CHROMSYSTEMS

VITAMINSTATUS
VITAMIN PROFILING
DOSAGE DE VITAMINES
PROFILO DELLE VITAMINE
PERFIL DE VITAMINAS

Instruction Manual for HPLC Analysis

Vitamin B6 in plasma/serum
Vitamin B6 in whole blood

31000

CE 0123 IVD

Incident reporting:
Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Chromsystems Instruments & Chemicals GmbH is certified according to ISO 13485 (including MDSAP). Products are produced and put into circulation according to regulation (EU) 2017/746 on in vitro diagnostic medical devices (IVDR).

You can download the declaration of conformity according to IVDR from the download centre of our website.

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Contents.....Page

1 Ordering information 4
1.1 .... Kits 4
1.2 .... Individual components 5

2 Introduction 7
2.1 .... Background information 7
2.2 .... Principle of the assay 8
2.3 .... Intended purpose 9
2.4 .... Clinical limitations 9

3 HPLC system 10
3.1 .... Additionally required equipment 10
3.1.1 ... Essential equipment 10
3.1.2 ... Optional/recommended equipment 10
3.2 .... Instrument parameters 11
3.3 .... HPLC column 11
3.4 .... System start-up 11
3.5 .... System shutdown 12

4 Chromatographic separation 12

5 Sample preparation 12
5.1 .... Collection and storage of patient specimens 13
5.2 .... Reconstitution of the calibrators 14
5.3 .... Reconstitution of the controls 15
5.4 .... Sample preparation procedure 16
5.5 .... Storage life of prepared samples 16
5.6 .... Handling of samples above the analytical measuring range 17

6 Quality control 17

7 Data acquisition and evaluation 18
7.1 .... Calibration of the analysis system 18
7.2 .... Examples of chromatograms 18
7.3 .... Conversion factors 20
7.4 .... Manual calculation 20

8 Storage and lifetime of the assay components 21

9 Waste disposal 21

10 Reference ranges 22

11 Interference testing 23
11.1 .. Interferences detected 27
11.2 .. No interference detected 27

12 Troubleshooting 29

13 Literature 30

Contents.....Page

Appendix I	Substance information	32
Appendix II	Analytical performance data	34
Appendix III	Clinical performance data	37
Appendix IV	Traceability of the calibrators	40
Appendix V	Symbols	44
Appendix VI	Version history	45

1 Ordering information

1.1 Kits

Reagent kit for the analysis of plasma and serum:

31000/S	**HPLC Reagent Kit** **Vitamin B6 in plasma/serum** with Plasma Calibration Standard	
	Kit content for 100 analyses:	
	Mobile Phase	1 x 1000 mL
	Plasma Calibration Standard	5 x 1.0 mL (lyoph.)
	Precipitation Reagent	1 x 30 mL
	Neutralisation Reagent	1 x 25 mL
	Derivatisation Reagent	1 x 10 mL
	Reaction Vials 1.5 mL, amber colour (light protection)	2 x 100 pcs.

The following CE/IVD products and accessories are not included in the kit 31000/S but are required for the application of the method:

31100	HPLC Column (equilibrated, with test chromatogram)	1 pc.
0038	Plasma Control Level I	5 x 2.0 mL (lyoph.)
0039	Plasma Control Level II	5 x 2.0 mL (lyoph.)

Reagent kit for the analysis of whole blood:

31000/WB	**HPLC Reagent Kit** **Vitamin B6 in whole blood** with Whole Blood Calibration Standard	
	Kit content for 100 analyses:	
	Mobile Phase	1 x 1000 mL
	Whole Blood Calibration Standard	5 x 1.0 mL (lyoph.)
	Precipitation Reagent	1 x 30 mL
	Neutralisation Reagent	1 x 25 mL
	Derivatisation Reagent	1 x 10 mL
	Reaction Vials 1.5 mL, amber colour (light protection)	2 x 100 pcs.

The following CE/IVD products and accessories are not included in the kit 31000/WB but are required for the application of the method:

31100	HPLC Column (equilibrated, with test chromatogram)	1 pc.
0023	Whole Blood Control Level I	5 x 2.0 mL (lyoph.)
0024	Whole Blood Control Level II	5 x 2.0 mL (lyoph.)

Basic kit for sample preparation

31000-BK	**HPLC Basic Kit** **Vitamin B6 in plasma/serum/whole blood**	
	Kit content for 100 sample preparations:	
	Precipitation Reagent	1 x 30 mL
	Neutralisation Reagent	1 x 25 mL
	Derivatisation Reagent	1 x 10 mL
	Reaction Vials 1.5 mL, amber colour (light protection)	2 x 100 pcs.

The following CE/IVD products and accessories are not included in the kit 31000-BK but are required for the application of the method:

For the analysis of plasma and serum

31100	HPLC Column (equilibrated, with test chromatogram)	1 pc.
31001	Mobile Phase	1000 mL
36005	Plasma Calibration Standard Vitamin B6 in plasma/serum	5 x 1.0 mL (lyoph.)
0038	Plasma Control Level I	5 x 2.0 mL (lyoph.)
0039	Plasma Control Level II	5 x 2.0 mL (lyoph.)

For the analysis of whole blood

31100	HPLC Column (equilibrated, with test chromatogram)	1 pc.
31001	Mobile Phase	1000 mL
31003	Whole Blood Calibration Standard Vitamin B6 in whole blood	5 x 1.0 mL (lyoph.)
0023	Whole Blood Control Level I	5 x 2.0 mL (lyoph.)
0024	Whole Blood Control Level II	5 x 2.0 mL (lyoph.)

1.2 Individual components

CE/IVD products and accessories

For sample preparation:

31004	Precipitation Reagent	30 mL
31005	Neutralisation Reagent	25 mL
31006	Derivatisation Reagent	10 mL

For chromatography:

31001	Mobile Phase	1000 mL
31002	Mobile Phase	10 x 1000 mL
31100	HPLC Column (equilibrated, with test chromatogram)	1 pc.
15011	PEEK-encased Prefilter 2 µm	5 pcs.
18010	Precolumn Cartridge 4/10	1 pc.

For calibration and quality control:

31003	Whole Blood Calibration Standard Vitamin B_6 in whole blood	5 x 1.0 mL (lyoph.)
0022	Whole Blood Control Bi-Level (I+II)	2 x 5 x 2.0 mL (lyoph.)
0023	Whole Blood Control Level I	5 x 2.0 mL (lyoph.)
0024	Whole Blood Control Level II	5 x 2.0 mL (lyoph.)
36005	Plasma Calibration Standard Vitamin B_6 in plasma/serum	5 x 1.0 mL (lyoph.)
0031	Plasma Control Bi-Level (I+II)	2 x 5 x 2.0 mL (lyoph.)
0038	Plasma Control Level I	5 x 2.0 mL (lyoph.)
0039	Plasma Control Level II	5 x 2.0 mL (lyoph.)

Non-CE/IVD products

33005	Reaction Vials 1.5 mL, amber colour (light protection)	100 pcs.
15010	PEEK Prefilter Housing	1 pc.
18001	Precolumn Cartridge Holder 4/10	1 pc.

2 Introduction

2.1 Background information

B vitamins play an essential role in a number of basic metabolic pathways. They are among the water-soluble vitamins that are poorly stored in the body, which is why a regular supply by food or dietary supplements is essential to avoid deficiency symptoms.

Vitamin B6
The term vitamin B6 (Figure 1) encompasses three naturally occurring pyridine derivatives: pyridoxine, pyridoxamine and pyridoxal as well as their 5'-phosphate esters pyridine 5'-phosphate, pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate. The most important active coenzyme form is the pyridoxal 5'-phosphate (PLP), which is also the main component of the variations listed.

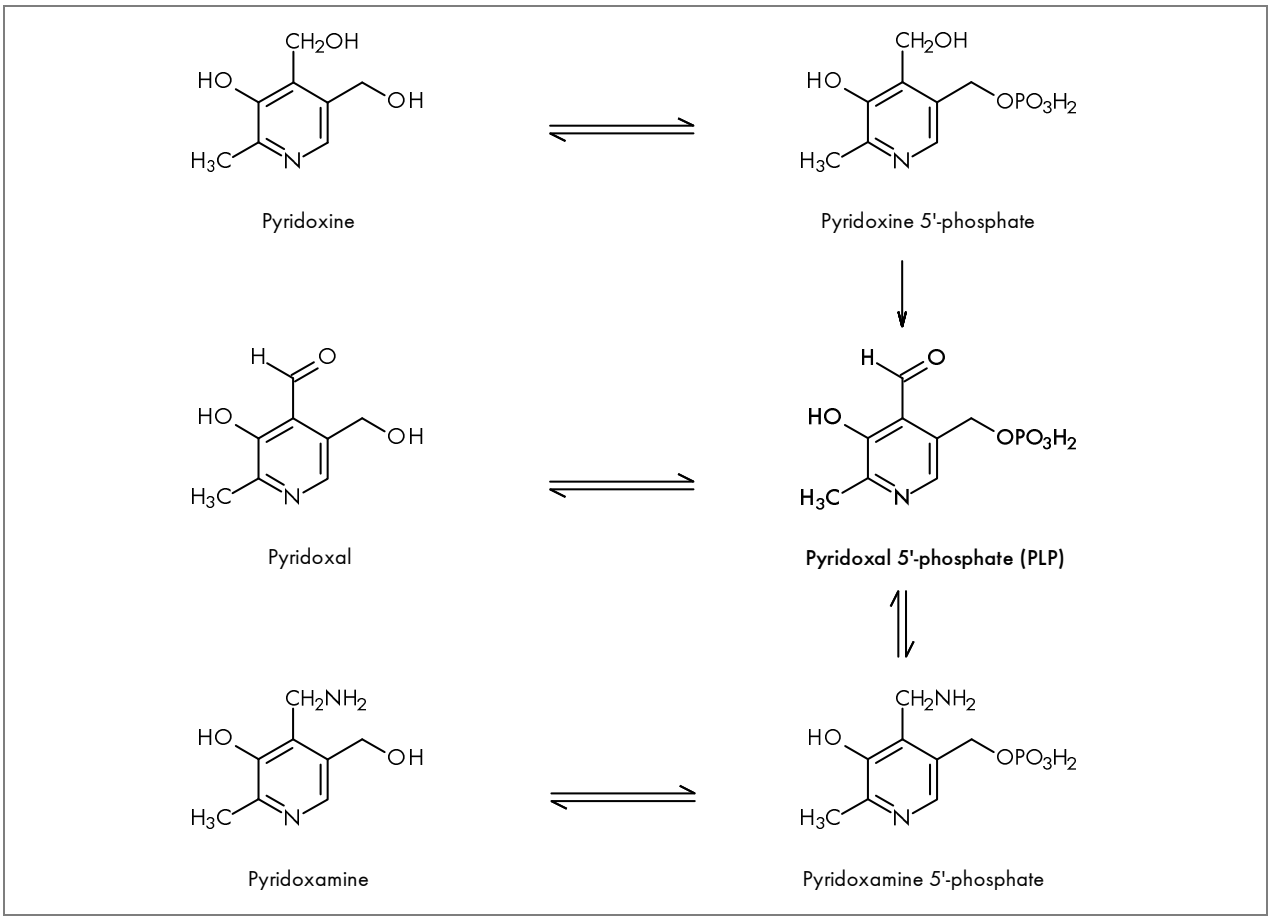

Figure 1: The Vitamin B6 group (modified after [1])

The substances of the vitamin B6 group are ingested with plant and animal food; phosphorylated compounds are converted into their free forms in the small intestine with the help of alkaline phosphatase and absorbed. The active coenzyme forms are mainly generated in the liver by ATP-dependent phosphorylation. PLP is released into the blood stream, largely bound to albumin. Alkaline phosphatase catalyses the hydrolysis of the PLP-albumin complex into free pyridoxal. Upon entering the cells, pyridoxal is phosphorylated to PLP by pyridoxal kinase. PLP is then available for cellular metabolism [2].

