

Application Note

► Sensitive determination of vitamin B6 in blood samples via fluorescence detection

Category	Clinical
Matrix	Blood samples
Method	UHPLC
Keywords	Vitamin B6, pyridoxal, pyridoxine, blood analyses, derivatization, bisulfite
Analytics ID	VCL0009N_P_E, 08/13



PLATIN blue

Summary

This Application note shows the sensitive determination of vitamin B6 in blood samples using bisulfite derivatization and fluorescence detection. Applying the PLATINblue UHPLC system in combination with a fluorescence detector, the analysis of vitamin B6 in less than 5 minutes becomes possible without the need for an additional postcolumn derivatization pump. The low detection limit and the high speed of the HPLC method make it well suited for routine analyses in clinical blood testing labs and hospitals.

Introduction

Vitamin B6 is the general term for active 3-hydroxy-2-methylpyridine derivatives. It is an essential precursor of pyridoxal (PL) and pyridoxamine phosphate coenzymes of a wide variety of enzymes of intermediary metabolism.¹ Pyridoxal 5'-phosphate (PLP) is the major form found in plasma, while PLP and pyridoxamine 5'-phosphate (PMP) predominate in the cell. Widely used methods for the detection of vitamin B6 are various assays like microbiological, enzyme, radioimmunoassays and chemical assays. But unfortunately, for the quantification of vitamin B6 and metabolic studies, none of these is really suitable.² Plasma PLP concentration is considered one of the better indicators of vitamin B6 status and is reported to be well correlated with tissue PLP concentrations. As a conclusion, the best way to analyze vitamin B6 in blood seems to be the use of an HPLC method instead of one of the mentioned assays.³ Hence, especially the detection of very low vitamin B6 concentrations found in plasma (typically nanomoles per liter) are of interest.² Many HPLC methods are already described including cation exchange and reversed phase separations with pre- or postcolumn derivatization and fluorimetric detection to ensure the required detection limits. These methods also differ in sample preparation techniques and HPLC method parameters.⁴

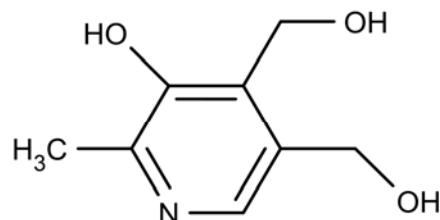


Fig. 1

Chemical structure of vitamin B6

Vitamin B6 (pyridoxine)

In this application note, a simple method for the sensitive determination of vitamin B6 in blood samples is described. With its short run time, good reproducibility and low detection limits, this method is well suited for routine analyses in clinical labs. In dependence on a method already described in the literature, the shown method does not need an additional post column derivatization step, because the bisulfite adduct formation is already realized via the mobile phase.^{4,5}

**Experimental:
preparation of
standard solution**

About 1 mg/ml vitamin B6 hydrochloride was weighed in exactly and dissolved in water. Vitamin B6 hydrochloride has a molecular weight of 205.69 g/mol, what means this solution has a molar concentration of 4.68 mmol/l. With this stock solution, calibration standards were prepared in the range of 26 – 260 nmol/l by diluting it again with water.

**Experimental:
sample preparation**

Blood samples were collected in EDTA and received already prepared by the customer. Sample preparation contained the following steps:
Deproteinization, centrifugation at 10,000 g and collecting and filtering the supernatant through a 0.45 µm RC membrane syringe filter.
These samples were analyzed pure and also spiked with the vitamin B6 standard.

