective reference intervals is used for calibrator preparation, linear extrapolation is only necessary for (nearly) deficient patients, which should be treated anyway.

3.2. Sample preparation

The procedure to prepare the samples for analysis is simple. Regular vortexing during precipitation is required for complete protein removal. It is also possible to scale down the volume of patient material, i.e. 50 μL EDTA whole blood instead of 250 μL and adjust the amounts of internal standards and TCA solution accordingly. For logistical reasons only the latter was validated.

3.3. LC-ESI–MS/MS

A Symmetry C18 column was used for the chromatographic separation of TPP and PLP. Although this column is not specifically designed for UPLC applications, it previously has been successful in the quantification of PLP alone and was therefore selected for this method too [24]. At least 15 injections were necessary to prime the column and the rest of the system, before Gaussian peak shapes without tailing were obtained. This may be caused by the presence of phosphate groups in the analytes, which are known to react with iron parts within the LC–MS system [25]. Furthermore, they may interact with free silanol groups present in a separation column, which results in peak tailing to at least some extent, dependent on pH of the mobile phase [26].

Regular acidic conditions (0.1% FA in water and methanol) were applied to achieve separation. Since these eluents are also used for other assays in our laboratory, it is a convenient choice for routine diagnostics. The application of acidic conditions is in contrast to previous publications, in which NH_4HCO_3 was used as aqueous buffer, resulting in highly alkaline conditions [15,16]. In our experience, the LC–MS/MS probe suffers from the formation of CO_2. Moreover, a column with extreme pH resistance is required to withstand such harsh conditions.

The method is fast, with a total run time of less than 2 min and a retention time of 1.11 min for TPP and 1.26 min for PLP (see Fig. 2). To realize this rapid separation, a relatively high flowrate of 0,5 mL/min was applied during mass spectrometric detection. Only after the LC flow was set to waste, to prevent oversupply of the ion source, a flowrate of 0.8 mL/min was applied to quickly wash and regenerate the column conditions.

The appropriate mass spectrometric conditions were established by direct infusion of a standard solution containing TPP and PLP (see Table 1). Collision induced dissociation of the protonated molecules resulted in daughter ions with m/z 121.85 and 303.9 for TPP and m/z 149.9 and 93.8 for PLP. The mass transition m/z 425.1 > 121.85 gave the better S/N ratio for TPP and was therefore used as the quantifier ion, for PLP this was m/z 247.9 > 149.9. The other mass transitions were used as the qualifier ions. For the deuterated molecules, no qualifier ions were measured, due to the narrow peaks and short run time, which makes the amount of possible measuring points during the elution of the peaks too limited. It is worth mentioning, that at a cone voltage of 35 V, which was initially used for PLP, an interfering peak was observed with the same mass as PLP and having the same fragment ion as the quantifier ion (data not shown). At a lower cone voltage of 20 V this phenomenon was not observed, where the cause of the peak could be due to in source fragmentation of a larger molecule at higher cone voltages.

3.4. Linearity and LLOQ

The calibration curves were linear over the analytical range of 12–4870 nmol/L as deduced from a lack of fit of 0.19 for TPP and 6–4850 nmol/L for PLP [20]. The mean linear correlation coefficients were $r^2 > 0.994$ for both calibration curves.

The LLOQ for TPP and PLP were 12 and 6 nmol/L respectively, determined from the concentrations at which the CV did not exceed 20%. The lowest concentration at which the response was at least ten times that of the blank was 4 nmol/L for both vitamers (Fig. 3).

With these characteristics, the clinically relevant ranges for TPP and PLP are covered. The LLOQs are well below the borders that define deficiency and even the exceptionally high values of PLP that can be found in hypophosphatasia patients are usually not higher than the upper limit of the analytical range measured [27]. TPP appears to accumulate to a much lesser extent, as patients with Leigh syndrome that receive large amounts of thiamine (100–900 mg/day), hardly ever reach TPP values above 500 nmol/L [28].