Pyridoxal 5'-phosphate is a co-factor in many enzymatic reactions of carbohydrate, protein and lipid metabolism and for synthesis of neurotransmitters, in particular, the formation of serotonin from tryptophan in the brain and of nicotinamide from tryptophan. It also plays a central role in amino acid synthesis and degradation [3,4].

Vitamin B6 deficiency can cause skin diseases including seborrheic dermatitis with cheilosis and glossitis, angular stomatitis as well as brain-specific symptoms such as irritability, depression and dementia [3]. Because of the direct involvement of vitamin B6 in the conversion of the amino acid homocysteine to cysteine, vitamin B6 deficiency leads to a rise in the concentration of homocysteine and an increased risk of arteriosclerosis.

Vitamin B6 deficiency
Our modern diet differs from the evolutionary diet, which was mainly plant-based consisting of vegetables, fruits and nuts, along with small amounts of fish and meat. Energy-rich, easily digestible and micronutrient-poor nutrition leads to vitamin deficiency even in developed societies and is linked to obesity, cardiovascular diseases and dementia [5]. Evaluation of the vitamin status should also be considered in the case of unbalanced diets.

In addition, certain diseases are associated with vitamin deficiency. Patients with diabetes mellitus type 2 are often deficient in vitamin B6. Particularly in the case of incipient nephropathy, pronounced changes in vitamin B6 metabolism and low plasma PLP concentrations can be detected [6].

People with alcohol-use disorder, renal dialysis patients, elderly, pregnant women, as well as patients after surgery are at high risk for vitamin B6 deficiency. Disturbances in absorption, e.g. in inflammatory bowel diseases or surgical procedure such as bariatric surgery also lead to a reduced supply of important nutrients [3,7].

Some medications e.g. chemotherapeutic agents, diuretics, isoniazid, and metformin interfere with vitamin B metabolism and may contribute to deficiency [3,8].

Vitamin B6 excess
Excess amounts of water-soluble B vitamins are excreted in the urine. Large oral supplemental doses of vitamin B6 can cause sensory neuropathy with ataxia or areflexia, impaired cutaneous and deep sensation and dermatological lesions [3].

Patients with hypophosphatasia, a rare inherited disorder of bone and mineral metabolism, have high levels of PLP which can be at least five-fold higher than in healthy individuals. Due to low activity of tissue alkaline phosphatase (TNSALP), there is an extracellular accumulation of the three TNSALP-substrates PLP, inorganic pyrophosphate (PPi) and phosphoethanolamine (PEA) [9].

2.2 Principle of the assay

The present reagent kit allows the rapid, easy and specific determination of vitamin B6 (pyridoxal 5'-phosphate, PLP) using a simple isocratic HPLC system. Instead of the usual, technically demanding post-column derivatisation, simple precolumn derivatisation is used. **Plasma/serum as well as whole blood specimens (after addition of EDTA or heparin as anticoagulant) are suitable for this kit.**

The sample clean-up procedure includes an effective protein precipitation and extraction step with optimised conditions to liberate PLP from its bound status. Subsequent derivatisation (water bath, 60 °C) produces a fluorescent PLP-derivative. The chromatographic determination is run on an isocratic HPLC system with fluorescence detection. By this sample clean up procedure highly stable extracts are achieved, allowing the analysis of large batches.

2.3 Intended purpose

The Chromsystems reagent kit 31000/S "Vitamin B6 in plasma/serum" is an in vitro diagnostic medical device for professional use in clinical laboratories for the quantitative detection of the physiologically active form of vitamin B6, pyridoxal 5'-phosphate, in human serum or plasma samples.

Sample preparation is carried out manually, and analytic separation is done via high performance liquid chromatography (HPLC).

The test kit is intended to be used for screening and/or monitoring of vitamin B6 levels where indicated
- in patients with suspected Vitamin B6 deficiency,
- in patients with suspected Vitamin B6 excess, and/or
- in patients under Vitamin B6 supplementation therapy.

The Chromsystems reagent kit 31000/WB "Vitamin B6 in whole blood" is an in vitro diagnostic medical device for professional use in clinical laboratories for the quantitative detection of the physiologically active form of vitamin B6, pyridoxal 5'-phosphate, in human whole blood samples.

Sample preparation is carried out manually, and analytic separation is done via high performance liquid chromatography (HPLC).

The test kit is intended to be used for screening and/or monitoring of vitamin B6 levels where indicated
- in patients with suspected Vitamin B6 deficiency,
- in patients with suspected Vitamin B6 excess, and/or
- in patients under Vitamin B6 supplementation therapy.

2.4 Clinical limitations

There are no universally applicable reference ranges for pyridoxal 5'-phosphate. Results obtained using different test methods cannot be compared. Laboratories should indicate the method used for analysis to enable accurate interpretation of the results.

Users must specify their own reference ranges based on clinical assessment. Conversion factors between different methods of analysis should not be used to predict results for a specific patient.

3 HPLC system

Caution: When using the reagents comply with the hazard information in Appendix I.

3.1 Additionally required equipment

In addition to the components included in the Chromsystems reagent kit (order no. 31000/S or 31000/WB), the HPLC analysis of Vitamin B_6 in plasma/serum or whole blood requires the following materials:

3.1.1 Essential equipment

Products directly associated with the kit, available from Chromsystems:

31100 HPLC Column (equilibrated, with test chromatogram)

Required for the analysis of vitamin B_6 in whole blood:
0022 Whole Blood Control Bi-Level (I+II)
0023 Whole Blood Control Level I
0024 Whole Blood Control Level II

Required for the analysis of vitamin B_6 in plasma/serum:
0031 Plasma Control Bi-Level (I+II)
0038 Plasma Control Level I
0039 Plasma Control Level II

Laboratory devices for HPLC analysis, only partially available from Chromsystems:
- Isocratic HPLC system
- Autosampler
- Fluorescence detector

Laboratory devices for sample preparation, not available from Chromsystems:
- Suitable centrifuge
- Vortex mixer
- Water bath

3.1.2 Optional/recommended equipment

Accessories, available from Chromsystems:
15011 PEEK-encased Prefilter 2 µm
18010 Precolumn Cartridge 4/10

General lab-equipment (non-CE/IVD products), available from Chromsystems:
15010 PEEK Prefilter Housing
18001 Precolumn Cartridge Holder 4/10

The PEEK Prefilter Housing (order no. 15010) is intended for combination with the PEEK-encased Prefilter 2 µm (order no. 15011), the Precolumn Cartridge Holder 4/10 (order no. 18001) for combination with the Precolumn Cartridge 4/10 (order no. 18010).

Laboratory devices:

- Thermostatted column compartment

3.2 Instrument parameters

The substances are separated chromatographically using an analytical column (order no. 31100). Keep the Mobile Phase closed or covered even when in use. The use of a thermostatted column compartment will avoid temperature variations and ensure optimal stability and reproducibility of the chromatographic separation.

Instrument settings:

Autosampler:	Magazine should be light protected, amber coloured glass vials
Injection volume:	50 µL
Run time:	8 min
Flow rate:	1.0 mL/min
Column temperature:	+20 to +25 °C
Fluorescence detector:	EX 320 nm, EM 415 nm
Needle rinsing solution for the injector:	Ultrapure water (HPLC grade) with 5–10 % methanol

3.3 HPLC column

The HPLC column for the analysis of pyridoxal 5'-phosphate (PLP) is supplied equilibrated and tested, and is ready for use. The backpressure of a new column at a flow rate of 1.0 mL/min is about 60 to 70 bar and may increase with column age and/or use. As long as the separations are satisfactory, a raised backpressure is of no consequence.

Note:
We recommend the use of a prefilter (PEEK-encased Prefilter 2 µm, order no. 15011) in order to enhance the column life-time. A pre-column (Precolumn Cartridge 4/10, order no. 18010) can be used for additional protection of the column.

The column should only be rinsed with the solutions specified in this instruction manual. Other solvents could irreversibly damage the column.

3.4 System start-up

Before starting a sequence, prepare the HPLC system as follows:

1. Rinse the system with approx. 30 mL of Mobile Phase before installing the HPLC column
2. Install the column and equilibrate the system at a flow rate of 1.0 mL/min for about 10 min with Mobile Phase, until the baseline has stabilised
3. Inject the prepared calibrator several times until the retention times and peak areas/heights are consistent
4. Compare the chromatogram with those in chapter 7.2
5. Start the sequence

Thereafter, the Mobile Phase can be recirculated for approximately 100 injections.

For proper use of your HPLC system, read the instruction manual of your HPLC system. If you have any questions, ask the device manufacturer. Training from the device manufacturer may be required.

3.5 System shutdown

For interruptions in operation of up to 3 days, pump the Mobile Phase at a low flow rate (0.2 mL/min). The HPLC column remains connected in the system. To protect the lamp, turn off the detector.

For longer periods of disuse, the HPLC column should be disconnected. Rinsing or conservation is not necessary. Store the column in the Mobile Phase at +18 to +30 °C. Insert a union to replace the column and rinse the HPLC system using about 50 mL of ultrapure water (HPLC grade)/methanol (80/20).

4 Chromatographic separation

The following table shows the retention time of pyridoxal 5´-phosphate (PLP) at a flow rate of 1.0 mL/min:

Table 1: Retention times

Substance	Retention time (ca.)
Pyridoxal 5'-phosphate (PLP)	3.1 min

Retention times may vary slightly, for instance if there is a change in ambient temperature, if you use a new batch of Mobile Phase or if you replace the HPLC column. Therefore, use a calibration chromatogram to determine current values.

5 Sample preparation

Caution:
When using the reagents comply with the hazard information in Appendix I.

Ensure that within a sequence the used batch of reagents for sample preparation as well as the batch of the calibrator and the controls are not changed.

5.1 Collection and storage of patient specimens

For the analysis of pyridoxal 5'-phosphate (PLP) use whole blood, plasma or serum.

Important notes:
- Blood samples should be taken in the morning on an empty stomach, prior to any medication and in absence of recent alcohol consumption [10].
- Rapid plasma separation after blood sample collection and frozen storage is recommended to ensure reliable PLP measurement [3].
- In certain diseases such as hypophosphatasia, an altered vitamin B_6 level is indicative. In these cases, the intake of vitamin B_6 supplements should be avoided for at least 2 weeks before blood withdrawal as elevated PLP levels caused by supplementation may be misleading [9].
- It should also be noted that PLP levels in plasma or serum might be lower in patients with low albumin (e.g. during states of increased inflammation) or altered alkaline phosphatase activity and long-term NSAID user [3,8].

Both EDTA (K3-EDTA and K2-EDTA) and heparin (lithium, sodium and ammonium heparin) are suitable as anticoagulants. EDTA whole blood/plasma is recommended because heparin whole blood/plasma is much more susceptible to enzymatic falsification of results (both increased and decreased analyte concentrations are possible) and is more prone to clotting, which may reduce pipetting precision.

Sample stability
Storage/transport temperature is the main factor likely to affect sample stability. Other factors identified in preliminary tests include the type of anticoagulant (EDTA or heparin) and differences between donors or blood samples, probably due to differing enzyme status. Stability differences between EDTA types (K2-EDTA, K3-EDTA) or heparin types (lithium heparin, sodium heparin, ammonium heparin) have not been identified. In addition, a donor-dependency was excluded for serum and plasma.