Method parameters

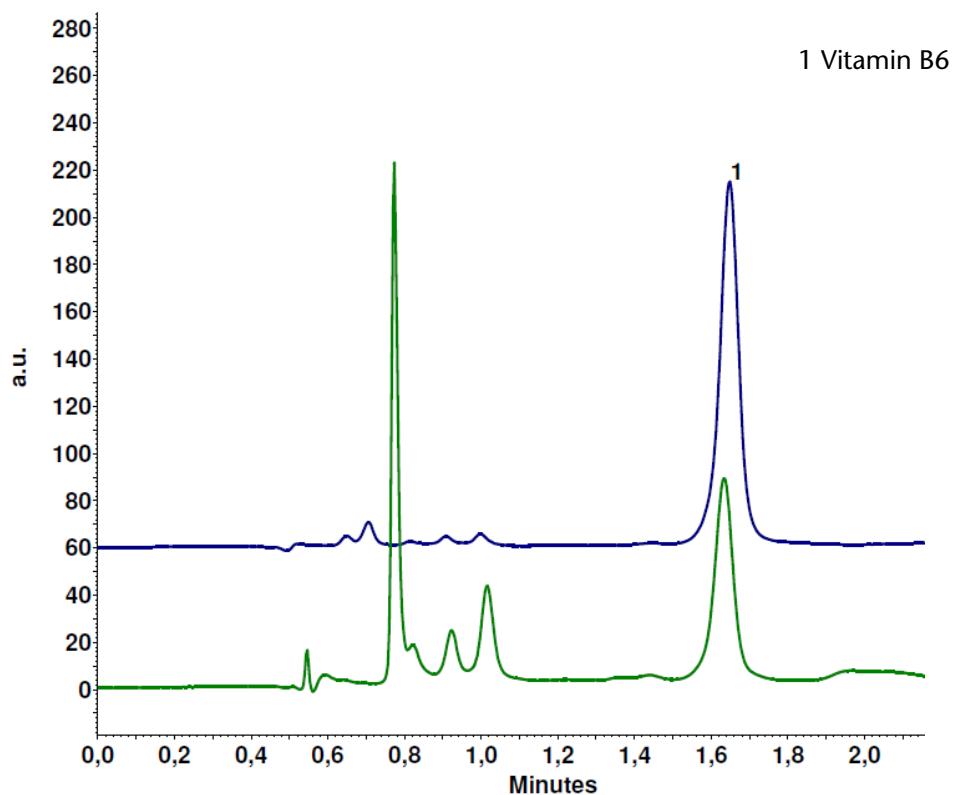
Column	ProntoSIL 120-5 C18 AQ, 125 x 3 mm ID with integrated precolumn		
Eluent A	100 mM KH ₂ PO ₄ , 100 mM NaClO ₄ , 0,5 g/l NaHSO ₃ /acetonitrile 97:3 (v/v)		
Eluent B	Acetonitrile		
Gradient	Time [min]	% A	% B
	0.00	100	0
	1.60	100	0
	1.70	50	50
	2.70	50	50
	2.80	100	0
	5.00	100	0
Flow rate	1.2 ml/min		
Injection volume	10 µl		
Column temperature	30 °C		
Detection	Fluorescence RF-20AXs, 5 Hz, 0.1 s, Ex 290, Em 400, Sensitivity: High, Gain x16		
Run time incl. washing step	5 min		

The gradient may not be raised to more than 50 % B because the buffer of the mobile phase A precipitates at higher organic content.

It is also possible to run the analyses with pure mobile phase A during one working day if an isocratic run is needed or preferred caused by the HPLC equipment. But then a washing step at the end of every day, or maybe two times a day depending on the matrix of the samples, is needed. The complete run time for one analysis is than shortened to about 2 minutes, but the column washing must be run additionally.

Results

A post-column derivatization was not needed, because the eluent already contains the substances needed for the derivatization. Vitamin B6 forms in this case adducts with bisulfite from a sodium bisulfite solution. With this given mobile phase, the separation was no problem on the ProntoSIL C18 AQ column. Figure 1 shows the chromatograms of the vitamin B6 standard in water and a sample spiked with vitamin B6.



The analysis itself runs isocratically with pure mobile phase A. The washing step starting after the elution of vitamin B6 is nevertheless recommended to wash the entire matrix from the samples off the column. Figure 3 shows a full chromatogram with the washing step to show that this step is really needed to clean the column, because a lot of matrix is eluting from the column at higher organic content of the mobile phase.