3.5. Precision

Inter- and intra-day precision determinations are presented in Table 2. Inter-day variation expressed as CV was determined at two different concentrations for each vitamer and ranged from 6.2% to 8.9% for TPP and 7.7% to 4.6% for PLP. Intra-day variation of those samples ranged from 2.7% to 4.3% and 3.5% to 3.3%, respectively. All coefficients of variation fall within the acceptance criteria of < 15% CV and are equal to or better than the %CV's determined by other simultaneous measurements of TPP and PLP by LC–MS/MS [15–17]. However, compared to our LC–MS/MS method for the measurement of PLP alone, the precision observed in this method for the combined analysis of TPP and PLP is not as good. This is probably caused by the analytical concessions that had to be made in developing a method for two molecules with opposite pH requirements for good separation within an extremely short total run time.

3.6. Recovery and matrix effects

Recovery was calculated as the difference in vitamer concentration between pre and post sample-preparation spiked samples. The mean recovery after correcting for endogenous TPP was 99% (range: 93–107%) and showed a CV of 3.8%. For PLP the mean recovery after correction was 94% (range: 86–101%) and showed a CV of 4.1%. By calculating recovery in this way, only the influence of different whole blood matrices on the sample preparation is established and the matrix effect on ionisation is left aside.

The matrix effect on ionisation was established separately using the analyte:internal standard response correction to determine the absolute and relative effect. The absolute matrix effect is a direct comparison of the MS/MS response of TPP and PLP in water versus a whole blood extract which may contain compounds that result in ionisation suppression or enhancement. The relative matrix effect is the variability in

