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A multicenter comparison of whole blood vitamin B6 assays

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Abstract

Background: The aim of this study was to compare different analytical methods that are currently in use in the Netherlands for the measurement of whole blood vitamin B6.
Methods: This method comparison study consisted of two separate parts. (1) Four laboratories participated in a pilot study in which the commercial Chromsystems and INstruchemie method, and a laboratory developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method and HPLC method were compared. Sixty-nine frozen whole blood samples and six lyophilized whole blood samples were used for comparison. (2) In the nationwide part of the study, 49 laboratories participated in the analysis of three identical sets of two whole blood samples of which one set was freshly analyzed, one set was analyzed after storage at –20 °C and one set was analyzed after lyophilization.
Results: In both parts of the study, the HPLC and LC-MS/MS methods showed equivalent results for all sample types tested. The Chromsystems method showed a positive bias of 45% (pilot study) and 30% (nationwide study) towards the LC-MS/MS method when fresh or frozen samples were used. The measurement of lyophilized samples showed no differences between the methods. The results of the INstruchemie method were inconclusive due to the low number of participants.
Conclusions: The different analytical methods for measuring vitamin B6 produce different results when whole blood patient samples are measured. The recognition of a reference method or the development of suitable reference materials and quality control materials might serve as a first step towards improved standardization or harmonization of the whole blood vitamin B6 assay.

Keywords: HPLC; liquid chromatography-tandem mass spectrometry (LC-MS/MS); method comparison; pyridoxal-5′-phosphate (PLP); vitamin B6; whole blood.

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Introduction

Malnutrition, alcohol abuse and renal insufficiency can lead to low levels of vitamin B6 [1–3]. Vitamin B6 deficiencies have been associated with an increased risk of stroke and coronary heart disease [4, 5]. The detection of vitamin B6 deficient patients is part of the Dutch clinical guidelines for the general practitioner in the differential diagnosis of dementia [6]. Since the measurement of vitamin B6 was added to these guidelines, the number of analyses of vitamin B6 in Dutch clinical laboratories has risen sharply. In most parts of the world it is common to determine the vitamin B6 status by the measurement of plasma PLP (pyridoxal-5′-phosphate), however, in a number of countries, like France, Australia and the Netherlands, whole blood is used. The rationale behind this is that in patients suffering from systemic inflammatory diseases, reduced plasma-PLP levels are found, even after supplementation. In these cases, whole blood PLP levels are 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 [7]. The analysis of vitamin B6 is mostly conducted with distinct commercial or laboratory developed liquid chromatography methods with either a

fluorescent detection [8–16] or, albeit to a lesser extent, a mass spectrometric detection [7, 17, 18]. For an unequivocal interpretation of test results in clinical guidelines and clinical practice, interchangeability and equivalence of test results between different methods are necessary. In the literature, studies comparing different vitamin B6 methods are sparse but the ones that have been published show large discrepancies between methods for both plasma- and whole blood vitamin B6 methods [19, 20]. The same studies also show poor precision profiles, with a reported coefficient of variation (CV) for some methods of >20% thereby indicating a lack of robustness for these methods. Unfortunately, it has not been possible yet to standardize or harmonize the measurement of vitamin B6 in whole blood due to the lack of a true reference method or a suitable reference material. Also, studies towards the commutability between methods for different sample materials have not been conducted and still need to be performed.

The aim of this study was to assess the comparability of the different analytical methods that are currently in use in the Netherlands for the routine measurement of vitamin B6 in whole blood.

Materials and methods

Four currently used methods in the Netherlands for the measurement of whole blood vitamin B6 (measured as PLP) were included in the study. These are the commercially available HPLC testkits from Chromsystems Instruments & Chemicals GmbH (Munich, Germany), and INstruchemie (Delfzijl, The Netherlands), a laboratory developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [7] and a laboratory developed HPLC method with a semicarbazide derivatization [16]. The study consisted of two separate method comparison parts.

