Validation of a new fully automated assay of Thiamine-PyroPhosphate and Pyridoxal-5'-Phosphate in whole blood using LC-MS/MS

Joep Vuurpijl[1], Dennis van den Heuvel[2], Chantal Wilbers[1], Tom Stokhof de Jong[2], Rein Hoedemakers[1]
[1]Jeroen Bosch Ziekenhuis, 's-Hertogenbosch, The Netherlands; [2]Shimadzu Benelux, 's-Hertogenbosch, The Netherlands

1. Introduction

•The biological active form of vitamin B1 is Thiamine-Pyrophosphate (TPP). This water-soluble vitamin acts as a coenzyme for the enzymatic degradation of glucose in the citric acid cycle. A non-varied diet or malnutrition can quickly lead to a deficiency.
•The biological active form of vitamin B6 in the human cell is Pyridoxal-5'-phosphate (PLP). A PLP deficiency can occur due to chemotherapy, alcoholism, pregnancy and kidney failure.

Due to the rising numbers of patient samples in clinical laboratories there is need for a simple and fast chromatographic method without excessive sample preparation. The aim of this study was therefore to set up a simple, fast and automated (U)HPLC-MS/MS test for TPP and PLP, with minimal sample preparation.

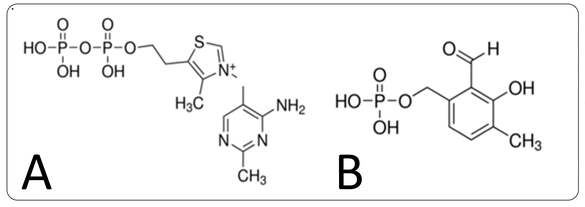
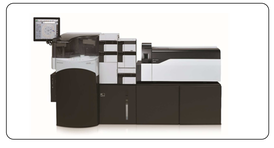


Figure 1 Structural formulae for (A) Thiamine-Pyrophosphate and (B) Pyridoxal-5'-Phosphate

2. Materials and Methods

For the analysis of TPP and PLP the IVD certified kit was purchased at Diagnotix (Appingedam, The Netherlands). The analysis of TPP and PLP was performed using a fully automatic LCMS preparation Unit (CLAM-2030, Shimadzu Corporation, Kyoto, Japan) online with a LCMS system (Nexera X3 + LCMS-8050, Shimadzu Corporation, Kyoto, Japan).



Automatic Sample Pretreatment
High Speed Mass Spectrometer
Ultra Fast Polarity Switching
- 5 msec

Ultra Fast MRM
- Max. 555 transition /sec

Ultra Fast Scanning
- Max. 30.000 MRM/sec

Figure 2 CLAM-LCMS-8050 triple quadrupole mass spectrometer

2.1 Automated Sample Preparation

Before placing the whole blood sample on the sample preparation module, the sample was frozen at -20°C and thawed at room temperature. The system was programmed to perform sample extraction and protein precipitation followed by filtration and sample collection. The volumes from the kit were adapted to fit the automated sample preparation procedure. The filtrated sample was then automatically transported to the HPLC for LC-MS/MS analysis and no human intervention was required (Fig 2.)

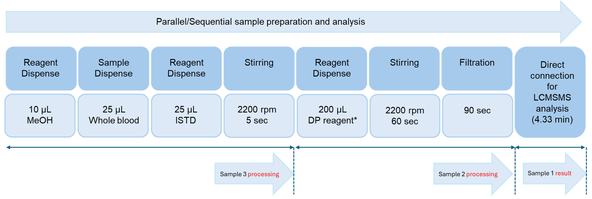


Figure 3 Schematic Automatic Sample Preparation and Analysis

2.2 (U)HPLC conditions (Nexera X3 system)

	Value
Column	**Xbridge BEH C_{18} 3.0 x 75 mm**
Temperature [°C]	**30**
Mobile Phase	**A: Diagnotix A, Water phase B: Diagnotix B, Organic phase**
Flow rate [mL/min]	**0.600**
Injection volume [µL]	**20**

Table 1 (U)HPLC conditions for the analysis of TPP and PLP in whole blood

2.3 MS conditions (LCMS-8050)

	Value
Nebulizer gas [L/min]	**2.5 (N_2)**
Heating gas [L/min]	**15 (Air)**
Drying gas [L/min]	**5 (N_2)**
Interface Temperature [°C]	**400**
Desolvation Temperature [°C]	**650**
Heat block Temperature [°C]	**400**
Interface Voltage [kV]	**2.8**
Ionization	**ESI+**

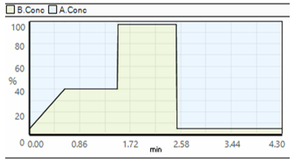


Table 2 Interface conditions for the analysis of TPP and PLP in whole blood

Compound	Precursor m/z	Product m/z	CE
TPP	**425.0**	**122.1**	**-22**
		304.1	-19
TPP-d_3	**427.0**	**125.2**	**-23**
PLP	**247.9**	**150.2**	**-17**
		94.2	-28
PLP-d_3	**250.8**	**153.0**	**-16**

Table 3 MRM settings for the analysis of TPP and PLP in whole blood

3. Results

For both TPP and PLP two transitions were optimized for quantification. The second transition of each compound was used as extra identification.