Stability of PLP was determined in K3-EDTA blood (representing both types of EDTA blood) and in lithium heparin blood (representing all three types of heparin blood) from five different donors. For PLP stability in EDTA plasma, heparin plasma and serum a donor pool was applied.

Deviations in analyte concentrations of ≤ 15% from the reference were accepted.

Table 2: Analyte stability in patient samples

Storage temperature	Whole blood (EDTA and Heparin)	EDTA plasma	Heparin plasma	Serum
+20 to +25 °C	24 hours	7 days	2 days	2 days
+2 to +8 °C	4 weeks	4 weeks	7 days	7 days
below –18°C	3 months	3 months	1 month	1 month
Freeze-thaw cycles	1 cycle	1 cycle	1 cycle	1 cycle

There are no restrictions on the use of haemolytic, lipaemic and icteric samples (see chapter 11).

Note:
It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine specific stability criteria for its laboratory.

5.2 Reconstitution of the calibrators

The Chromsystems Plasma Calibration Standard (order no. 36005) and Whole Blood Calibration Standard (order no. 31003) are intended for the calibration of your analysis system. The lyophilised calibrators are single point calibrators. They are based on human plasma or human whole blood. After reconstitution, they are handled in the same manner as a patient sample and are analysed under routine conditions analogous to the respective test procedure.

Prior to sample preparation, reconstitute the plasma or whole blood calibrator as follows:

1. Pipette 1.0 mL high-purity water into the original vial
2. Reconstitute for 10 to 15 min at +20 to +25 °C, swirling repeatedly

Check that the vial contents are homogeneous. If undissolved substances are still visible, extend the reconstitution time. Avoid exposure to direct sunlight.

The calibrator levels are traceable to certified reference material (see Appendix IV). The analyte concentrations in the calibrator are batch-dependent. Individual levels are given in the calibrator leaflet.

Caution
This product is manufactured from pooled human plasma or human whole blood which has been tested by the manufacturer and found negative for infections by the human immunodeficiency virus (HIV), the hepatitis B virus (HBV), the hepatitis C virus (HCV) and the bacterium *Treponema pallidum*. Nevertheless, a potential risk of infection cannot be entirely excluded. Consider all products containing human source material as potentially infectious and exercise the same care in the handling of this product as in the handling of potentially infectious patient samples.

Storage life of the calibrators after reconstitution:
The calibrators dissolved in water have the following storage lives, but not beyond the date indicated on the label:

Table 3: Stability of the plasma calibrator (order no. 36005) after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	24 hours	Light protection, tightly closed
+2 to +8 °C	5 days	Light protection, tightly closed
below –18 °C	6 weeks	Light protection, tightly closed
Freeze-thaw cycles	3 cycles	—

Table 4: Stability of the whole blood calibrator (order no. 31003) after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	24 hours	Light protection, tightly closed
+2 to +8 °C	7 days	Light protection, tightly closed
below –18 °C	3 months	Light protection, tightly closed
Freeze-thaw cycles	3 cycles	—

To avoid unnecessary freeze-thaw cycles, aliquot calibrators before freezing.

5.3 Reconstitution of the controls

The Chromsystems Plasma Controls (order no. 0038, 0039) or Whole Blood Controls (order no. 0023, 0024) are intended to monitor the accuracy and precision of each analytical sequence. They are available in two different concentration levels. The lyophilised controls are based on human plasma or human whole blood. After reconstitution, they are handled in the same manner as a patient sample and are analysed under routine conditions analogous to the respective test procedure.

Prior to sample preparation, reconstitute the plasma or whole blood controls as follows:

1. Pipette 2.0 mL high-purity water into the original vial
2. Reconstitute for 10 to 15 min at +20 to +25 °C, swirling repeatedly

Check that the vial contents are homogeneous. If undissolved substances are still visible, extend the reconstitution time. Avoid exposure to direct sunlight.

The analyte concentrations in the controls are batch-dependent. Individual levels are given in the leaflet accompanying each control.

Caution:
This product is manufactured from pooled human plasma or human whole blood which has been tested by the manufacturer and found negative for infections by the human immunodeficiency virus (HIV), the hepatitis B virus (HBV), the hepatitis C virus (HCV) and the bacterium *Treponema pallidum*. Nevertheless, a potential risk of infection cannot be entirely excluded. Consider all products containing human source material as potentially infectious and exercise the same care in the handling of this product as in the handling of potentially infectious patient samples.

Storage life of the controls after reconstitution:
Controls dissolved in water have the following storage lives, but not beyond the date indicated on the label:

Table 5: Stability of the plasma controls (order no. 0038, 0039) after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	24 hours	Light protection, tightly closed
+2 to +8 °C	5 days	Light protection, tightly closed
below –18 °C	6 weeks	Light protection, tightly closed
Freeze-thaw cycles	3 cycles	—

Table 6: Stability of the whole blood controls (order no. 0023, 0024) after reconstitution

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	24 hours	Light protection, tightly closed
+2 to +8 °C	7 days	Light protection, tightly closed
below –18 °C	3 months	Light protection, tightly closed
Freeze-thaw cycles	3 cycles	—

To avoid unnecessary freeze-thaw cycles, aliquot controls before freezing.

5.4 Sample preparation procedure

Important:
Sufficient light protection during the whole sample preparation is essential. The samples must not be exposed to direct sunlight.

Before sample preparation, allow reagents/calibrators/controls/samples that are stored frozen or refrigerated to reach ambient temperature and mix thoroughly.

To prepare patient samples, controls and calibrators for analysis, work through the following steps in the order given:

1. To 200 µL of sample/calibrator/control add 300 µL Precipitation Reagent (order no. 31004) in an amber coloured Reaction Vial (order no. 33005), vortex-mix for at least 30 s
2. Incubate for 10 min at +2 to +8 °C, then centrifuge for 5 min at 16000 x g
3. Transfer 250 µL supernatant into a new amber coloured Reaction Vial (order no. 33005)
4. Add 250 µL Neutralisation Reagent (order no. 31005), mix briefly (a precipitation will form, do not centrifuge)
5. Add 100 µL Derivatisation Reagent (order no. 31006), mix briefly
6. Incubate for 20 min at 60 °C (water bath)
7. Cool the sample in cold water, then incubate for 10 min at +2 to +8 °C
8. Centrifuge for 2 min at 16000 x g
9. Transfer supernatant into an amber coloured autosampler glass vial, inject 50 µL into the HPLC system.

5.5 Storage life of prepared samples

Samples prepared for analysis as indicated in chapter 5.4 have the following storage life:

Table 7: Storage life of the prepared samples

Storage temperature	Storage life	Other storage conditions
+20 to +25 °C	5 days	Light protection, tightly closed, glass vials
+2 to +8 °C	7 days	Light protection, tightly closed, glass vials
below –18 °C	2 weeks	Light protection, tightly closed, glass vials
Freeze-thaw cycles	1 cycle	—

5.6 Handling of samples above the analytical measuring range

Patient samples whose analyte concentrations are above the analytical measuring range (see Appendix II) should be handled as follows:

Plasma and serum:
Prior to sample preparation, dilute the original patient sample with high-purity water at a ratio up to 1:100 so that the analysis result regardless of the dilution factor is within the measuring range of the method. When calculating the analyte concentrations of the samples, the dilution factor must be taken into account.

Whole blood:
Prior to sample preparation, dilute the original patient sample with Whole Blood Control Level I (order no. 0023) at a ratio up to 1:100. The measured analysis result of this mixture has to be within the measuring range of the assay.

When calculating the analyte concentrations of the samples, the dilution factor and the analyte concentration of the quality control must be taken into account. This is done using the following formula:

$$Conc.\ sample = \left\{value\ measured - \left(1 - \frac{1}{dilution\ factor}\right) \cdot target\ value\ quality\ control\right\} \cdot dilution\ factor$$

Example for the calculation of a 1:100 dilution:

$$Conc.\ sample = \left\{value\ measured - \left(1 - \frac{1}{100}\right) \cdot target\ value\ quality\ control\right\} \cdot 100$$

6 Quality control

Monitor precision and accuracy of the analyses by including additional controls (plasma controls, order no. 0038, 0039, or whole blood controls, order no. 0023, 0024) in each analytical run. If the analysis of these controls yields values outside the range given on the accompanying information leaflet, check the system. If the discrepancy continues to exist, re-calibrate the system.

Monitor quality of chromatographic separation by comparison of retention times and chromatographic peak shapes of the analyte with the chromatogram of the column certificate or with an example chromatogram (chapter 7.2). In case of a column in use, compare to preceding analytical runs of the same assay (e.g. in the course of system start-up, chapter 3.4). Significant deviations might be due to decreasing performance of the prefilter, precolumn and/or analytical column. Typical indicators would be tailing of the peaks.

For more information, see chapter 12 Troubleshooting.

7 Data acquisition and evaluation

7.1 Calibration of the analysis system

Run a calibration of the analysis system for each series of measurements. Use the plasma calibrator (order no. 36005) for the analysis of plasma/serum samples and the whole blood calibrator (order no. 31003) for the analysis of whole blood samples. The concentration of the analyte in the calibrator is batch-dependent. Exact levels are given in the package leaflet.

Calibration curves are constructed by calculating the analyte peak area or peak height on the y-axis against calibrator concentration on the x-axis. Then plot a calibration curve through origin (single point calibration).

Select the external standard method for calibration in your analysis system.

7.2 Examples of chromatograms

The following graphs provide examples of chromatograms created using this method.

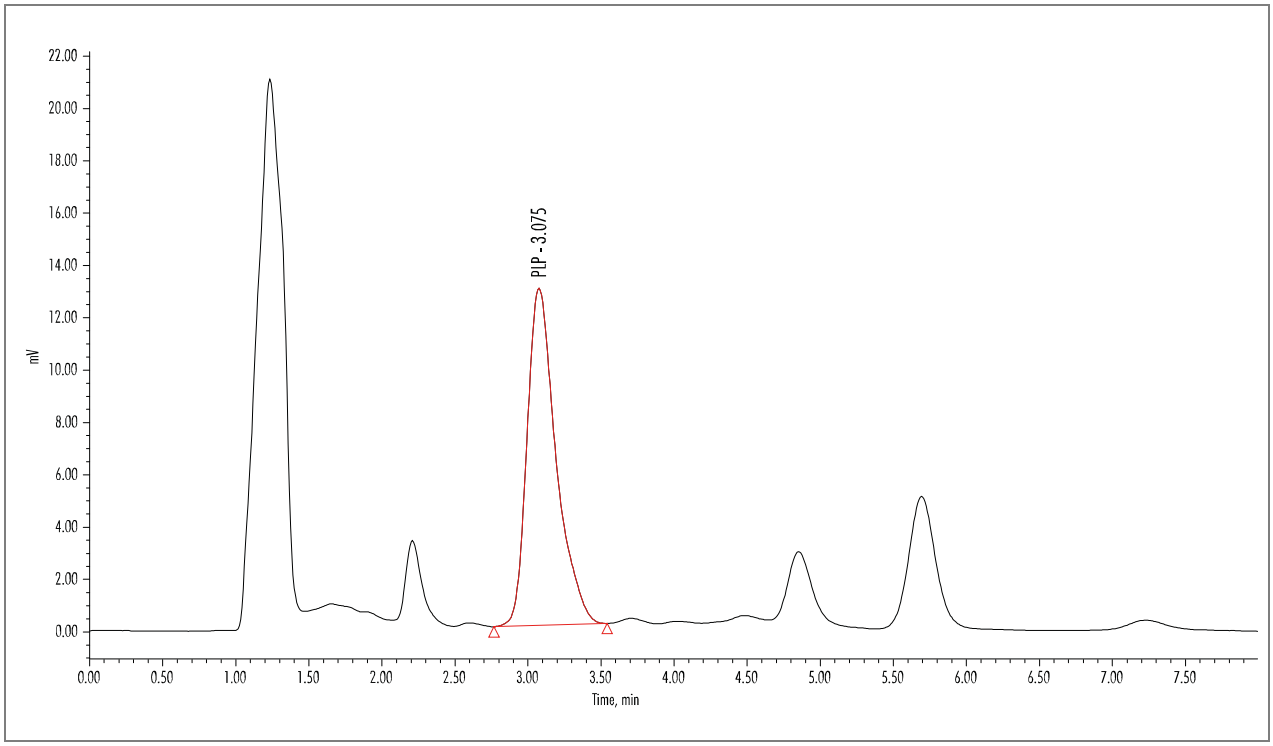

Figure 2: Chromatogram of a plasma calibrator
Concentration of the analyte: Pyridoxal 5'-phosphate (PLP): 12.5 µg/L, flow rate: 1.0 mL/min