Part one – pilot study

Four laboratories, each with a different method, participated in the pilot study. 1) Twenty fresh whole blood samples (leftovers from routine analysis) and six reconstituted lyophilized whole blood samples (external quality control, SKML, Nijmegen, The Netherlands) were aliquoted four-fold and stored at –80 °C until shipment on dry ice to the participating laboratories for the subsequent analysis of vitamin B6. 2) Preliminary results indicated a large proportional bias between the Chromsystems method and the LC-MS/MS method. This bias was validated by the analysis of another 49 frozen whole blood samples with both the Chromsystems method and the LC-MS/MS method. 3) To investigate the potential influence of sample freezing on the outcome of method comparability, 20 freshly drawn whole blood samples were aliquoted two-fold and the vitamin B6 concentration was measured within 2 h using the Chromsystems method and the LC-MS/MS method. 4) Seventeen whole blood samples (divided across methods) were analyzed directly after sample drawing and were re-analyzed after 24, 48 and 72 h to investigate sample stability. During these periods the samples were kept at room temperature and in the dark.

The laboratories that performed the commercial assays, analyzed the samples according the manufacturer's instructions. The laboratories that performed the laboratory developed methods, analyzed the samples according their in-house approved standard operating procedures.

The Erasmus MC guidelines state that, for the use of anonymous leftover samples, no permission from the Ethical Committee is needed.

Part two – nationwide study

The second part of the method comparison study consisted of a nationwide survey, organized by the Dutch EQA foundation SKML (Stichting Kwaliteitsbewaking Medische Laboratoriumdiagnostiek). In this survey, six samples, comprising two fresh-, two frozen- and two lyophilized whole blood samples were sent to 58 laboratories within the Netherlands that measure whole blood vitamin B6 on a regular basis. These samples were labeled sample A and sample B, in which the latter was identical to sample A but spiked with 100 nmol/L of PLP (Sigma-Aldrich, Zwijndrecht, The Netherlands). First, each laboratory received four fresh whole blood samples (2× sample A and 2× sample B) that were sent at room temperature, two of which (1× sample A and 1× sample B) were analyzed within 72 h of sampling after being kept in the dark and at room temperature until analysis and two (1× sample A and 1× sample B) were put at –20 °C immediately after receipt. Secondly, after 2 weeks, each laboratory received a lyophilized version of sample A and sample B. These samples were kept at 4 °C until they were reconstituted and analyzed together with the sample A and sample B that were stored at –20 °C. Sample storage conditions and reconstitution procedures were extensively described in an accompanying instruction leaflet. Each laboratory was asked to report the results within 3 weeks after receiving the lyophilized samples. The participants were also asked to fill-out a questionnaire (Supplemental Data 1) to get more insight into the methods of the individual laboratories. Measurements at each laboratory were performed either according to manufacturer's protocol or approved in-house standard operating procedures.

Statistics

The comparability between the Chromsystems, INstruchemie, HPLC and LC-MS/MS method in the pilot study was evaluated using Passing and Bablok regression analysis with a 95% confidence interval.

The assessment of sample stability and the within-method comparability of the different sample types in the nationwide study was calculated using a repeated measures ANOVA test with a post-hoc Bonferroni multiple comparison test. The between-method comparability of the different samples in the nationwide study was calculated using a one-way ANOVA test with a post-hoc Bonferroni multiple comparison test. p-Values <0.05 were considered significant.

As preliminary data showed equivalent results between the HPLC method and the LC-MS/MS method, and to control the abundance in number of comparisons, the LC-MS/MS method was used as method of comparison.

The recoveries were calculated as: (([PLP] sample B–[PLP] sample A)/[PLP] spiked)×100%.

A ranking list was made, based on the recovery and precision performance of the individual participants. Each laboratory was given a recovery-score based on the mean recovery of their fresh and frozen sample results, whereby the laboratory with the recovery closest to 100% received the best score and the laboratory whose recovery deviated most from 100% received the worst score. A precision-score was given to the laboratories as determined by the mean CV of sample A (fresh/frozen) and sample B (fresh/frozen). Laboratories with the lowest mean CV received the best score and laboratories with the highest mean CV received the worst score. The calculated mean of the recovery-score and the precision score resulted in a final performance score, which determined the position on the ranking list for all participants. The ranking list was composed using the fresh and frozen sample results, as these sample types are commonly used in daily laboratory practice. The laboratories that failed to measure the fresh and/or frozen samples were not given a performance ranking.

