Analysis of Thiamine Pyrophosphate and Pyridoxal-5'-phosphate in whole blood using a fully automated sample preparation LC/MS/MS system (CLAM-2000 + LCMS-8045)

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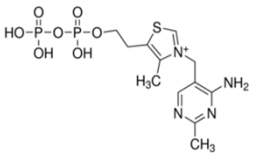
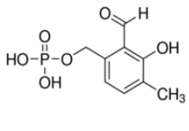
1. Introduction

Vitamin B1, thiamin, plays an important role in the metabolic pathway of the human body. The biological active form is Thiamine Pyrophosphate (TPP). The 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 which can result in inter alia depression, muscle weakness and tachycardia.
Vitamin B6 has multiple forms. The biological active form in the human cell is Pyridoxal-5'-phosphate (PLP). The water soluble vitamin acts as a coenzyme in the formation of aminoacids, amines and peptides. In case of a deficiency the other B vitamins will also be deficient. PLP deficiency can occur due to chemotherapy, alcoholism, pregnancy and kidney failure. These two vitamins (TPP and PLP) are predominantly analysed with HPLC and fluorescence detection. These methods are performed with excessive sample preparation including pre- or postcolumn derivatisation using toxic reagents and have relatively long analysis times. Due to the rising numbers of patient samples in clinical laboratories and to prevent variability in sample preparation, there is need for a simple and fast chromatographic method without excessive sample preparation. We report a fully automated platform for the quantitation of TPP and PLP in whole blood samples with no need of any manual sample preparation and automated LC-MS/MS analysis.



Thiamine PyroPhosphate



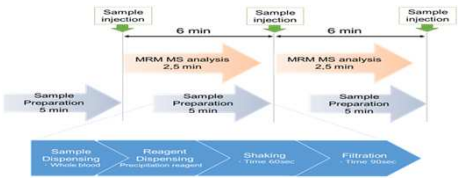
Pyridoxal-5'-Phosphate

2. Methods

The analysis of TPP and PLP was performed using a fully automatic LCMS preparation unit (CLAM-2000, Shimadzu) inline with a LC-MS/MS system (Nexera X2 – LCMS-8045, Shimadzu) directly from whole blood samples using the Vitamin B1&B6 kit (Instruchemie, The Netherlands).

Figure 1 CLAM-2000 sample preparation system inline with Nexera X2 and LCMS-8045.

The CLAM-2000 was programmed to perform sample extraction and protein precipitation followed by filtration and sample collection. Only 25 µL of whole blood sample was transferred to a disposable micro-vial with filter. 200 µL of precipitation reagent and 25 µL of deuterated internal standard mix was added automatically. The disposable micro-vial and filter were mixed and filtration was performed using a vacuum. The sample was automatically transferred into the autosampler and 20 µL was injected on the Shimadzu Nexera X2 binary UHPLC system (consisting of two LC-30AD pumps, SIL-30AC cooled automated injector, CTO-20AC column oven, FVC20AH2 divert valve) coupled to a Shimadzu LCMS-8045 tandem quadrupole mass spectrometer with electrospray ionization (ESI-MS/MS). Reversed phase chromatography was performed using a Shimpack GIST C_{18}-AQ column and a fast gradient was performed using mobile phase A and B from the kit. The column was thermostated at 30ºC. Because of the design of the CLAM-2000, sample preparation was performed parallel to LC-MS/MS analysis (see figure 2).



Compound	Quantifier	Qualifier
TDP	425.0>122.2	425.0>303.2
PLP	248.1>150.2	248.1>94.2
d_3-TDP	428.0>125.2	
d_3-PLP	251.1>153.2	

MS/MS METHOD	
Nebulizer gas [L/min]	3 (N_2)
Heating gas [L/min]	10 (Air)
Drying gas [L/min]	10 (N_2)
Interface temperature [°C]	300
Desolvation line [°C]	250
Heat block temperature[°C]	400
Interface voltage [kV]	5
Dwell time [ms]	25
Pause time [ms]	3
Ionization	ESI, positive
Scan Type	MRM

Figure 2 CLAM-2000 fully automated sample preparation and analysis. Due to the overlapped sample preparation the throughput of the instrument was 1 results every 6 minutes for TDP/PLP quantitation.

3. Results and discussion

The linearity and accuracy of the method was evaluated using 6 reference whole blood calibrators (Instruchemie 3168). For both compounds linearity and accuracy were within the analytical acceptable range (85% - 115%). In order to estimate the precision of the method, reference whole blood samples (Instruchemie 3159, 3160, 3161) spanning from low to high concentration level were analyzed in triplicate. For both analytes the CV% were within acceptable analytical ranges.

Compound	R2	Accuracy [%]	
		min	max
TDP	0,997	85,1	111,5
PLP	0,997	89,00	106,3

Table 1 Linearity and accuracy for TDP and PLP for 6 reference whole blood calibrators.

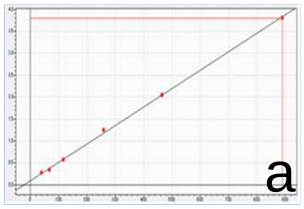


Figure 3 Calibration curve TPP (39–890nmol/L) (a); Calibration curve PLP (21-550 nmol/L) (b)

Compound	Precision [CV%]		
	low	middle	high
TDP	-8,7	1,6	2,7
PLP	-0,4	1,60	-2,2

Table 2 Coefficient of variation for TDP and PLP at three levels

Compound	Bias [%]		
	low	middle	high
TDP	0,6	-9,6	1,1
PLP	-1,5	-7,90	2,8

Table 3 Accuracy for TDP and PLP at three levels

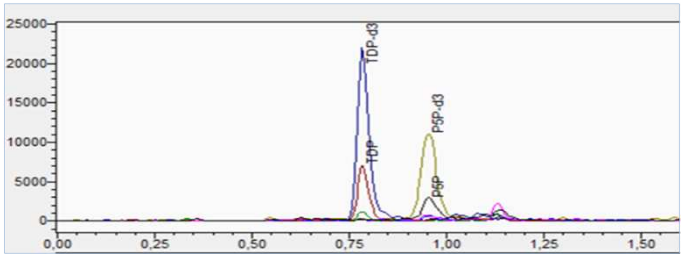


Figure 4 Representative chromatogram for a refence whole blood sample at low level TDP/PLP 49/31 nmol/L

4. Conclusion

- The fully automated sample preparation CLAM-2000-LCMS-8045 system in combination with the Instruchemie vitamin B1&B6 kit can eliminate the risk of error-of-variability introduced by manual sample preparation, which is often a problem for whole blood analysis.
- Fully automated sample preparation procedure proved to be suitable for the quantitation of TDP and PLP by elimination of all manual sample preparation steps.
- The quick and high precision analytical workflow of the CLAM-2000 system in combination with the LCMS-8045 is a perfect solution for the high amount of whole blood samples that are analysed for TDP and PLP in the clinical chemistry.