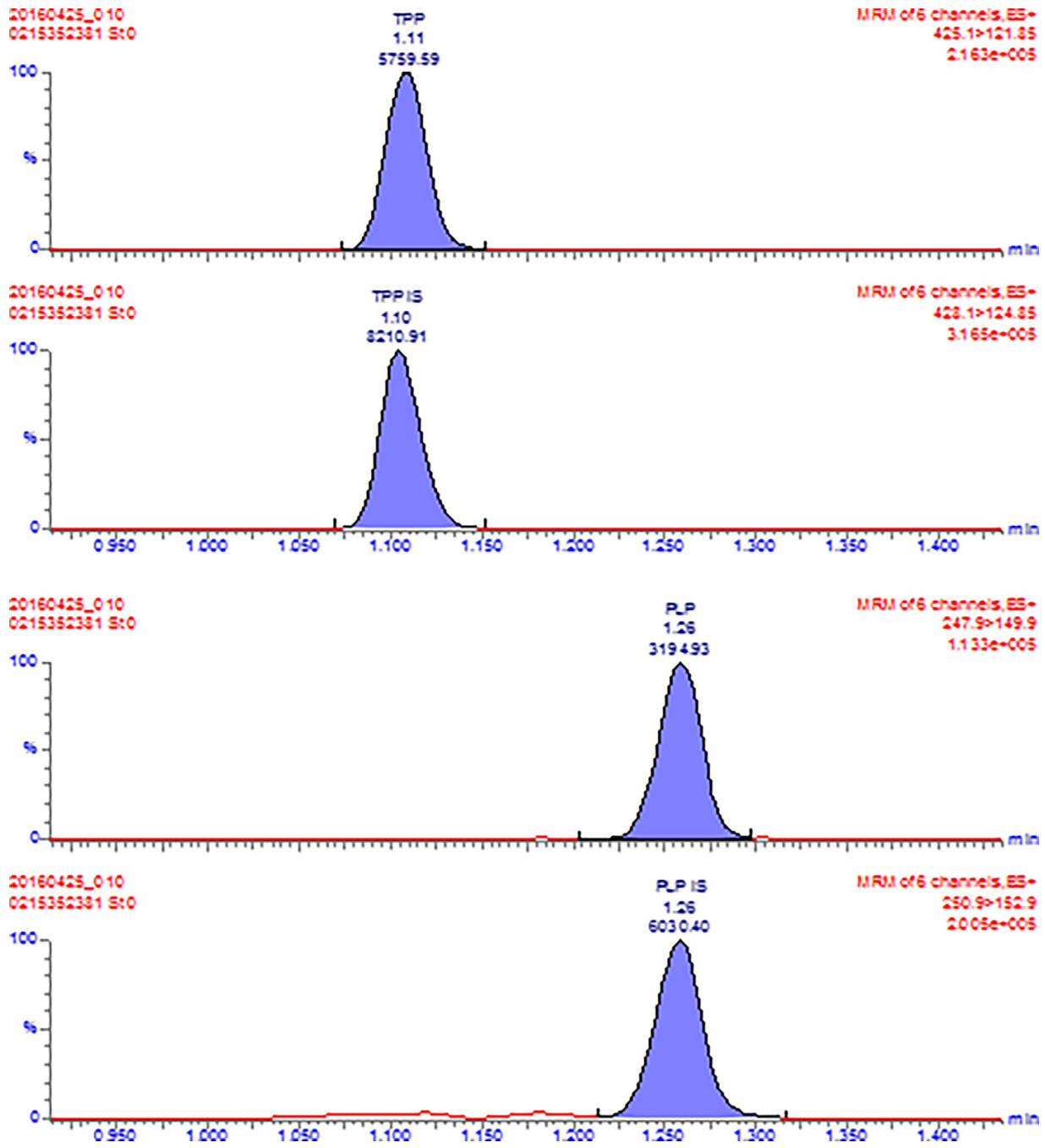

Fig. 2. Example of the chromatograms, showing the TPP and TPP-d3 peaks (upper) and the PLP and PLP-d3 peaks (lower) for the mass transitions of the respective quantifier ions at the lowest concentration of the calibration curve.

response between different samples of the same matrix (whole blood) originating from different subjects. This is the most meaningful matrix effect, since routine analysis is always performed in different whole blood patient samples and not in surrogate solutions. The mean absolute matrix effect of the TPP-assay was 107% (range: 102–114%) with a coefficient of variation of 3.3%, yielding a relative matrix effect of 100–3.3 = 96.7%. For PLP the mean absolute matrix effect was 101% (range: 87–115%) with a coefficient of variation of 6.8%, yielding a relative matrix effect of 100–6.8 = 93.2%. The necessity of the internal standards became evident when calculating the absolute and relative matrix effects using analyte area alone without correction. These values showed the occurrence of ion suppression for TPP (absolute 72%, relative 89.2%) and to a greater degree for PLP (absolute 26%, relative 79.3%). However, the matrix effect for the respective internal standards alone showed the same pattern, producing relative matrix effects for both TPP and PLP that were smaller than our acceptance criteria of 10%, which is acceptable for vitamins. Puts and coworkers did investigate the matrix effect on their method. Unfortunately, however, their simplistic approach does not allow a quantitative expression of the matrix effect, making it difficult to directly compare the results. Given that a 1:1 dilution of whole blood samples with water gives increased recoveries of 120% for TPP and 110% for PLP, this would indicate a matrix effect is observed [15].

3.7. Carry-over

Carry-over for TPP was calculated to be 0.04% and 0.12% for PLP, so no significant carry-over of material was observed.

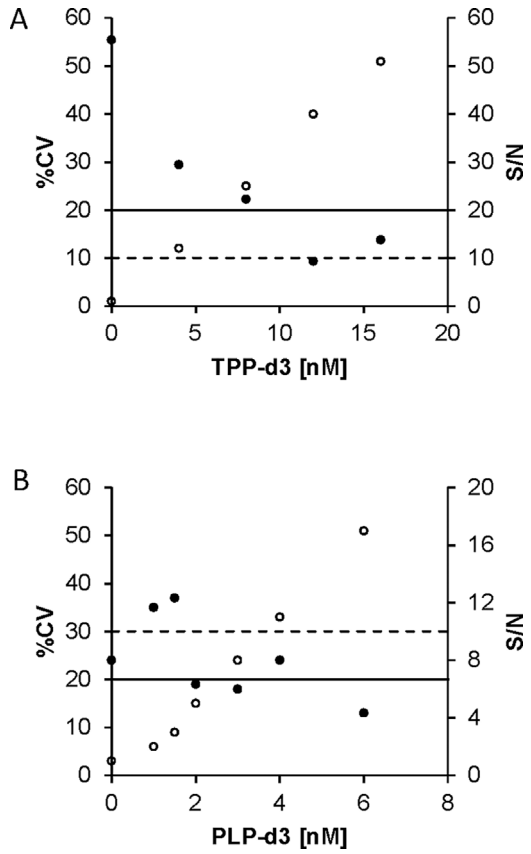

Fig. 3. Lower limit of quantification of (A) TPP and (B) PLP. Closed circles and solid line represent %CV and its criterion of ≤20%, open circles and dashed line represent S/N ratio and its criterion of ≥10x.