The Analyse-It software package v2.30 for Excel was used for Passing and Bablok analysis. Graphpad Prism v5.03 was used for ANOVA and Bonferroni testing. The INstruchemie and LC-MS/MS methods were not subjected to ANOVA and Bonferroni testing as the number of laboratories using these methods was too low. The results from laboratories that stored the samples under aberrant conditions were omitted from statistical analysis. Reported results based on Chromsystems calibrator batch number 0713 were also omitted from statistical analysis because of an erroneous assignment of the calibrator value.

Results

Part one – pilot study

The method comparison using frozen whole blood samples showed a varying degree of agreement with the LC-MS/MS method. The Passing and Bablok regression analysis indicated that the INstruchemie and HPLC method were comparable to the LC-MS/MS method as shown by the respective slopes of 1.03 [CI: 0.97–1.17] and 1.01 [CI: 0.92–1.07], whereas the Chromsystems method showed a significant positive bias, as indicated by the slope of 1.45 [CI: 1.38–1.55], towards the LC-MS/MS method (Figure 1). The resulting intercept of the comparison of the Chromsystems, INstruchemie and HPLC method with the LC-MS/MS method showed small but significant absolute biases of –9.5, –5.3 and –14.2 (Figure 1). In order to validate the proportional bias between the Chromsystems- and the LC-MS/MS method, an extra batch of 49 frozen whole blood samples was analyzed with both methods. The separate results of both batches were statistically identical (Preliminary: Chromsystems=1.51×LC-MS/MS–10.6, validation: Chromsystems=1.46×LC-MS/MS–11.5) and therefore combined

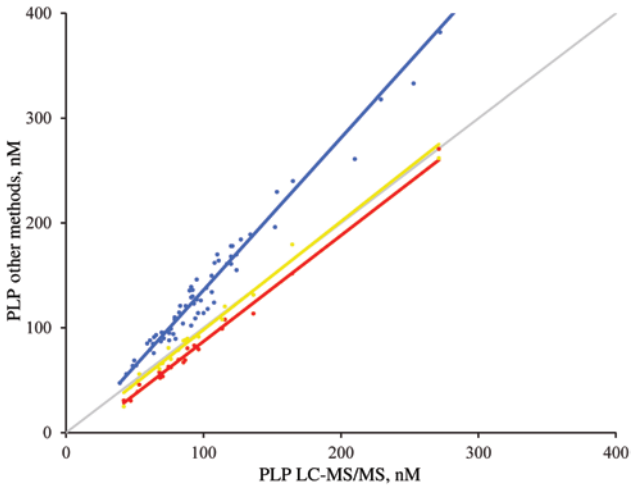

Figure 1: Comparison of PLP results from frozen samples between the LC-MS/MS method and the Chromsystems method, HPLC method and the INstruchemie method with a Passing and Bablok fit and confidence intervals.
Chromsystems: y=1.45 [CI: 1.38–1.55]x–9.5 [CI:–19.8 to –5.6] (blue line). HPLC: y=1.01 [CI: 0.92–1.07]x–14.2 [CI:–19.8 to –6.9] (red line). INstruchemie: y=1.03 [CI: 0.97–1.17]x–5.3 [CI:–15.4 to –0.5] (yellow line). y=x (grey line).

(Chromsystems=1.45×LC-MS/MS–9.5, Figure 1). The correlation coefficient between the LC-MS/MS method and the other methods was >0.98 in all cases.

To exclude that the differences in result were due to sample-storage at –80 °C, fresh whole blood samples were analyzed directly after sampling using the Chromsystems method and the LC-MS/MS method. The result of this fresh sample comparison was identical to the frozen sample comparison for these methods (fresh samples: Chromsystems=1.43×LC-MS/MS–5.1, frozen samples, Chromsystems=1.45×LC-MS/MS–9.5), thereby ruling out a confounding effect caused by sample freezing.