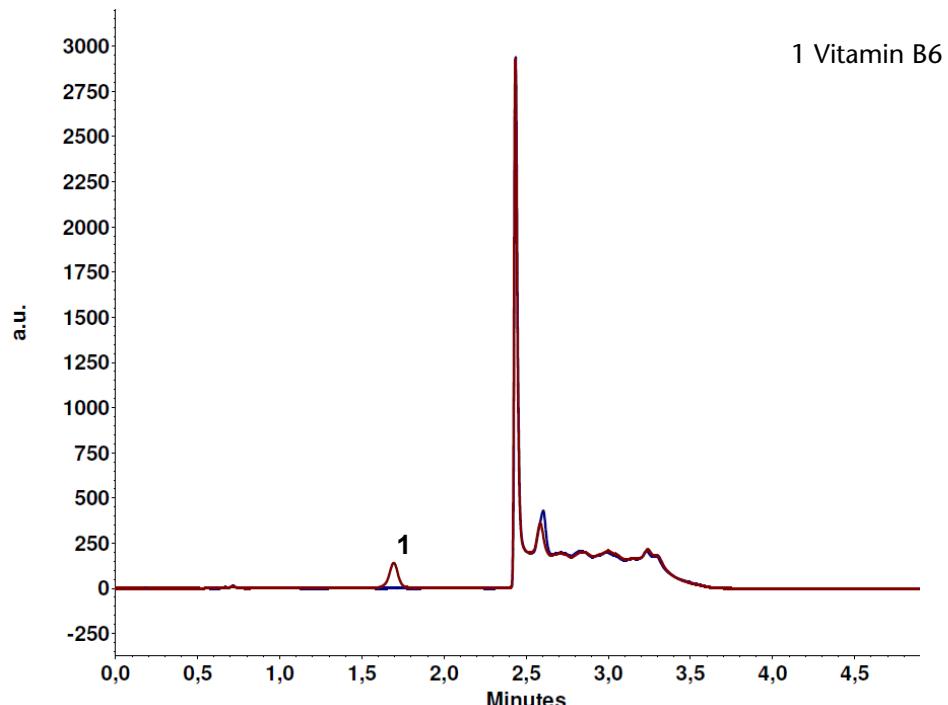


Figure 4 shows the customer's sample pure and spiked with the vitamin B6 standard. It is obvious that no other substance elutes at the same time as vitamin B6. This proves that the target compound is well separated from the matrix.

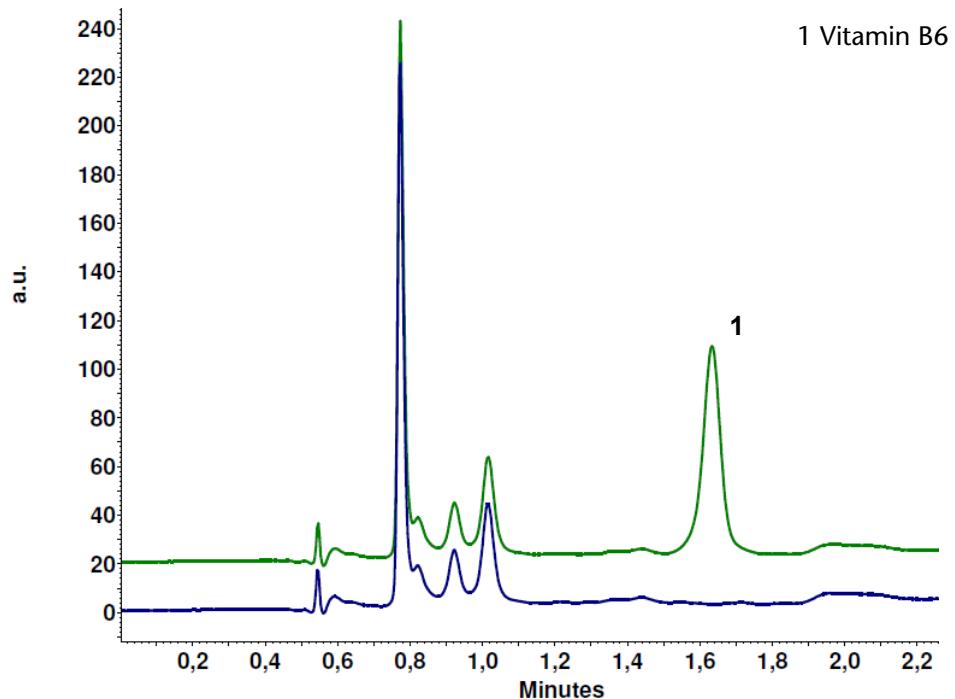


Fig. 4

Chromatograms for the vitamin B6 analysis,
blue: sample,
green: sample spiked with
vitamin B6.