Table 2
Summary of precision.

	Intra-day (n = 20)		Inter-day (n = 10)	
	Mean (nmol/L)	CV (%)	Mean (nmol/L)	CV (%)
TPP medium	96	2.7	102	6.2
TPP high	155	4.3	160	8.9
PLP medium	68	3.5	71	7.7
PLP high	175	3.3	181	4.6

3.8. Method comparison

The Passing & Bablok method comparison using patient samples yielded the following equation for vitamin B1: TPP LC–MS/MS = 0.97 [0.86-1.10] × total thiamine HPLC − 10.61 [−27,77 − 2,70] (r^2 = 0.94). The LC–MS/MS method in which TPP is determined showed a negative total bias of −12.9%, compared to the HPLC method that measures total thiamine (Fig. 4). This bias is in accordance with our expectations, since about 85–90% of the total amount of thiamine in whole blood is present in the form of TPP [29]. When the reference interval for total thiamine (70–140 nmol/L) is adapted to obtain a reference interval for TPP (61–122 nmol/L) using this factor of 0.87, all individual patients get the same diagnosis in terms of having sufficient vitamin B1 (27 out of 30) or a level above the upper limit of normal (3 out of 30), independent of the method used. For vitamin B6, the Passing & Bablok equation is: PLP LC–MS/MS new = 1.01 [0.98–1.04] × PLP LC–MS/MS old – 1.58 [−4.04 to 0.67] (r^2 = 0.99) and the total bias is −1.0% (Fig. 4), meaning that the correlation of both methods is excellent.

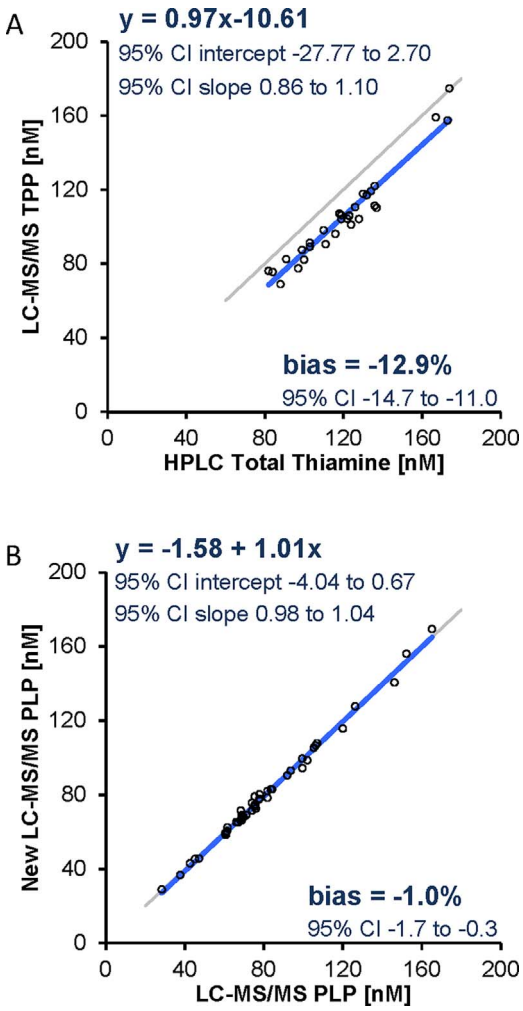

Fig. 4. Method comparison by Passing & Bablok regression analysis of (A) the current HPLC-method for total thiamine and (B) the old LC–MS/MS method for PLP with the newly developed LC–ESI–MS/MS-method for the simultaneous determination of TPP and PLP.

4. Conclusions

This LC–ESI–MS/MS method for the simultaneous determination of TPP and PLP in EDTA whole blood is characterized by short run times and simple sample preparation, making it suitable for high throughput applications. The linearity, precision, recovery and matrix effects were evaluated and these parameters all met the applied acceptance criteria. Comparison with the current methods is excellent. The new LC–MS/MS method is a convenient method to determine TPP and PLP for both clinical routine and research applications.

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