Remarkably, the method-comparison using reconstituted lyophilized samples showed no significant differences between methods. The Passing and Bablok regression analysis yielded non-significant slopes of 1.02 [CI: 0.86–1.22], 0.95 [0.83–1.13] and 1.03 [CI: 0.96–1.07] for the comparison between the INstruchemie, HPLC and Chromsystems methods with the LC-MS/MS method (Figure 2). The accompanying intercepts of these comparisons showed small insignificant biases of –5.6, –8.2 and –6.3, respectively. The correlation coefficient between methods was >0.99 in all cases.

The study towards room temperature stability of whole blood vitamin B6, showed a mean increase of 11% ($p<0.001$) between results obtained directly after blood sampling (t0) and results obtained 24 h after sampling (Figure 3). Forty-eight hours after sampling, a mean

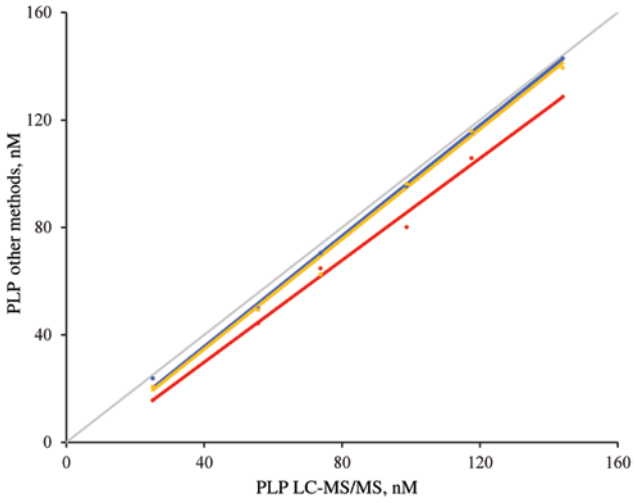

Figure 2: Comparison of PLP results of lyophilized samples between the LC-MS/MS method and the Chromsystems method, HPLC method and the INstruchemie method with Passing and Bablok fit and confidence intervals.
Chromsystems: y=1.03 [CI: 0.96–1.07]x–5.6 [CI: –10.1 to 0.3] (blue line). HPLC: y=0.95 [CI: 0.83–1.13]x–8.2 [CI: –22.6 to 0.7] (red line). INstruchemie: y=1.02 [CI: 0.86–1.22]x–6.3 [CI: –25.8 to 6.5] (yellow line). y=x (grey line).

increase of 10% (p<0.001) was found and 72 h after sampling a non-significant increase of 5% was found with respect to the results at t0.

Part two – nationwide study

This part of the method comparison study consisted of a nationwide survey which was conducted with the purpose

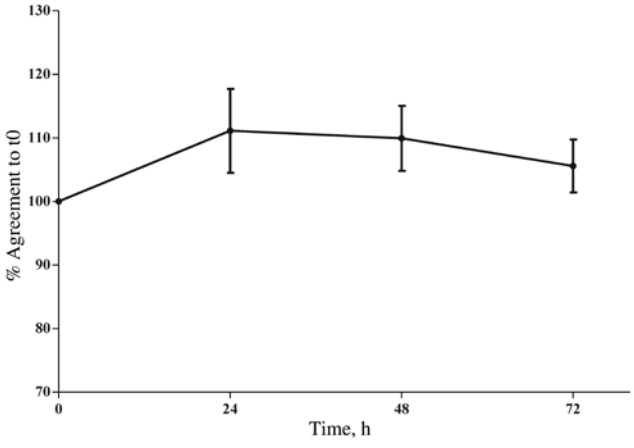

Figure 3: Whole blood PLP stability results in nmol/L, as measured after sample storage for 24, 48 and 72 h (20 °C in the dark), compared with the results obtained directly after blood sampling (t0). Vertical lines at each time point represent the standard deviation.

of validating the results as found in the pilot study. Of the 58 invited Dutch laboratories, 49 agreed to participate (84%), of which 33 laboratories employed the Chromsystems method (67%), 12 laboratories used a laboratory developed HPLC method (24%), three laboratories used the INstruchemie method (6%) and one laboratory used a laboratory developed LC-MS/MS method (2%). All participants reported the results and the filled-out questionnaire within the set deadline. The vitamin B6 results are presented in Table 1 and in Supplemental Table 1, where the routinely found CV, method of calibration, used reference values and other details of each participating laboratory are also reported.

