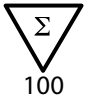
Manual

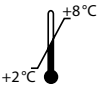
Vitamin B_6 HPLC Kit

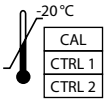
For the determination of vitamin B_6 in plasma, serum, whole blood and dried blood spots

Valid from 2023-07-31

REF **KCR2100**







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Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: +49 6251 70190-0 Fax: + 49 6251 70190-363
e.mail: info@immundiagnostik.com www.immundiagnostik.com

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1. INTENDED USE

This HPLC application is intended for the quantitative determination of vitamin B_6 in plasma, serum, EDTA whole blood and dried blood spots (DBS). or research use only. Not for use in diagnostic procedures.

2. SUMMARY AND EXPLANATION OF THE TEST

Vitamin B_6 is the term for pyridoxin, pyridoxal and pyridoxamin and the appropriate phosphate products. All forms can be transformed into the active form pyridoxal-5-phosphate (here referred to as vitamin B_6).

Vitamin B_6 functions as a coenzyme and is essential for more than 50 reactions in the protein metabolism thereby synthesizing, transforming or degrading amino acids. Vitamin B_6 supports the resorption of amino acids and their transport into the cells. Furtheron vitamin B_6 contributes to the synthesis of neuro transmitters and amine products (histamin).

Vitamin B_6 is involved in a variety of different metabolic reactions. This leads to a wealth of possible research fields around the topic of vitamin B_6.

Possible fields of research:

- determination of vitamin B_6 status
- homocysteinaemia
- skin diseases
- movement disorders
- Anaemia, depression

3. PRINCIPLE OF THE TEST

The first step in the determination of vitamin B_6 includes the sample preparation with additional derivatisation. During the precipitation, higher molecular substances are removed. The supernatant is used for derivatisation (5 min at 60 °C), thereby transforming the vitamin B_6 into a fluorescent product. The sample is then injected into the HPLC system.

The separation via HPLC follows an isocratic method at 30 °C using a reversed phase column; one run lasts about 7 minutes. The quantification is performed with the delivered calibrator; the concentration is calculated via integration of the peak area/ heights.

Summary

This HPLC application for the determination of vitamin B_6 allows to determine the vitamin in an easy, fast and precise method. The kit includes all reagents in ready to use form for preparation and separation of the samples with exception of the columns.

As for many other parameters, the advantage of HPLC analytics is the simultaneous handling of many analytes in a single test. The HPLC complete system enables even laboratories without experience in high performance liquid chromatography to use this technique for scientific chemical routines quickly and precisely. Mostly, a one-point calibration is sufficient for calibrating the test system – unlike immunoassays with up to 6 calibrators per test. It is possible to automate the sample application and calculation of the results so that even higher number of samples can be handled nearly without control. With short test series, the one-point calibration is much more economic than 6-point calibration for immunoassays.

4. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KR 0005.15	RECSOL	Reconstitution solution	1 x 15 ml
KCR0003.40	PREC	Precipitation reagent (serum, plasma, whole blood, DBS)	1 x 40 ml
KCR2100	MOPHA	Mobile phase, ready to use (can be recirculated)	1 x 1 000 ml
	CAL	Calibrator; lyophilised (see specification data sheet for concentration)	4 x
	DER	Derivatisation solution (contains KCN)	1 x 26 ml
	CTRL1	Control1; lyophilised	4 x
	CTRL2	Control2; lyophilised	4 x

For reorders of single components, use the catalogue number followed by the label as product number.

The HPLC column (KCR2100RP), can be ordered separately from Immundiagnostik. To extend the lifetime of your HPLC column, pre-columns (KCR2100VS) are highly recommended. These and also the pre-column holders (KCR2100VH) can also be ordered from Immundiagnostik. In addition to the complete kits, all components can also be ordered separately. Please ask for our single component price list.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- 1.5 ml reaction tubes (e. g. Eppendorf)
- Centrifuge
- Various pipettes
- Vortex
- HPLC with fluorescence detector
- Reversed phase C_{18} column
- Thermoblock
- Ultrasonic bath

6. STORAGE AND PREPARATION OF REAGENTS

- **The lyophilised calibrator** (**CAL**) is stable at **-20 °C** until the expiry date stated on the label. Before use, the CAL has to be reconstituted in x ml (x = see the enclosed product specification for the volume needed) **reconstitution solution (RECSOL)**. The concentration of vitamin B_6 slightly changes from lot to lot, please refer to the enclosed product specification sheet for the exact amount.
- **The lyophilised controls 1 and 2** (**CTRL 1 and CTRL 2**) are stable at **-20 °C** until the expiry date stated on the label. Before use, they have to be reconstituted with each x ml (x = see the enclosed product specification for the volume needed) **reconstitution solution (RECSOL)**. The concentration of vitamin B_6 slightly changes from lot to lot, the exact concentration is stated on the specification data sheet.
- All other test reagents are ready-to-use. Test reagents are stable until the expiry date (see label) when stored at **2–8 °C**.