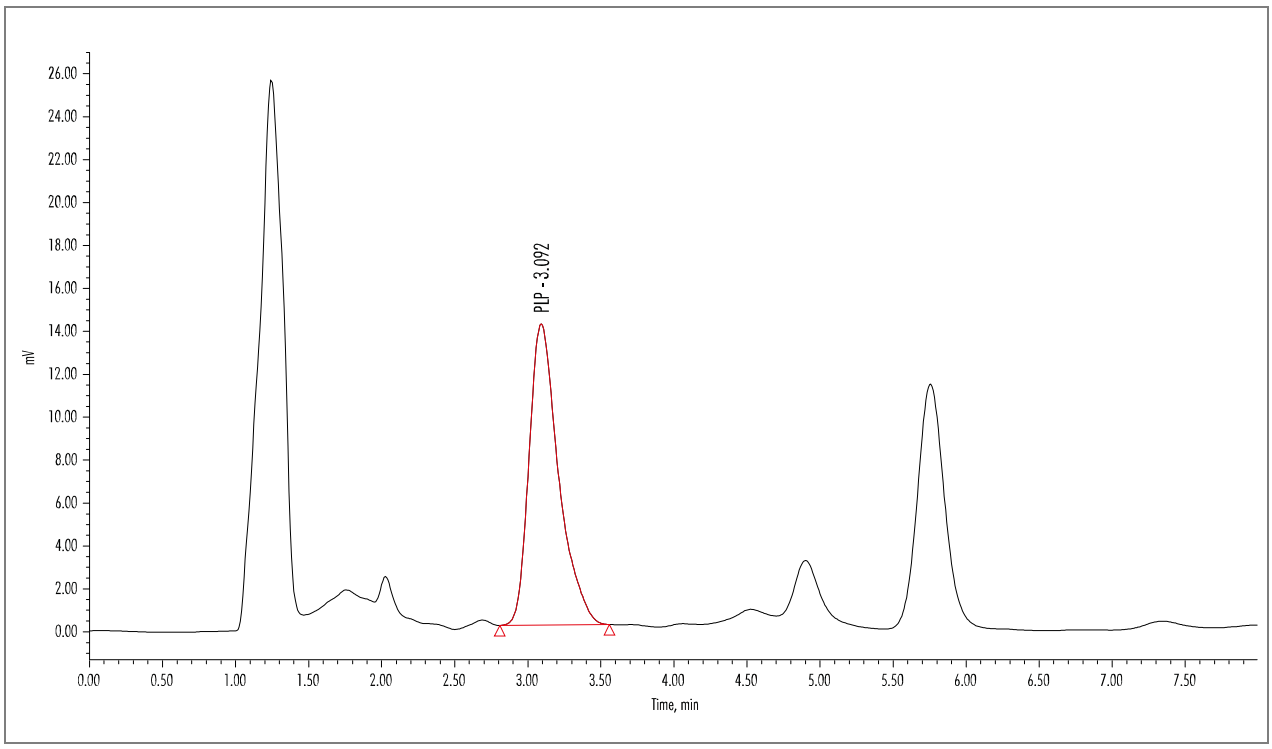

Figure 3: Chromatogram of a plasma patient sample
Concentration of the analyte: Pyridoxal 5'-phosphate (PLP): 13.9 µg/L, flow rate: 1.0 mL/min

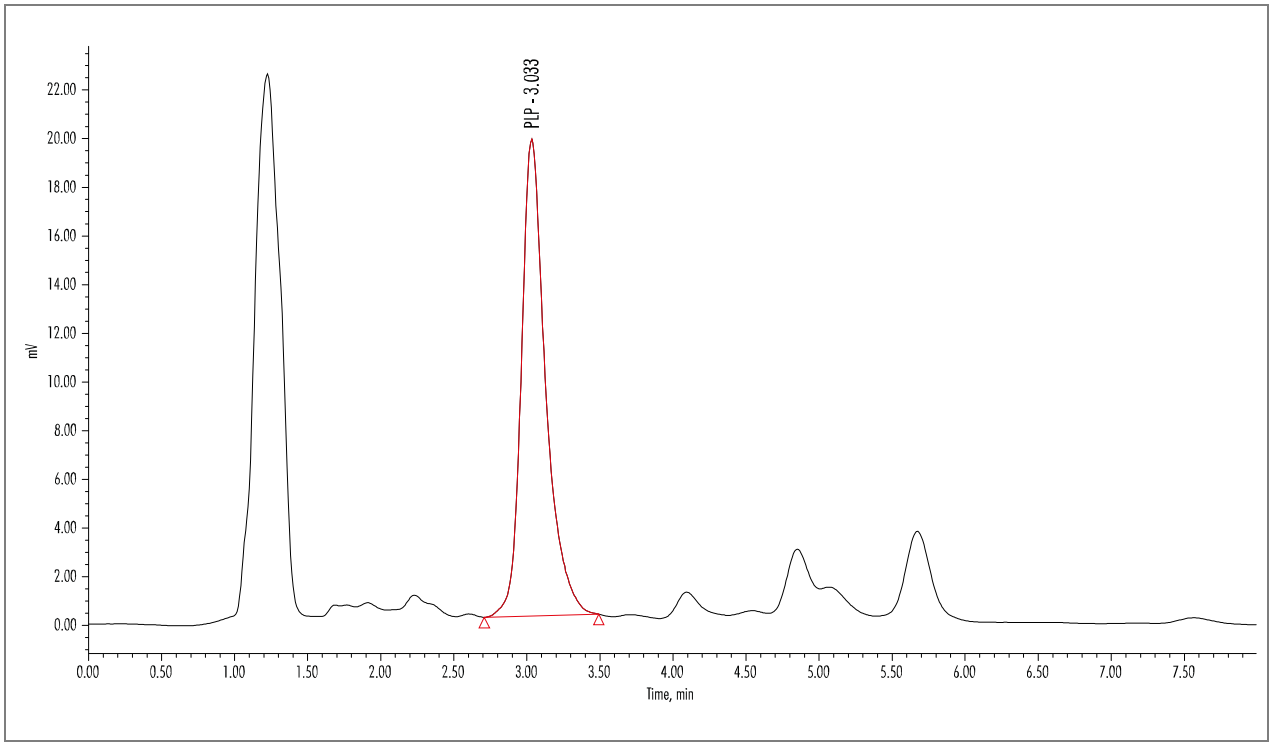

Figure 4: Chromatogram of a whole blood calibrator
Concentration of the analyte: Pyridoxal 5'-phosphate (PLP): 19.4 µg/L, flow rate: 1.0 mL/min

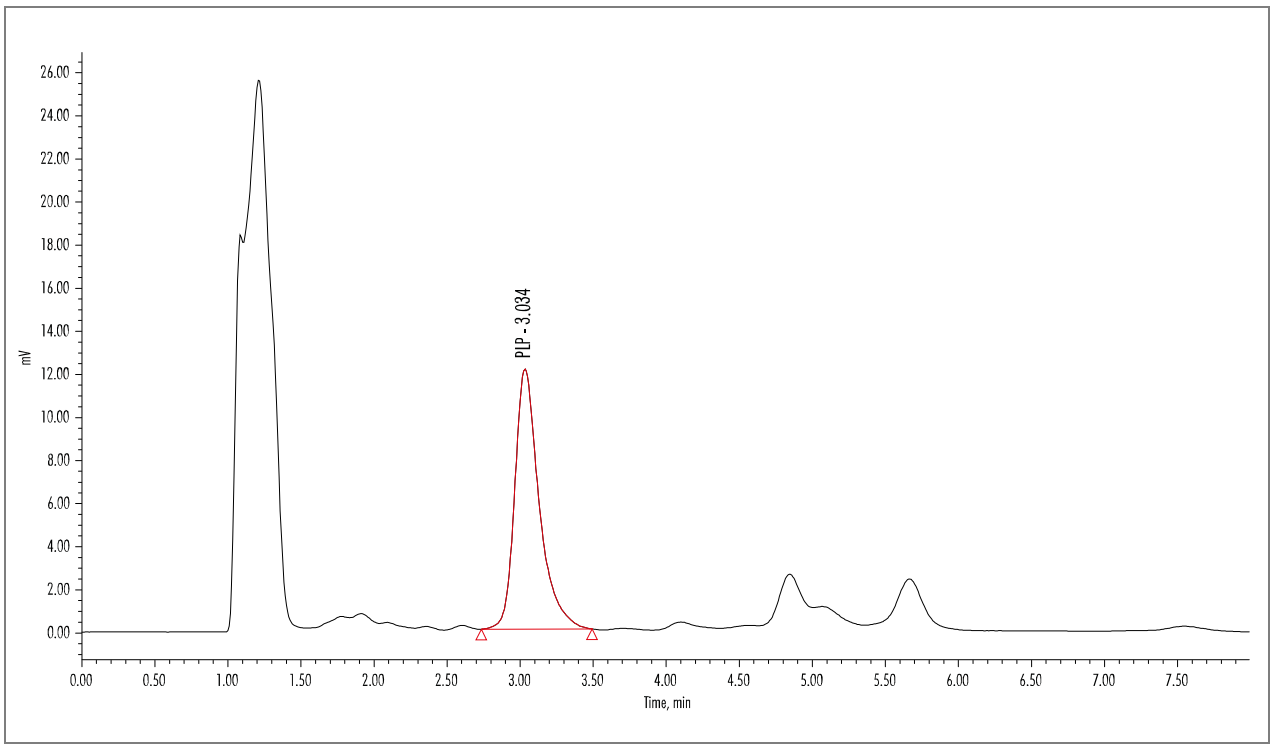

Figure 5: Chromatogram of a whole blood patient sample
Concentration of the analyte: Pyridoxal 5'-phosphate (PLP): 13.0 µg/L, flow rate: 1.0 mL/min

7.3 Conversion factors

The following table lists conversion factors between mass and molar concentrations and conversely.