The within-method comparison showed equal results for fresh and frozen samples for the Chromsystems, HPLC and LC-MS/MS methods (Table 1). The INstruchemie method seems to yield higher results for the frozen samples than for the fresh samples, but this could not be statistically proven due to the low number of INstruchemie users (n=3). The Chromsystems method showed 20% higher results for the fresh and frozen samples compared to the lyophilized samples (p<0.001). The HPLC and LC-MS/MS method showed identical results between these sample types, with only a significant higher recovery for the fresh sample compared to the lyophilized sample for the HPLC method (p<0.05; Table 1).

The between-method comparison yielded results for the fresh, frozen and lyophilized samples that were equivalent for the HPLC and the LC-MS/MS methods (Table 1), which was in concordance with the results of the pilot study. The associated recoveries of these samples were also comparable for these methods and never deviated more than 10% from a perfect recovery of 100%. The results of the fresh and frozen samples for the Chromsystems method were 30% higher compared with the HPLC and LC-MS/MS methods and this difference was statistical significant (Chromsystems vs. HPLC) for three out of four samples (Table 1). This confirmed the proportional bias for these sample types between the Chromsystems method and the other methods as found in the pilot study, although to a less explicit degree. The result of lyophilized sample A in the Chromsystems assay was comparable with the result of the other methods, while lyophilized sample B showed a not significantly higher result for the Chromsystems assay compared with the HPLC and LC-MS/MS methods. The calculated recoveries for the Chromsystems assay were higher compared with the HPLC and LC-MS/MS methods, but were never significantly different from the HPLC method. The INstruchemie method led to higher results for the frozen and lyophilized samples compared with the LC-MS/MS method, but identical results for the fresh samples.

Table 1: PLP results and recoveries from two samples, represented in three sample types, as measured by four different PLP-methods.

Sample type	Sample A		Sample A	Sample B		Sample B		Recovery	Recovery	
Method	**Fresh**	**Frozen**	**Lyophilized**	**Fresh**	**Frozen**	**Lyophilized**	**Fresh**	**Frozen**	**Lyophilized**
Chromsystems n=33	71 [38–126][a,b]	75 [51–89][a,c]	60 [49–71]	193 [99–258][a]	204 [160–250][a,c]	168 [117–197]	120 [61–158][a]	127 [95–170][a]	108 [64–132]
CV	26%	12%	9%	20%	11%	10%	19%	14%	12%
INstruchemie n=3	54 [51–63]	63 [56–70]	67 [55–69]	159 [156–170]	186 [184–199]	171 [167–189]	107 [102–108]	128 [116–136]	112 [102–122]
CV	11%	11%	12%	5%	4%	7%	3%	8%	9%
HPLC n=12	50 [44–73]	53 [41–76]	57 [42–74]	161 [127–234]	164 [127–248]	152 [129–191]	110 [83–164][d]	109 [86–172]	96 [81–124]
CV	17%	18%	15%	20%	18%	12%	23%	19%	12%
LC-MS/MS n=1	55	54	58	163	157	148	108	103	90
CV	NA	NA	NA	NA	NA	NA	NA	NA	NA

The median and the ranges of the PLP results in nmol/L and recovery in %, including the CV for each sample per method. Sample B is identical to sample A, but spiked with 100 nmol/L PLP. NA, Not applicable. [a]$p<0.001$ within method significance compared to lyophilized sample; [b]$p<0.01$ between method significance compared to HPLC method; [c]$p<0.001$ between method significance compared to HPLC method; [d]$p<0.05$ within method significance compared to lyophilized sample.

The CV within the Chromsystems users group was very high for the freshly measured samples, with a mean CV for sample A and sample B of 23% (Table 1). The frozen and lyophilized samples performed much better in this assay, with a mean CV of 12% and 10%, respectively. The CV within the HPLC group seemed independent of the sample type with a total mean CV of 17%, with only a slightly better performance for the lyophilized samples. The CV found within the INstruchemie group was also independent of sample type, but showed a difference in mean CV between the low level sample (11%) and the high level sample (5%). There was only one LC-MS/MS user implying that the CV for this method could not be calculated.