With these results the method was found to be well suited for the analysis of vitamin B6. To prove the robustness, the next step was to evaluate the reproducibility. 5 replicate runs were performed and analyzed statistically according the retention time and peak area of vitamin B6. An overlay of the 5 runs can be seen in figure 5 and table 1 shows the statistical evaluation of the vitamin B6 peak.

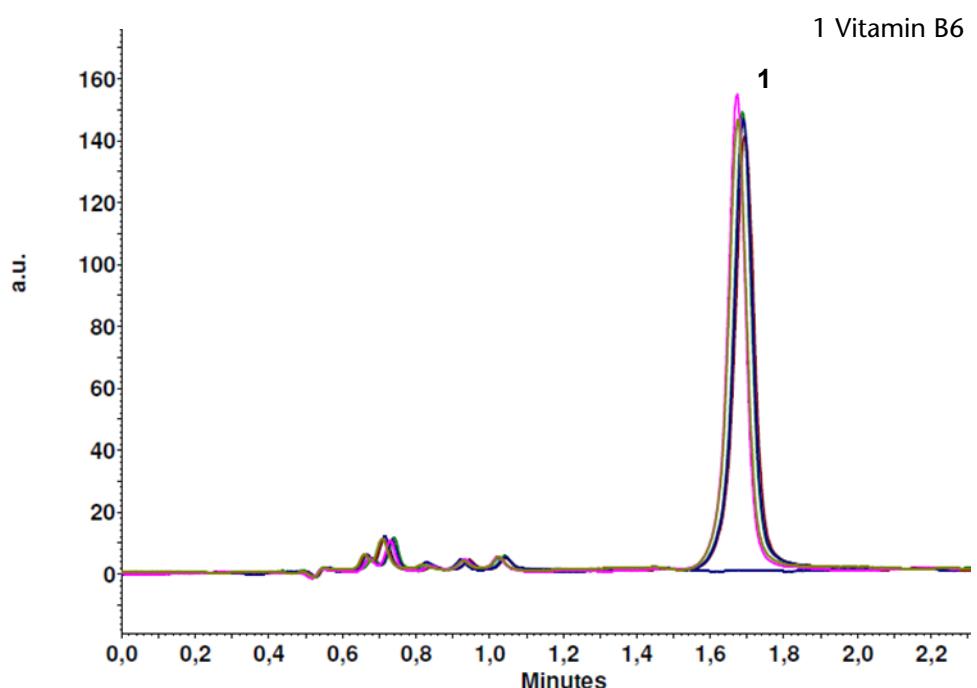


Fig. 5

Overlay of the chromatograms of the 5 replicate runs and a blank measurement of pure water (blue).

Table 1

Statistical evaluation of the vitamin B6 peak regarding retention time and peak area to prove the method's robustness.