7. SPECIMEN COLLECTION AND PREPARATION

Plasma, serum, EDTA-whole blood, CPDA-whole blood and dried blood spots are suitable for use in the assay.

Sample storage

Vitamin B_6 is light- and temperature-sensitive; therefore, samples have to be protected from light, cooled and centrifuged immediately.

The samples are stable in the dark at 2–8 °C for one week. For longer storage, samples can be frozen and kept at -20 °C for up to 6 months.

Dried blood spots

Collection and storage of dried blood spots

50 µl whole blood dripped on a dried sample carrier cleared by Immundiagnostik AG are suitable as sample material after complete drying. We recommend DrySpot-ID (catalogue no. DZR9020ID or DZR9021ID) as dried blood spot carrier. The moistened cards are stable for 7 days at room temperature. For longer storage, store at -20 °C in a dry place.

8. ASSAY PROCEDURE

Test procedure

Plasma, serum and whole blood	**DBS**
Pipet **100 µl calibrator**, **control 1 and 2 and samples** into 1.5 ml reaction tubes (e. g. Eppendorf).	Cut each **DBS** into 4 parts and put into a 1.5 ml reaction tube (e. g. Eppendorf).
Add **300 µl precipitation reagent** (PREC) and **vortex** for at least **30 s.**	Add **400 µl precipitation reagent** (PREC) and **cover the DBS completely.**
	Extract for **5 min** in the ultrasonic bath.
Centrifuge for **5 min** at **10 000 *g*.**	
Add **250 µl derivatisation solution** (DER) to **150 µl supernatant** and mix.	
Incubate for **5 min** at **60 °C.**	
The **derivatised solution** is stable for 5 days at 2–8 °C in the dark.	
Inject 50 µl of the solution into the HPLC.	**Inject 100 µl** of the solution into the HPLC.

Chromatographic conditions

Column material:	Prontosil Eurobond, 5 µm	
Column dimension:	125 × 4 mm	
Flow rate:	1.2 ml/min	
	Please refer to the quality certificate of the column	
Fluorescence detection:	Excitation:	320 nm
	Emission:	415 nm
Temperature:	26 °C	
Running time:	6 min	

9. TREATMENT OF THE COLUMN

It is recommended to use a guard column to extend column life.

After analysis, the column should be flushed with 30 ml ultrapure water (1 ml/min) and stored in 50 % methanol in water (~ 30 ml, flow 0.5 ml/min). Before use, the system should be equilibrated with ~ 30 ml mobile phase (MOPHA).

10. RESULTS

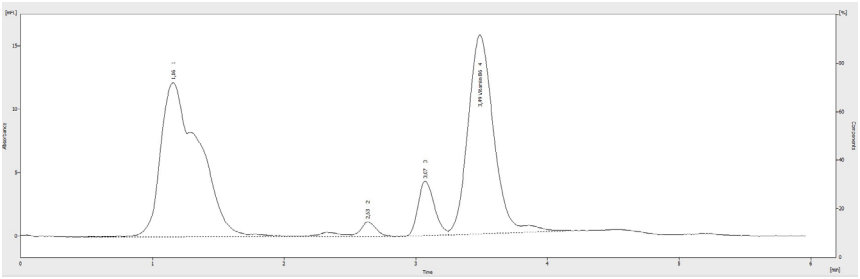
Calculation

$$\text{Sample concentration} = \frac{\text{Peak height sample} \times \text{calibrator concentration*}}{\text{Peak height calibrator}}$$

* see label

Tip: Alternatively, the peak area instead of the peak height can be used for quantification.

Typical chromatogram



11. QUALITY CONTROL

We recommend each laboratory to establish its own reference range.

Controls

Control samples should be analysed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the samples may not be valid if within the same assay one or more values of the quality control sample are outside the acceptable limits.

12. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra assay (n = 3 x 6)

Sample	CV [%]
CAL	4.8
C1	3.8
C2	4.3

Intra assay DBS (n = 3 x 6)

Sample	CV [%]
VB1	5.3
VB2	8.9
VB3	7.5

Inter assay (n = 3 x 12)

Sample	CV [%]
CAL	3.3
C1	4.7
C2	5.6

Inter assay DBS (n = 3 x 12)

Sample	CV [%]
VB1	7.4
VB2	7.2
VB3	6.5

Analytical sensitivity

Lower measuring limit 1 µg/l

Upper measuring limit 400 µg/l

Linearity is given over the entire measuring range. Non-linearity is less than 10 % over this range.

Trueness

n = 2 x 12

Sample	Recovery [%]
C1	99
C2	97

n = 3 x 12 DBS

Sample	Recovery [%]
VB1	96
VB2	107
VB3	87

13. DISPOSAL

The derivatisation solution **(DER)** must be oxidised with hydrogen peroxide, the pH value adjusted to 6–8, and disposed as aqueous salt solution. The mobile phase **(MOPHA)** and the precipitation reagent **(PREC)** must be neutralised with NaOH to neutral pH and disposed as salt solution.

Important: Reaction will produce heat, be careful!

Please refer to the appropriate national guidelines.

14. TROUBLESHOOTING

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signal cord and connection
	Detector lamp is too old	Change lamp
No peaks	Injector is congested	Check injector
Double peaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase
	Air in the system	Check pump, purge the system
	Auto sampler vials contaminated	Use new vials or clean them with methanol

Problem	Possible reason	Solution
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump conveys inaccurately	Check pump, purge the system
	System is not in steady state yet	Rinse system mobile phase for 15 min
Baseline is drifting	Detector lamp did not reach working temperature yet	Wait
	Detector lamp is too old	Renew lamp
	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump conveys inaccurately	Check pump, purge the system
Baseline is not smooth	Pump conveys inaccurately	Check pump, degas the system
	Detector flow cell is dirty	Clean flow cell

15. PRECAUTIONS

- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- As the derivatisation solution (DER) contains KCN, it should be pipetted under a fume hood.
- The precipitation reagent consists of an acid. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped up immediately with copious quantities of water. Do not breath vapour and avoid inhalation.
- The test components contain organic solvents. Contact with skin or mucous membranes must be avoided.

16. GENERAL NOTES ON THE TEST

- All reagents in the kit package are for research use only.
- Do not use reagents beyond the expiration date stated on the kit label.
- Do not interchange different lot numbers of any kit component within the same assay.
- Follow the guidelines for laboratories.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which has not been consulted with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.
- Warranty claims and complaints regarding deficiencies must be lodged within 14 days after receipt of the product. The product should be sent to Immundiagnostik AG along with a written complaint.
- Analyze controls with each run.
- Always perform the assay according to the enclosed manual.
- Serious incidents are to be reported to Immundiagnostik AG and the national regulatory authorities. Warranty claims and complaints regarding deficiencies must be logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.
- The assay should always be performed according to the enclosed manual.

17. REFERENCES

1. Ambrosch, A. et al., 2001. Relation between homocysteinaemia and diabetic neuropathy in patients with Type 2 diabetes mellitus. *Diabetic medicine : a journal of the British Diabetic Association*, **18**(3), pp.185–92.
2. Dierkes, J., Domröse, U., et al., 2001. Homocysteine lowering effect of different multivitamin preparations in patients with end-stage renal disease. *Journal of renal nutrition : the official journal of the Council on Renal Nutrition of the National Kidney Foundation*, **11**(2), pp.67–72.
3. Dierkes, J., Westphal, S., et al., 2001. Vitamin supplementation can markedly reduce the homocysteine elevation induced by fenofibrate. *Atherosclerosis*, **158**(1), pp.161–4.

Symbol explanation:


Temperature limitation

For research use only


Manufacturer

Lot number


Contains plasma derivatives or human blood

Consult specification data sheet

Unique Device Identification


Medicinal substance

Catalogue number

To be used with


Content sufficient for <n> tests


Use by


Consult instructions for use


Do not re-use

Contains material of animal origin

Contains material of human origin

Immundiagnostik AG

Stubenwald-Allee 8a
64625 Bensheim, Germany

Tel.: +49 6251 70190-0
Fax: +49 6251 70190-363

info@immundiagnostik.com
www.immundiagnostik.com