Each laboratory was given a ranking based on the performance of the recovery and precision. The 20 highest ranked laboratories consisted of eight HPLC users, 10 Chromsystems users, 1 INstruchemie user and 1 LC-MS/MS user (Supplemental Table 1). Interestingly, the Chromsystems users were highly ranked because of either a good recovery-score or a good precision-score, but never a good score for both. This is in contrast to the HPLC and LC-MS/MS users where there is more balance between recovery-score and precision-score. The single LC-MS/MS user and seven out of 11 HPLC users had a recovery-score and a precision-score <20.

Discussion

When different methods are being used for the measurement of an analyte, the results those methods produce should preferably be interchangeable. In this study, we compared different vitamin B6 whole blood assays and demonstrated that the laboratory developed HPLC and LC-MS/MS method produce equivalent results irrespective of the sample type. In contrast, the commercial Chromsystems method showed results for lyophilized samples that were comparable to the HPLC and LC-MS/MS method, while the Chromsystems method yielded distinctly higher results for fresh and frozen whole blood sample compared with the other methods. The INstruchemie method showed ambiguities for which elucidation is required.

In the pilot study, the INstruchemie method and the LC-MS/MS method appeared to be fully comparable for frozen and lyophilized samples, while in the nationwide study, differences between these methods were found for the same sample types. Inconsistent results between the different parts of the study were witnessed for the INstruchemie method only and a more thorough validation of this method is needed before a solid assessment of

this method can be made, preferably by the expansion of the number of INstruchemie users.

The Chromsystems method yielded results for fresh and frozen whole blood samples that were 30%–45% higher than the LC-MS/MS method, whereas the results for the lyophilized samples were equivalent. Similarly, the Chromsystems method measured higher values for the fresh and frozen samples compared with the matching lyophilized samples. This indicates that in the Chromsystems method, lyophilized whole blood behaves differently than fresh or frozen whole blood. It might be that during the process of lyophilization, the sample-matrix changes in such a manner that it causes the Chromsystems method to react differently towards lyophilized samples than to either fresh or frozen samples. All Chromsystems users employ a lyophilized whole blood calibrator (Table 2) with the consequence that these laboratories report falsely elevated results for fresh or frozen whole blood patient samples (Figure 1; Table 1), this could most likely be avoided if a liquid calibrator was applied in the Chromsystems assay. The choice of either a lyophilized or liquid calibrator is not an issue for the HPLC and LC-MS/MS methods, as these methods do not show matrix dependent results. The huge differences in interlaboratory CV for the Chromsystems method (26% for fresh blood and 9% for lyophilized blood for sample A) suggest that the stability of fresh whole blood is an issue within the Chromsystems assay. It is recommended that the chromatography or the sample preparation procedure of the Chromsystems method is adapted in order to eliminate the observed sample matrix dependent differences. It is remarkable though, that the processed (i.e. lyophilized) materials show the most comparable results between methods and that the 'real' patient samples show discrepancies. This phenomenon is poorly understood and needs clarification.

Differences between vitamin B6 methods as witnessed in our study, have been described before. A vitamin B6 interlaboratory comparison study conducted by the Center of Disease Control (CDC, Atlanta, GA, USA) in 2005 for serum samples showed positive biases, a high degree of imprecision and poor standard addition recoveries for several methods including the Chromsystems method [19]. In a study towards reference values for several clinical parameters, Steen et al. [21] reported reference values for vitamin B6 for the Chromsystems method of 51–183 nmol/L, which are approximately 50% higher than the commonly used reference values of 35–110 nmol/L, thereby indicating a similar positive bias for the Chromsystems method as found in our study. Likewise, about half of the Dutch Chromsystems users have adopted reference values that are similar to the reference values by Steen et al. [21] (Supplemental Table 1).