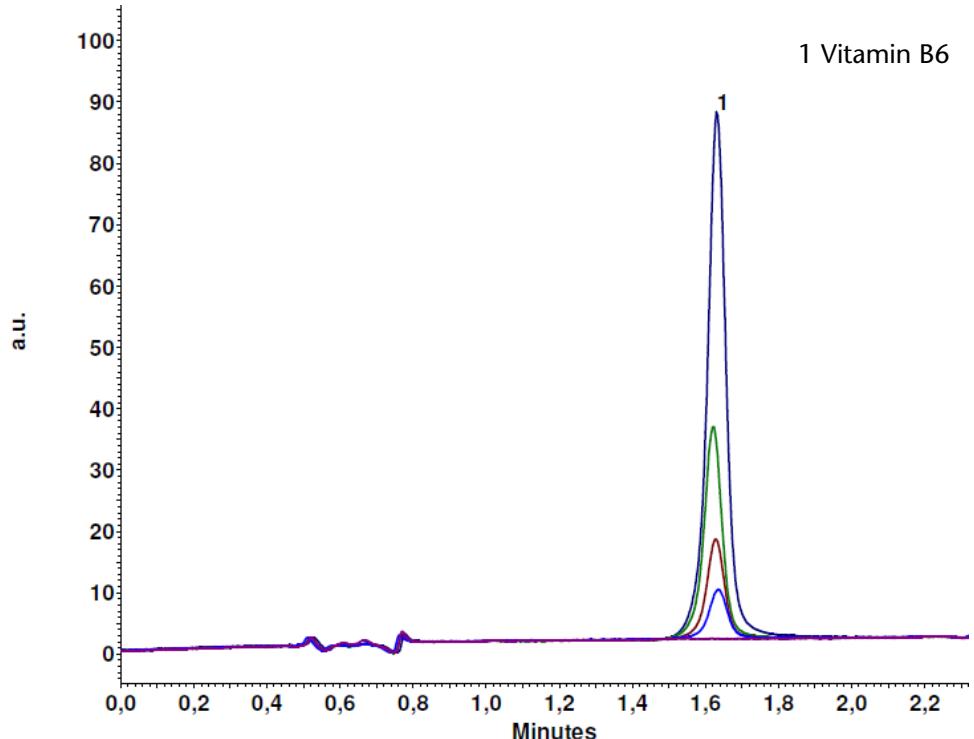
The relative standard deviation lies far below 1 % for both retention time and peak area. With this result, the method can be regarded as robust and reliable.

RF-20A/Axs [290/400 nm]	Vitamin B6	Vitamin B6
Data Filename	Retention Time	Area
130717_VitB6_M5_PS_C18AQ_125x3_probe_gespiked_1µl_001.dat	1,687	554696
130717_VitB6_M5_PS_C18AQ_125x3_probe_gespiked_1µl_002.dat	1,673	548481
130717_VitB6_M5_PS_C18AQ_125x3_probe_gespiked_1µl_003.dat	1,673	547238
130717_VitB6_M5_PS_C18AQ_125x3_probe_gespiked_1µl_004.dat	1,690	554950
130717_VitB6_M5_PS_C18AQ_125x3_probe_gespiked_1µl_005.dat	1,693	551819
	Min:	1,673
	Max:	547238
	Mean:	1,683
	Std Dev:	0,009
	%RSD:	0,56
		554950
		551437
		3517
		0,64

Calibration:

To evaluate the detection limit of this method, a calibration was done. Standards were prepared from the vitamin B6 stock solution by dilution in deionized water. The resulting concentrations were 26, 52, 104 and 260 nmol/l. An Overlay of the corresponding chromatograms can be seen in figure 6.

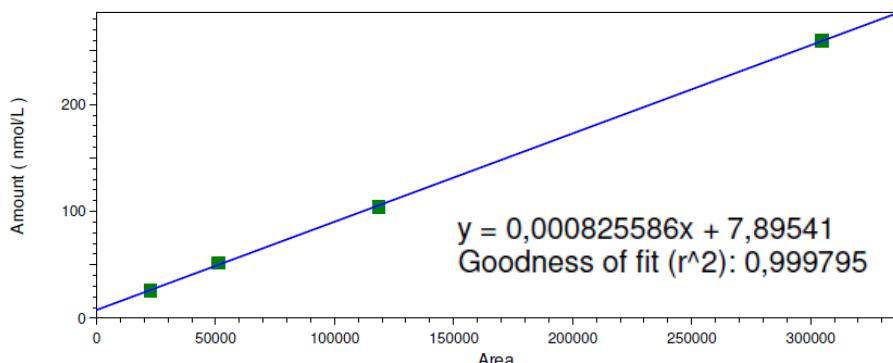
With these results, a calibration curve was evaluated and the result is shown in figure 7. The goodness of fit was in the range of > 0,999 so that a linear calibration could be fitted very well.