Table 8: Conversion factors

Substance	µg/L to nmol/L	nmol/L to µg/L
Pyridoxal 5'-phosphate (PLP)	x 4.047	x 0.2471

7.4 Manual calculation

For the manual calculation the following data are required:

- Peak area/height of substance A in the chromatogram of the sample =A_{Sample}
- Peak area/height of substance A in the chromatogram of the calibrator =$A_{Calibrator}$
- The concentration of substance A in the calibrator =$C_{Calibrator}$

The concentration of the substance A in the sample C_{Sample} is then calculated as follows:

$$C_{Sample} = \frac{A_{Sample}}{A_{Calibrator}} \times C_{Calibrator}$$

8 Storage and lifetime of the assay components

Unopened, and provided that transport and storage conditions are met, the assay components are stable until the expiry date stated on the label. Transport and store the components under the following conditions:

Table 9: Transport conditions for the reagent kit and components

Product	Transport temperature
Reagent kit (order no. 31000/S, 31000/WB)	ambient
Basic kit (order no. 31000-BK)	ambient
All other components listed in chapter 1	ambient

Immediately unpack components after transport and store individually as stated below:

Table 10: Storage conditions for the reagents, calibrators and controls

Product	Storage temperature
Mobile Phase (order no. 31001, 31002)	+18 to +30 °C
Precipitation Reagent (order no. 31004)	+18 to +30 °C
Neutralisation Reagent (order no. 31005)	+18 to +30 °C
Derivatisation Reagent (order no. 31006)	+18 to +30 °C
Plasma Calibrator (order no. 36005)	below –18 °C
Plasma controls (order no. 0031, 0038, 0039)	below –18 °C
Whole blood calibrator (order no. 31003)	below –18 °C
Whole blood controls (order no. 0022, 0023, 0024)	below –18 °C

Close the reagents immediately after use and store them at the specified temperature. The in-use shelf-life is one year but does not extend beyond the stated expiry date. Details of the stability of the reconstituted calibrators and controls are given in chapters 5.2 and 5.3.

The HPLC column, precolumn and laboratory materials not listed here can be stored at +18 to +30 °C.

The in-use shelf-life of HPLC column, precolumn and prefilter is dependent on the individual conditions these components are used under (i.a. frequency of use, number of samples, type of samples, injection volume). Consider quality control measures (chapter 6) to identify decreasing chromatographic performance.

9 Waste disposal

Hazardous waste
The Precipitation Reagent (order no. 31004) contains a strong, fire-promoting acid. Neutralise product residues and dispose of into a collection container for salt solutions.

Residues of patient samples, prepared samples, controls (order no. 0023, 0024, 0038, 0039) and calibrators (order no. 31003, 36005) as well as laboratory consumables contaminated with human material must be collected and disposed of as potentially infectious waste.

Hazardous waste must not be disposed together with domestic waste. Do not circulate into the main water supply. Dispose of in compliance with Directive 2008/98/EC on Waste and national and local requirements. The waste containers must be stored appropriately and only access permitted to authorised parties.

Non-hazardous waste
Mobile Phase (order no. 31001, 31002), Neutralisation Reagent (order no. 31005), Derivatisation Reagent (order no. 31006) and non-contaminated laboratory consumables are not classified as hazardous. Dispose of in compliance with Directive 2008/98/EC on Waste and national and local requirements.

10 Reference ranges

Note:
Reference ranges listed below are representing levels found in a proportion of a healthy population (e.g. of 95 % of the investigated healthy blood-donors). Consequently, they might or might not align with clinically relevant decision levels or cut-off-values regarding the intended purpose of this device. For clinical performance data, including values expected in affected and non-affected persons, see Appendix III.

The stated reference ranges are based on the literature [11,12]. They may differ from other published data. As the levels vary depending on patient population and measurement method, determine specific reference ranges for your laboratory. When determining ranges, make sure that you comply with local national requirements.

Lithium heparin blood samples from at least 15 men and 15 women of various age groups (21–30, 31–40, 41–50, 51–60, 61–70 and > 70 years old) were collected for the G. Steen and M. van der Zwaal study [11]. A questionnaire was administered to check for the following exclusion criteria: use of oral vitamin or iron supplements, anticoagulant use, diabetes, pregnancy, and oral contraceptive use. Testing was done using HPLC fluorescence assay after derivatisation.

For the study of K. K. Panton et al. [12] EDTA plasma samples were collected from 65 males aged 22 to 66 years (median 44 years) and 55 females aged 21 to 67 years (median 44 years). All participants declared themselves healthy, medications of the last 4 weeks were checked and donors excluded dependant on their medication. Vitamin supplementation was not an exclusion criterion in this study. The analysis was done by HPLC fluorescence measurement after derivatisation.

In the National Health Nutrition and Examination Survey (NHANES, data from 2005-2006) [13] in total 8311 US Americans were examined. Vitamin supplementation was not an exclusion criterion in this study. Analysis was done by HPLC with post-column derivatisation and fluorometric detection.

Table 11: Reference ranges for pyridoxal 5'-phosphate (PLP)

	Whole blood	Plasma	Serum
Reference range	**12.6–45.2 µg/L 51–183 nmol/L**	**5.7–55.1 µg/L 23–223 nmol/L**	**2.8–75 µg/L; deficiency < 4.9 µg/L 11.3–302 nmol/L; deficiency < 20 nmol/L**
Number of results obtained	246	120	8311
Statistical method of the reference range	Removal of upper and lower 2.5 % of results	Removal of upper and lower 2.5 % of results	Removal of upper and lower 2.5 % of results
Correlation between results obtained and years of age	Decrease with increasing age 21–30 years: 138 nmol/L (men) and 113 nmol/L (women); > 70 years: 79 nmol/L (men) and 84 nmol/L (women) Weak effect: no age-related reference ranges introduced	No influence on the upper reference range but decrease of the lower reference range with increasing age Weak effect: no age-related reference ranges introduced	Detailed reference ranges by age groups are provided. No age-related deficiency concentration was set.
Correlation between results obtained and gender	None	No influence on the lower reference range but influence on the upper reference range by gender (males > females) Weak effect: no gender-related reference ranges introduced	Detailed reference ranges by gender are provided. No gender-related deficiency concentration was set.
Source	[11]	[12]	[13]

11 Interference testing

Structure-related compounds, water soluble vitamins and drugs were spiked into whole blood and plasma samples or prepared whole blood samples prior derivatisation at the highest expected concentrations (see tables below) and tested for interferences.

In addition, different sample conditions were simulated and their influence on the test was examined. Different sampling systems were also tested for interference.

Table 12: Tested substances and their concentrations - Structure-related compounds

Substance	Test concentration in µg/L
Pyridoxal	7.50
Isopyridoxal	30.6
Pyridoxamine	0.600

Substance	Test concentration in µg/L
Pyridoxamine 5'-phosphate	5.00
Pyridoxine	1.80
Pyridoxine 5'-phosphate	2.50
4-Pyridoxic acid	1.70

Table 13: Tested substances and their concentrations - water soluble vitamins

Substance	Test concentration
Ascorbic acid (vitamin C)	72.0 mg/L
Biotin (vitamin B_7)	21.0 µg/L
Cobalamin (vitamin B_{12})	1.68 µg/L
Folate (vitamin B_9)	360 µg/L
Niacin (nicotinic acid, vitamin B_3)	10.8 mg/L
Pantothenic acid (vitamin B_5)	4.20 mg/L
Riboflavin (vitamin B_2)	0.960 mg/L

Table 14: Tested substances and their concentrations - drugs

Substance	Test concentration in mg/L
Acetaminophen	156
Acetylsalicylic acid	30.0
Allopurinol	60.0
Alprazolam	0.258
Amlodipine	0.0750
Amoxicillin	54.0
Amphetamine/dexamfetamine	0.330
Apixaban	0.315
Atenolol	9.00
Atorvastatin	0.750
Azithromycin	11.1
Bisoprolol	0.258
Brivaracetam	8.20
Bupropion	0.396
Candesartan	0.551
Carbamazepine	29.0
10-OH-Carbamazepine	92.6
Carbamazepine-diol	28.4
Carbamazepine-10,11-epoxide	17.8
Carvedilol	0.233
Cefuroxime	543
Citalopram	5.43

Substance	Test concentration in mg/L
Clonazepam	0.300
Clopidogrel	0.00169
Cyclobenzaprine	0.102
N-Desmethylmesuximide	92.4
Diclofenac	24.0
Duloxetine	0.162
Edoxaban	1.01
Empagliflozin	0.929
Enalaprilat	0.819
Ethosuximide	230
Felbamate	276
Fenoterol	0.00358
Fluoxetine	1.42
Fluticasone	0.00126
Formoterol	0.000273
Furosemide	15.9
Gabapentin	52.8
Glimepiride	1.64
Glipizide	3.00
Hydrochlorothiazide	1.13
Hydrocodone	0.0720
Ibuprofen	219
Ipratropium bromide	0.00300
Isoniazide	60.0
Lacosamide	22.4
Lamotrigine	35.0
Levetiracetam	175
Lercanidipine	0.0407
Levothyroxine	0.429
Lisinopril	0.246
Lorazepam	0.720
Losartan	0.836
Meloxicam	6.00
Metamizole/dipyrone as 4-methylaminoantipyrine	206
Metformin	12.0
Methocarbamol	123
Methylphenidate	0.108
Metoclopramide	2.25
Metoprolol	1.50
Montelukast	1.65

Substance	Test concentration in mg/L
Nebivolol	0.0300
Noscapine	0.810
Omeprazole/esomeprazole	8.40
Oxcarbazepine	7.32
Pantoprazole	30.0
Perampanel	3.42
Phenobarbital	117
Phenprocoumon	15.0
Phenylethylmalonamide (PEMA)	21.0
Phenytoin	45.8
Pravastatin	0.207
Prednisolone	1.20
Prednisone	0.0990
Pregabalin	26.6
Primidone	38.2
Propranolol	1.01
Ramipril	0.156
Ranitidine	10.5
Retigabine	4.40
Rivaroxaban	2.70
Rosuvastatin	0.111
Rufinamide	70.4
Salbutamol/albuterol	0.0450
Sertraline	0.927
Simvastatin	0.0831
Sitagliptin	1.15
Spironolactone	0.555
Stiripentol	50.8
Sultiame	30.4
Tamsulosin	0.0338
Theophylline	44.4
Tiagabine	0.510
Tilidine as nortilidine	0.368
Topiramate	59.2
Torasemide	83.1
Tramadol	3.14
Trazodone	14.7
Valproic acid	232
Valsartan	11.7
Venlafaxine	0.696

Substance	Test concentration in mg/L
Vigabatrin	87.6
Zolpidem	0.816
Zonisamide	95.0

11.1 Interferences detected

Isoniazid, an antibiotic drug, interacts with pyridoxal 5'-phosphate (PLP) and leads to falsely low determination of pyridoxal 5'-phosphate (PLP).

Late eluting substances may be present in native whole blood samples potentially leading to interferences in subsequent analyses (e.g. in form of broad peaks or elevated baseline).

11.2 No interference detected

The following substances were tested and have a negligible influence on the quantitative results (deviation ≤ 15%).

Metabolites
Isopyridoxal, pyridoxal*, pyridoxamine*, pyridoxamine 5'-phoshate*, 4-pyridoxic acid, pyridoxine*, pyridoxine 5'-phosphate*
* These metabolites can be converted into each other and into PLP by enzymatic reactions. They are not analytical but rather pharmacokinetic interferences.