3.1 Linearity

The response ratio of TPP and TPP-d_3 and the response ratio of PLP and PLP-d_3 was plotted against the concentration. Linear regression with 1/x weighing was used for calculation. Both compounds showed good linearity (r^2 > 0.999) in a clinically relevant concentration range.

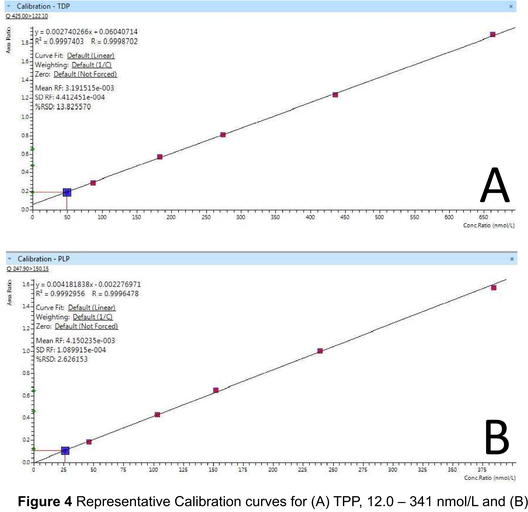


Figure 4 Representative Calibration curves for (A) TPP, 12.0 – 341 nmol/L and (B) PLP, 17.0 – 1592 nmol/L

3.2 Precision and accuracy

	Target [nmol/L]	Measured TPP [nmol/L]	Difference [%]	Target [nmol/L]	Measured PLP [nmol/L]	Difference [%]
Low	49.0	**47.8**	**-2.4**	30.4	**30.8**	**1.3**
High	234.0	**227.8**	**-2.6**	166.6	**165.5**	**-0.7**
Pool	153.6	**158.8**	**3.4**	109.4	**111.8**	**2.2**

Table 4 Precision data in a clinically relevant range for TPP and PLP

	N	SD [nmol/L]	CV [%]	X_{mean} [nmol/L]
Low	5	**TPP: 1.8 PLP: 0.6**	**TPP: 3.8 PLP: 1.9**	**TPP: 47.6 PLP: 31.1**
High	5	**TPP: 2.9 PLP: 4.9**	**TPP: 1.3 PLP: 3.0**	**TPP: 228.3 PLP: 161.6**
Pool blood	5	**TPP: 3.1 PLP: 4.3**	**TPP: 1.9 PLP: 3.7**	**TPP: 160.2 PLP: 117.2**

Table 5 Run to run inter-assay data in a clinically relevant range for TPP and PLP

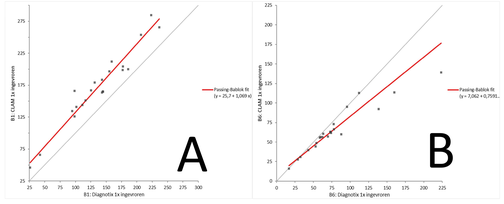


Figure 5 Passing-Bablock correlation for (A) TPP and (B) PLP, comparison manual and automatic sample preparation

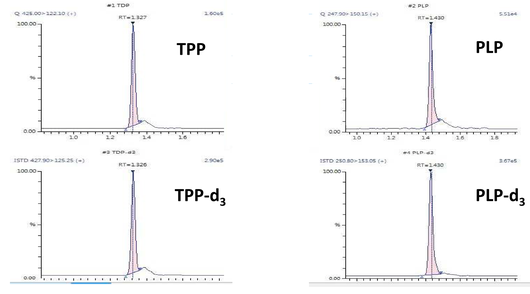


Figure 6 Representative chromatogram for a patient sample at 200 (TPP) and 130 (PLP) nmol/L

4. Conclusions

- Reference values were determined at 100 - 220 nmol/L for TPP and 35 - 110 nmol/L for PLP.
- An end-to-end to support quantification of Thiamine-PyroPhosphate and Pyridoxal-5'-Phosphate in whole blood.
- With this fully-automatic sample preparation and LC-MS/MS analysis, a reliable and cost-effective, time-saving method is developed, which minimizes human errors..