A poor precision for some of the vitamin B6 methods, as noted by the CDC, was also observed in an Australian quality assurance program, where CVs of 25% or higher were found for two out of five laboratories that used the Chromsystems method [20]. In our study, the observed CVs differed between the respective methods, and sometimes even between sample types. It is remarkable that for the Chromsystems assay, the CV in the results of the fresh samples (26% and 20%) is much higher than the CV in the results for the frozen (12% and 11%) or lyophilized samples (9% and 10%). As the poorest precision is observed for the fresh samples, one might postulate that the presence of intact red blood cells is the source of the high variation. The CV within the HPLC and INstruchemie methods is more equally distributed between the

Table 2: The origin and the number of calibrators used by the diagnostic laboratories for the different PLP measuring methods.

Calibrator	Methods			
	Chromsystems	INstruchemie	HPLC	LC-MS/MS
Chromsystems	n=28	n=0	n=2	n=0
INstruchemie	n=0	n=3	n=0	n=0
SKML	n=5	n=0	n=4	n=0
Recipe	n=0	n=0	n=1	n=0
PLP solution	n=0	n=0	n=4	n=0
PLP in whole blood	n=0	n=0	n=1	n=1
# of Calibrators:				
1	n=31	n=3	n=5	n=0
2	n=2	n=0	n=3	n=0
3 or >3	n=0	n=0	n=4	n=1

The commercial Chromsystems, SKML and Recipe calibrators are lyophilized whole blood calibrators. The INstruchemie calibrator is lyophilized PLP in an albumin matrix.

respective sample types. The CV in the results of the different sample types measured with the HPLC method is higher than 15% for five out of six samples. The individual laboratory precision scores, which is mainly a reflection of laboratory performance, indicate that these high CVs are largely caused by a small number of individual HPLC users that show problems with reproducibility, whereas the high CVs for the Chromsystems method are a more general problem, which is indicative of a method that is lacking in robustness (Supplemental Table 1). The possibility of producing high quality duplicate sample results is getting restricted for laboratories when using a method that shows poor method robustness. Hence, the precision score not only mirrors lab performance but is also dependent on method robustness. All individual laboratories with poor precision profiles, independent of the method employed, should undertake adequate measures to improve in that area. In that respect, the extraction efficiency of PLP from its whole blood binders might be one of the aspects to examine. Although difficult to prove, different experiments during validation of the LC-MS/MS method suggest that the sample preparation of this method for frozen blood produces complete extraction of PLP [7], while for the others methods this is currently unknown.

The stability study showed that vitamin B6 is not stable at room temperature with a maximum increase of 11% in measured vitamin B6 concentration at 24 h after sample drawing (Figure 3). The samples used in the nationwide study were sent to the participants at room temperature and some influence on the results caused by sample instability cannot be excluded. However, in the pilot study, stability issues were surpassed by the direct measurement or direct freezing of the samples and as the same outcome in method comparability was found in the pilot study and the nationwide study, it seems unlikely that sample (in) stability had a major influence on our results. For a proper judgment of whole blood vitamin B6 stability though, a more thorough study of the total vitamin B6 system is recommended for each method. This should encompass stability before sample preparation and also stability of the derivatized product and potential differences thereof between sample types.

Conclusions

This study showed that from the different analytical methods that are currently in use for the measurement of whole blood vitamin B6, the Chromsystems method clearly leads to higher patient results compared to the HPLC and LC-MS/MS methods as evidenced by the method comparison and recovery studies, whereas the INstruchemie method suffers from a low number of participants. The lack of a reference method or suitable certified reference material for the measurement of vitamin B6 in whole blood is impeding the standardization or harmonization of this assay. If in the future a method could be acknowledged as a reference method, the equivalency between methods can and should improve. Also, studies towards potential calibrators and quality control samples should be integrated in the implementation-phase of new commercial or laboratory developed tests and methods into the clinical laboratory, as this study demonstrated that matrix-dependent differences can occur. Provisionally, laboratories measuring vitamin B6, should use method specific reference values to circumvent the current methodological differences, in order to not misdiagnose vitamin B6 deficiencies. As there is no consensus yet in the Netherlands in the applied reference values for each method (Supplemental Table 1), it is recommended that for the measurement of whole blood vitamin B6, laboratories employing the Chromsystems method adopt higher reference values than the laboratories using the HPLC, INstruchemie or LC-MS/MS method, as described by Steen et al. [21].

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