Water soluble vitamins
Ascorbic acid (vitamin C), biotin (vitamin B7), cobalamin (vitamin B12), folate (vitamin B9), niacin (nicotinic acid, vitamin B3), pantothenic acid (vitamin B5), riboflavin (vitamin B2)

Drug substances
Acetaminophen, acetylsalicylic acid, allopurinol, alprazolam, amlodipine, amoxicillin, amphetamine/dexamfetamine, apixaban, atenolol, atorvastatin, azithromycin, bisoprolol, brivaracetam, bupropion, candesartan, carbamazepine, 10-OH-carbamazepine, carbamazepine-diol, carbamazepine-10,11-epoxide, carvedilol, cefuroxime, citalopram, clonazepam, clopidogrel, cyclobenzaprine, N-desmethylmesuximide, diclofenac, duloxetine, edoxaban, empagliflozin, enalaprilat, ethosuximide, felbamate, fenoterol, fluoxetine, fluticasone, formoterol, furosemide, gabapentin, glimepiride, glipizide, hydrochlorothiazide, hydrocodone, ibuprofen, ipratropium bromide, lacosamide, lamotrigine, levetiracetam, lercanidipine, levothyroxine, lisinopril, lorazepam, losartan, meloxicam, metamizole/dipyrone as 4-methylaminoantipyrine, metformin, methocarbamol, methylphenidate, metoclopramide, metoprolol, montelukast, nebivolol, noscapine, omeprazole/esomeprazole, oxcarbazepine, pantoprazole, perampanel, phenobarbital, phenprocoumon, phenylethylmalonamide (PEMA), phenytoin, pravastatin, prednisolone, prednisone, pregabalin, primidone, propranolol, ramipril, ranitidine, retigabine, rivaroxaban, rosuvastatin, rufinamide, salbutamol/albuterol, sertraline, simvastatin, sitagliptin, spironolactone, stiripentol, sultiame, tamsulosin, theophylline, tiagabine, tilidine as nortilidine, topiramate, torasemide, tramadol, trazodone, valproic acid, valsartan, venlafaxine, vigabatrin, zolpidem, zonisamide

Interference-free analysis is possible with the following sample conditions:

Haemolysis
Plasma and serum samples were spiked with haemoglobin to a haemoglobin concentration of 10 g/L and freshly collected whole blood samples were haemolysed by freezing and thawing. Analyte concentrations were compared against those of the original sample:

No significant interferences occurred (deviation ≤ 15 %).

Lipaemia
Whole blood, plasma and serum samples were spiked with different concentrations of a lipaemic emulsion (0.67 to 10 g/L) and analyte concentrations were compared against those of the original sample:

No significant interferences occurred (deviation ≤ 15 %).

Icterus
Whole blood, plasma and serum samples were spiked with unconjugated and conjugated bilirubin (each 0.4 g/L) and the analyte concentrations were compared against those of the original sample:

No significant interferences occurred (deviation ≤ 15 %).

The following sampling systems were tested without significant interference; the quantitative results were not affected (deviation ≤ 15%):

Table 15: Whole blood sampling systems causing no interferences

Type	Manufacturer	Order no.	Volume	Description	Lots tested
Whole blood EDTA (K3E)	Sarstedt	04.1951 01.1605.001	1.8 mL 7.5 mL	S-Monovette® 1.6 mg K3-EDTA/mL, spray-dried	7030611 1032921
Whole blood EDTA (K3E)	Greiner	454036 454036	4 mL 4 mL	Vacuette® K3-EDTA, spray-dried	C181033M A161036E
K2-EDTA whole blood (K2E)	Sarstedt	04.1915.100	2.7 mL	S-Monovette® 1.6 mg K2-EDTA/mL, spray-dried	8595111
K2-EDTA whole blood (K2E)	BD	368856 367525 367864	3 mL 10 mL 6 mL	Vacutainer® 1.8 mg K2-EDTA/mL, spray-dried	9347839 0307322 1096715
Li-heparin whole blood (LH)	Sarstedt	03.1628 03.1628 01.1604.100	5.5 mL 5.5 mL 7.5 mL	S-Monovette® 16 I.U./mL Li-heparin, granulate	9031311 7034611 9032011
Li-heparin whole blood (LH)	BD	368886	6 mL	Vacutainer® 17 I.U./mL Li-heparin, spray-dried	7066712
Na-heparin whole blood (NH)	Sarstedt	01.1613.100 01.1613.100	7.5 mL 7.5 mL	S-Monovette® 16 I.U./mL Na-heparin, granulate	7032211 9268311
Na-heparin whole blood (NH)	Greiner	454051 454051	4 mL 4 mL	Vacuette® Na-heparin, spray-dried	A171039Q A19033AM
NH4-heparin whole blood (AH)	Sarstedt	05.1105 05.1105	4.5 mL 4.5 mL	S-Monovette® 16 I.U./mL NH4-heparin, granulate	8033511 7033011
NH4-heparin whole blood (AH)	Greiner	455031	9 mL	Vacuette® NH4-heparin, spray-dried	A180936D

Table 16: Serum sampling systems causing no interferences

Type	Manufacturer	Order no.	Volume	Description	Lots tested
Serum	Sarstedt	02.1726.021	7.5 mL	S-Monovette® Serum	6032613
Serum Gel	Sarstedt	01.1601.001 04.1905.001	7.5 mL 2.6 mL	S-Monovette® Serum Gel Z	4033201 5035111
Serum CAT	BD	369032	4 mL	Vacutainer CAT	0230269
Serum SST	BD	367957	3.5 mL	Vacutainer SST II advance	0288531
Serum Clot Activator	Greiner	455071 456073	8 mL 5 mL	Vacuette Z CAT Serum Separator Clot Activator	A180635P D2110349

If you have any questions concerning interferences, contact your local Chromsystems representative or our Chromsystems support staff directly by calling our hotline +49 89 18930-111 or by e-mail at support@chromsystems.com.

12 Troubleshooting

Table 17: Troubleshooting

Problem	Possible cause	Remedy
Baseline drifts	Detector lamp not yet warm	Wait until baseline is stable
	Detector lamp old	Replace lamp
	System not yet in equilibrium	Inject calibrator repeatedly, until two successive chromatograms are identical
	Temperature drift	Use column oven
	Flow rate not constant	Check pump
Baseline unstable	HPLC pump	Check pump (air, seals)
	Air in the system	Degas Mobile Phase
	Detector cell contaminated	Clean detector cell
	Mobile Phase contaminated	Replace Mobile Phase
Interference peaks	Air in the system	Degas Mobile Phase
	Injector contaminated	Clean injector
	Injection syringe contaminated	Clean syringe with methanol
	Prefilter or precolumn contaminated	Replace prefilter or precolumn
	HPLC column contaminated	Replace column
	Late-eluting peaks from previous sample	Inject Mobile Phase several times
	Interference	Check sample for known interferences
Broad peaks, tailing	Prefilter or precolumn contaminated	Replace prefilter or precolumn
	HPLC column too old	Replace column
Double peaks	Dead volume in fittings	Replace fittings

Problem	Possible cause	Remedy
	Dead volume in precolumn	Replace precolumn
	Dead volume in HPLC column	Replace column
No peaks	System leaking	Check injector and fittings
Reduced sensitivity	Detector lamp ageing	Replace lamp
	Detector cell contaminated	Clean cell
	Defective injection valve	Check injector
Retention times changed	Temperature drift	Use column oven
	Unstable flow rate, pump pulsing	Check HPLC pump
	Leak	Check system for leaks
	Mobile Phase has evaporated	Replace Mobile Phase and keep it closed or covered even when in use
	System not in equilibrium	Repeat injections of calibrator until two successive chromatograms are identical
No signal	Connection to data system defective or interrupted	Check signal cable and connection
	Detector lamp	Check lamp, replace lamp if necessary
Quality control outside acceptable range	Interference	Check chromatogram for interference
	Incorrect sample preparation	Check reproducibility of incorrect results
	Insufficient light protection	Use amber coloured vials

If you have any questions concerning troubleshooting, contact your local Chromsystems representative or our Chromsystems support staff directly by calling our hotline +49 89 18930-111 or by e-mail at support@chromsystems.com.

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Appendix I Substance information

Hazardous substances
When using the reagents, note the following hazard information and take the relevant safety measures. More information can be gathered from our safety data sheets. These can be downloaded from our website www.chromsystems.com or are available upon request.

Table 18: Hazard and precautionary statements

Pictograms	Hazard and precautionary statements
Precipitation Reagent (order no. 31004)	Components: 10–25 % perchloric acid
	**Danger** H272 May intensify fire; oxidiser. H290 May be corrosive to metals. H314 Causes severe skin burns and eye damage. P280 Wear protective gloves/protective clothing/eye protection/face protection. P301+P330+P331 IF SWALLOWED: rinse mouth. Do NOT induce vomiting. P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P309+P311 IF exposed or if you feel unwell: Call a POISON CENTER or doctor/physician.
These components are not classified as dangerous according to European Union legislation:	
Mobile Phase (order no. 31001, 31002)	Components: aqueous solution
Neutralisation Reagent (order no. 31005)	Components: aqueous solution
Derivatisation Reagent (order no. 31006)	Components: aqueous solution
Whole Blood Calibration Standard (order no. 31003)	Components: human whole blood
Plasma Calibration Standard (order no. 36005)	Components: human plasma
Whole Blood Controls (order no. 0022, 0023, 0024)	Components: human whole blood
Plasma Controls (order no. 0031, 0038, 0039)	Components: human plasma

Active ingredients:

Table 19: Active ingredients

Order no.	Description	Active component	Specification
31001/ 31002	Mobile Phase	Phosphate buffer	0–2 %
		Water	98–100 %
31003	Whole Blood Calibration Standard	Analyte (pyridoxal 5´-phosphate)	Conc. see leaflet
36005	Plasma Calibration Standard	Analyte (pyridoxal 5´-phosphate)	Conc. see leaflet
31004	Precipitation Reagent	Perchloric acid	10–25 %
31005	Neutralisation Reagent	Phosphate buffer	40–50 %
31006	Derivatisation Reagent	Nitrilic oxidizing agent	< 0.1 %
31100	Analytical Column	Polymer based on silica	> 90 %
0031	Plasma Control Bi-Level	See 0038, 0039	–
0038	Plasma Control Level I	Analyte (pyridoxal 5´-phosphate)	Conc. see leaflet
0039	Plasma Control Level II	Analyte (pyridoxal 5´-phosphate)	Conc. see leaflet
0022	Whole Blood Control Bi-Level	See 0023, 0024	–
0023	Whole Blood Control Level I	Analyte (pyridoxal 5´-phosphate)	Conc. see leaflet
0024	Whole Blood Control Level II	Analyte (pyridoxal 5´-phosphate)	Conc. see leaflet

Appendix II Analytical performance data

The performance features were determined and verified on the following equipment:
- Shimadzu fluorescence detector (FLD) RF-20A
- Waters fluorescence detector (FLD) 2474

Users wishing to use the HPLC assay "Vitamin B_6 in plasma/serum" (order no. 31000/S) or "Vitamin B_6 in whole blood" (order no. 31000/WB) with an HPLC system other than the one specified here should validate the method on that device.

Peak areas were used to calculate results throughout the analytical performance evaluation.

Metrological traceability and trueness:
For the Plasma Calibration Standard (order no. 36005) and Whole Blood Calibration Standard (order no. 31003) metrological traceability was demonstrated and is available as traceability chain (see Appendix IV).