**Fig. 6**

Overlay of the chromatograms from the vitamin B6 calibration.

Fig. 7

Calibration curve from the vitamin B6 calibration



It is obvious that even the standard with the lowest concentration of 26 nmol/L could be analyzed well and therewith a really low detection limit could be reached. The calculated LOD ($S/N = 3$) lies in the range of 1 nmol/l and the LOQ ($S/N = 9$) in the range of 3 nmol/l.

Method performance

Limit of detection 1 nmol/L range ($S/N = 3$)

Goodness of linearity fit (r^2) > 0.999

Retention time precision* < 0.6 % RSD

Peak area precision* < 0.7 % RSD

*repeatability calculated over 5 replicate runs

Conclusion

The ProntoSIL 120-5 C18 AQ column is well suited for the analysis of vitamin B6. With the given method parameters, the user is able to run the analysis easily and in less than 5 minutes including a washing step of the column. The flow rate was adjusted to 1.2 ml/min to optimize the analysis time. If the method should be speed up more, it is also possible to use a smaller particle size column with a shorter length in a smaller column dimension. But also with the presented 5 μ m column particle and the 125 mm column length a high speed retention time of less than 2 min of the vitamin B6 is realizable.

The presented method is well suited for the analysis of vitamin B6 in blood samples. The robustness and the reliability were proven and a linear calibration as well as an excellent detection limit could be reached. No additional post column derivatization is needed what makes this method also very easy and uncomplicated. The target compound is already derivatized for fluorescence detection by the well-chosen mobile phase.

References

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Authors

Mareike Margraf, Columns and Applications Department, KNAUER

Dr. Silvia Marten, Head of Columns and Applications Department, KNAUER

Physical properties of recommended column



ProntoSIL C18 AQ with its unique bonding technology has been especially developed for use with aqueous mobile phases with an organic content below 10%. ProntoSIL C18 AQ gives excellent peak shapes in these mobile phases resulting in enhanced selectivities. The advantages of the AQ-packings can be demonstrated in applications of polar analytes.

Stationary phase	ProntoSIL 120-5 C18 AQ
USP code	L1
Pore size	120 Å
Pore volume	0.8 ml/g
Specific surface area	300 m ² /g
Particle size	5 µm
Form	spherical
% C	14
Endcapping	yes
Dimensions	125 x 3 mm with integrated precolumn
Order number	12XF184PSJ

Recommended instrumentation



This application requires the PLATINblue binary high pressure gradient UHPLC system equipped with degasser, autosampler, column thermostat and fluorescence detector. Other configurations are also available. Please contact KNAUER to configure a system that's perfect for your needs.

Description	Order No.
PLATINblue UHPLC Pump P-1 with SmartMix 100	A60013
PLATINblue UHPLC Pump P-1 with Degasser	A60014
PLATINblue HPG kit to connect Two P-1 to a HPG System	A60017
PLATINblue UHPLC Autosampler AS-1	A63501
PLATINblue Column Thermostat T-1	A63412
PLATINblue modular eluent tray	A60900
PLATINblue Accessory Kit with Flasks, Screwcaps and cable protective tubes	A64387
PLATINblue stainless steel capillary kit	A64386-1
PLATINblue Tool Kit	A64330
Fluorescence detector RF-20Axs 200 – 750 nm incl. Accessories	A59201
Interface Box IFU 2.1 USB	AZB00
Premium PC English Windows 7	A1322E
Monitor TFT 19" 4:3 Bild	A1612
Netgear ProSafe Router	A64808
OpenLAB CDS EZChrom Edition Workstation for one System	A2605-1

Contact information

Wissenschaftliche Gerätebau
Dr. Ing. Herbert Knauer GmbH
Hegauer Weg 38
14163 Berlin, Germany

Tel: +49 30 809727-0
Fax: +49 30 8015010
E-Mail: info@knauer.net
Internet: www.knauer.net