Trueness of measurement was demonstrated within the analytical performance evaluation process, due to the absence of a reference method or reference material based on following strategies (at least two strategies per matrix):
- Comparison with a CE/IVD method
- Determination of relative recovery
- Proficiency scheme participation (available in the download centre of our homepage: www.chromsystems.com/downloadcenter.html)

Relative recovery

Relative recovery was determined with the matrices of plasma and serum. Ten plasma and twelve serum samples were analysed. Every sample was spiked with two different concentrations of the analyte inside the analytical measuring range. Recovery is calculated using the following formula:

$$\text{Recovery [\%]} = \frac{\text{measured concentration in spiked sample} - \text{measured concentration in unspiked sample}}{\text{Spike concentration}} \times 100$$

Table 20: relative recovery rates in plasma and serum, determined with Shimadzu FLD RF-20A

Substance	Matrix	Recovery rate (concentration of substance)	
Pyridoxal 5'-phosphate (PLP)	Plasma	96.6 % (10.0 µg/L)	105 % (200 µg/L)
	Serum	95.3 % (10.0 µg/L)	102 % (200 µg/L)

Analytical measuring range
Lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ):
The lower limit of quantitation (LLOQ) has been determined by a defined dilution of samples with low endogenous analyte concentrations with PLP-free plasma. Upper limit of quantitation (ULOQ) was determined by spiking whole blood, plasma, and serum samples with defined quantities of standard substances.

The method is linear from the lower limit of quantitation (LLOQ) to the stated upper limit of quantitation (ULOQ).

Table 21: Lower and upper limit of quantitation, determined with Shimadzu FLD RF-20A[1)] (LLOQ in whole blood, plasma, and serum and ULOQ in serum) and with Waters FLD 2474[2)] (ULOQ in whole blood and plasma)

Substance	Matrix	LLOQ	ULOQ
Pyridoxal 5'-phosphate (PLP)	Whole blood	1.0 µg/L[1)]	250 µg/L[2)]
	Plasma	0.7 µg/L[1)]	250 µg/L[2)]
	Serum	0.9 µg/L[1)]	250 µg/L[1)]

Precision (intra-assay):
Determination of the intra-assay precision was done by 10 preparations of the same sample and determination of the analyte concentration at 3 different concentrations in whole blood and plasma:

Table 22: Intra-assay precision, determined with Waters FLD 2474

Substance	Matrix	Coefficient of variation (concentration of analyte)		
Pyridoxal 5'-phosphate (PLP)	Whole blood	0.9 % (11.0 µg/L)	1.4 % (23.2 µg/L)	0.7 % (34.1 µg/L)
	Plasma	1.0 % (7.67 µg/L)	0.6 % (13.5 µg/L)	0.9 % (21.2 µg/L)

Precision (inter-assay precision)
Determination of the inter-assay precision was done by 10 sample preparations and determination of the analyte concentrations in three whole blood and three plasma pools in 10 different test series:

Table 23: Inter-assay precision, determined with Waters FLD 2474

Substance	Matrix	Coefficient of variation (concentration of analyte)		
Pyridoxal 5'-phosphate (PLP)	Whole blood	4.0 % (10.5 µg/L)	1.7 % (23.0 µg/L)	3.8 % (34.3 µg/L)
	Plasma	2.0 % (7.53 µg/L)	1.0 % (13.6 µg/L)	1.9 % (21.7 µg/L)

Drift
To identify any drift of analyte concentration over time the analyte concentration in the three whole blood and three plasma samples was compared over a 10-day period. No drift was observed.

Precision (reproducibility)
The performance data were determined at 3 sites on the basis of 4 different samples for whole blood, plasma, and serum. All samples were prepared 5-fold on 5 different days. The procedure is based on CLSI EP05-A3 and corresponds to a 3 x 5 x 5 test design.

Table 24: Reproducibility, determined with Shimadzu FLD RF-20A

Substance	Sample	Mean	Reproducibility	
			Coefficient of variation	95 % confidence interval
Pyridoxal 5'-phosphate (PLP)	Whole blood native	16.3 µg/L	5.9 %	4.5–8.7 %
	Whole blood low	7.58 µg/L	9.6 %	7.3–14.2 %
	Whole blood medium	48.8 µg/L	5.1 %	3.9–7.1 %

Substance	Sample	Mean	Reproducibility	
			Coefficient of variation	95 % confidence interval
	Whole blood high	198 µg/L	6.4 %	5.1–8.4 %
	Plasma native	9.15 µg/L	5.4 %	3.8–9.2 %
	Plasma low	4.43 µg/L	7.4 %	5.8–10.2 %
	Plasma medium	72.0 µg/L	5.0 %	4.2–6.2 %
	Plasma high	214 µg/L	7.4 %	6.4–8.8 %
	Serum native	7.33 µg/L	7.3 %	5.4–11.2 %
	Serum low	2.94 µg/L	10.9 %	7.1–23.0 %
	Serum medium	60.2 µg/L	4.7 %	3.7–6.4 %
	Serum high	197 µg/L	6.0 %	4.9–7.7 %

Carry-over
A prepared plasma and a prepared whole blood sample with an analyte concentration in the range of the upper limit of quantitation was analysed in between several blank samples. The analyte concentrations of blank samples before and after the high-level samples were compared. In case of significant carry-over, the amount was calculated on a percentage basis in relation to the preceding sample.
Review of the data obtained showed no carry-over effects. In all cases the measured concentration of the blank sample was below the limit of quantitation.

Robustness
The effect of defined modifications in sample preparation and HPLC system setup were evaluated. The method is robust within the following tolerances provided the particular setup remains constant throughout a measurement series:

Table 25: Tolerance ranges HPLC system

HPLC system	Tolerance range
Column temperature	+20 to +30 °C
Flow rate	1.0 to 1.2 mL/min
Injection volume	25 to 50 µL

Table 26: Tolerance ranges sample preparation

Sample preparation (according to chapter 5.4)	Tolerance range
Step 2: ...centrifuge for 5 min at 16000 x g	...centrifuge for 5 min at 8000 to 16000 x g
Step 6: Incubate for 20 min at 60°C (water bath).	Incubate for 20 min at 57 to 63°C (water bath).
Step 8: Centrifuge for 2 min at 16000 x g	Centrifuge for 2 min at 8000 to 16000 x g

These data have been established in our laboratory solely in order to verify the performance of the reagent kit and to fulfil regulatory requirements. We particularly emphasize that these data are not suitable to compare the measurement systems used, nor to make any statement concerning their general performance.

Appendix III Clinical performance data

The Chromsystems reagent kits 31000/S "Vitamin B_6 in serum/plasma" and 31000/WB "Vitamin B_6 in whole blood are used to screen specific populations for abnormal levels or to monitor vitamin B_6 status in patients, e.g., under supplementation therapy. As vitamin B_6 status never serves to diagnose a disease directly, clinical performance parameters such as diagnostic specificity, diagnostic sensitivity, positive predictive value, negative predictive value or likelihood ratio are not applicable for these devices. Instead, relevant clinical parameters are expected values in normal and affected populations.

The table below exhibits clinical performance data using the assays described.

Clinical performance data for vitamin B_6

Monitoring of vitamin B_6 levels or screening are relevant in population groups who have shown an increased risk for deficiency or excess. These include

for vitamin B_6 deficiency:
- Patients with restrictive eating disorders
- Pregnant women
- Patients who undergo hemodialysis
- Elderly patients (with/without Hyperhomocysteinemia)
- Morbidly obese patients
- Patients after gastric bypass
- Patients with type II diabetes

for vitamin B_6 excess:
- Patients under vitamin B_6 supplementation therapy
- Patients with hypophosphatasia

Table 27: Clinical performance for vitamin B_6

Aim/research question of the study	Matrix used to determine PLP levels	Expected values in affected population	Expected values in non-affected population	Ref
Study to screen biochemical, nutritional and hormonal parameters for nutritional abnormalities in adolescent anorexia nervosa patients and to establish whether certain abnormalities persist after short-term refeeding	Whole blood	Deficiency: < 14.4 nmol/L	14.4–72.8 nmol/L	[14]
Study to describe changes in intake of selected micronutrients (including vitamin B_6) intake, their micronutrient status (B_6 etc.) and overall diet quality in women from preconception to the second trimester of pregnancy	Whole blood	No categorisation/diagnosis Pregnant women taking vitamin B_6 supplements were measured at 3 different time points. T0 (before pregnancy): 89.8 ± 3.4 nmol/L T1 (12th week of gestation): 88.7±2.9 nmol/L T2 (24th week of gestation): 80.0 ± 2.8 nmol/L		[15]

Aim/research question of the study	Matrix used to determine PLP levels	Expected values in affected population	Expected values in non-affected population	Ref
Study to measure the water-soluble vitamins and trace element in blood and dialysate in patients treated by online post-dilution	Whole blood	—	35–110 nmol/L	[16]
Study to examine association between vitamins, dietary intake, nutritional status and length of stay in elderly rehabilitation patients	Plasma	Deficiency: < 20 nmol/L	—	[17]
Study to assess bone strength and microarchitecture in adults with hypophosphatasia (HPP) (diagnosed by B_6 levels and other markers) by determining biochemical parameters and bone markers	Plasma	—	15–73 nmol/L	[18]
Retrospective hospital-based study to evaluate the role of laboratory markers for hypophosphatasia (including vitamin B_6)	Plasma	Affected by hypophosphatasia (elevation): > 134 nmol/L	Healthy population without hypophosphatasia: 40–110 nmol/L	[19]
Study to derive the reference limits for plasma PLP	Plasma	—	20.8–176 nmol/L (women) 24.7–278 nmol/L (men) 23.0-223 nmol/L (all) (2.5 and 97.5 percentiles)	[12]
Study to assess the vitamins A, B1, B2, B6, C, D, and E in morbidly obese Norwegian patients seeking weight reduction and comparison of the concentrations with those observed in a healthy control group	Serum	Deficiency: < 15 nmol/L (women) < 16 nmol/L (men)	15–111 nmol/L (women) 16–158 nmol/L (men)	[20]
Investigation to assess the changes in blood vitamin concentrations in patients who were taking predefined supplements after gastric bypass surgery compared to patients without prescribed supplements	Serum	Deficiency: < 15 nmol/L (women) < 16 nmol/L (men)•1	16–158 nmol/L (men)•1 •1 added for completeness from the study [20] as patients were participants in this study, males were also included	[21]
Study to assess Homocysteine (Hcy)-related vitamins and other plasma Hcy determinants in patients with acute porphyrias (AP)	Serum	Relative deficiency*: <45 nmol/L * the authors considered relative vitamin deficiency of serum B6 under 25th percentile of a sex-matched reference Italian healthy	34.4–170 nmol/L	[22]

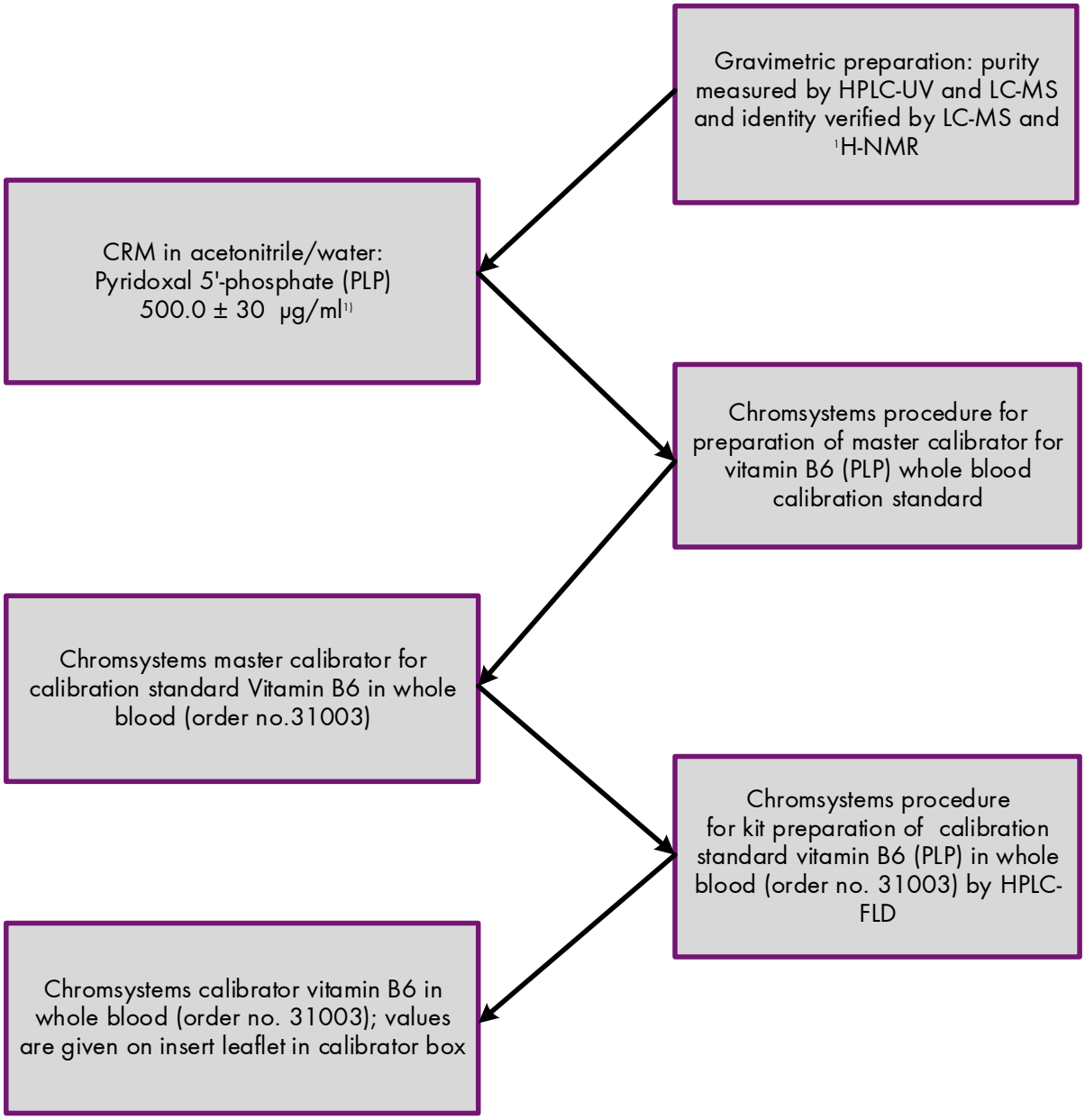
Aim/research question of the study	Matrix used to determine PLP levels	Expected values in affected population	Expected values in non-affected population	Ref
		population (300 subjects)		
Study to determine the prevalence of hyper-homocysteinemia (HHcy) by level determination of the homocysteine- related vitamin B_6 in elderly hospitalised patients.	Serum	Deficiency: < 14.6 nmol/L Hyper-homo-cysteinemia: Mean tHcy > 15 µmol/L	14.6–72.8 nmol/L	[23]
Measurement of fasting serum vitamin B_6 in type II diabetes patients with/without methionine tolerance to assess the effect of insulin administration on tHcy	Serum	Deficiency: < 34.4 nmol/L	34.4–170 nmol/L	[24]
Study to identify clinical and biochemical characteristics that would help distinguish Hypophosphatasia from other metabolic diseases in adults attending a metabolic bone clinic by comparing patients who have genetically confirmed HPP with group of patients with low bone mineral density (BMD)	Serum	Affected by hypophosphatasia (elevation) > 120 nmol/L	Normal PLP (general population) 14.6–177.9 nmol/L (women) 19.4–250.7 nmol/L (men) PLP in hypophosphatasia non-affected <120 nmol/L	[25]
To assess the effect of dietary folate intake on blood folate, vitamin B_6 and B12 and homocysteine status in terms of differences by sex and ethnicity	Serum	Deficiency: ≤ 20 nmol/L	> 20 nmol/L	[26]

Appendix IV Traceability of the calibrators

Whole Blood Calibration Standard (order no. 31003)

Vitamin B6 in whole blood

Version 1.0



The master whole blood calibrator (31003) was prepared gravimetrically by addition of pure pyridoxal-5'-phosphate (PLP, Vitamin B6) obtained from a commercial supplier. The concentration was determined in the manufacturer's laboratory using the HPLC reagent kit for vitamin B6 with CRM obtained from ISO 17025 (and ISO 17034) certified supplier (concentration is provided in the graph above). The purity and identity of CRM were determined by certified supplier using a minimum of two independent methods, as shown in the graph above.

The methodology for master whole blood calibrator (31003) is using Chromsystems reagent kit No. 31000 (reagent kit for determination of vitamin B_6 in serum/plasma and whole blood) by HPLC-FLD analysis.

The Chromsystems calibration standard vitamin B_6 in whole blood (working calibrator) (31003) has a concentration as indicated on the insert leaflet of each batch, which is determined by the manufacturer's laboratory using the Chromsystems reagent kit method as reference. The assay was calibrated using Chromsystems master calibrator with a concentration (including uncertainty) as shown in the diagram above. The methodology for working calibrator is using Chromsystems reagent kit no. 31000 (Vitamin B_6 in serum/plasma and whole blood) by HPLC-FLD analysis.

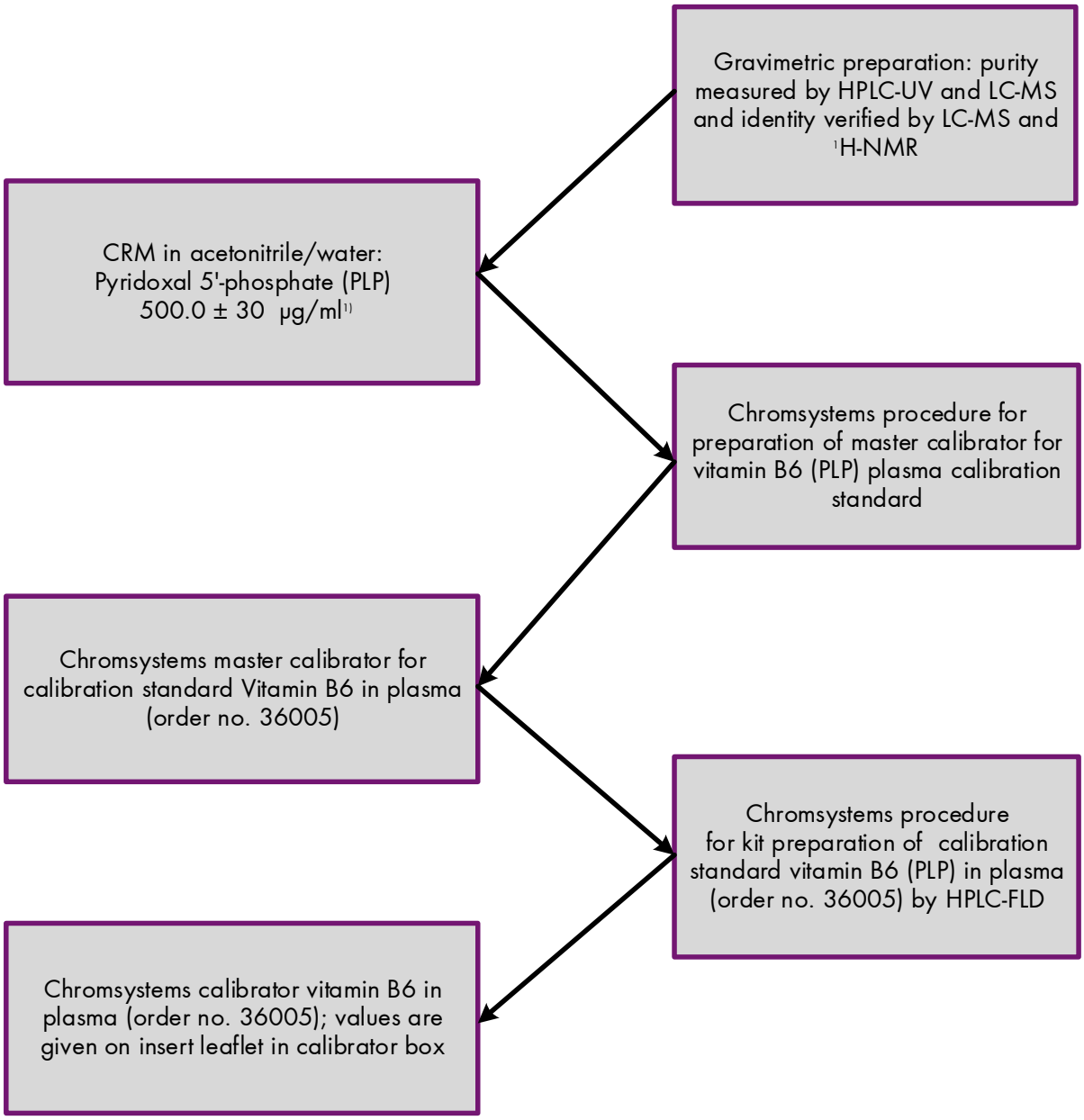
Homogeneity is checked for each batch by multiple analyses of several aliquots based on Chromsystems statistic rationale for sample size (based on ISO 13528 with a minimum set of 10 repeats and two runs).
The assigned values and corresponding uncertainties are provided on the insert leaflet for each calibrator.

[1)] Uncertainty of the concentration is expressed as an „expanded uncertainty" at the approximate 95 % confidence interval using a coverage factor of k=2.

Plasma Calibration Standard (order no. 36005)

Vitamin B6 in plasma

Version 1.0



The master plasma calibrator (36005) was prepared gravimetrically by addition of pure pyridoxal-5'-phosphate (PLP, Vitamin B6) obtained from a commercial supplier. The concentration was determined in the manufacturer's laboratory using the HPLC reagent kit for vitamin B6 with CRM obtained from ISO 17025 (and ISO 17034) certified supplier (concentration is provided in the graph above). The purity and identity of CRM were determined by certified supplier using a minimum of two independent methods, as shown in the graph above.

The methodology for master plasma calibrator (36005) is using Chromsystems reagent kit No. 31000 (reagent kit for determination of vitamin B6 in serum/plasma and whole blood) by HPLC-FLD analysis.

The Chromsystems calibration standard vitamin B6 in plasma (working calibrator) (36005) has a concentration as indicated on the insert leaflet of each batch, which is determined by the manufacturer's laboratory using the Chromsystems reagent kit method as reference. The assay was calibrated using Chromsystems master calibrator with a concentration (including uncertainty) as shown in the diagram above.

The methodology for working calibrator is using Chromsystems reagent kit no. 31000 (Vitamin B6 in serum/plasma and whole blood) by HPLC-FLD analysis.

Homogeneity is checked for each batch by multiple analyses of several aliquots based on Chromsystems statistic rationale for sample size (based on ISO 13528 with a minimum set of 10 repeats and two runs).

The assigned values and corresponding uncertainties are provided on the insert leaflet for each calibrator.

[1] Uncertainty of the concentration is expressed as an „expanded uncertainty" at the approximate 95 % confidence interval using a coverage factor of k=2.

Appendix V Symbols

We use EN ISO 15223-1 symbols on our labels, specifications and packaging. The meanings of each symbol are given in the table below:

Table 28: Symbols

Symbol	Meaning
	Manufacturer
	Date of manufacture
	Use by
	Order number
	Batch/lot code
	See instructions for use
	Upper temperature limit: Store below a certain temperature
	Temperature limit: Store within a certain temperature range
	*In-vitro* diagnostic medical device
	Sufficient for <n> appliances
	Caution
	Serial number
	CE marking of conformity with the relevant EU legislation
	CE marking of conformity with the relevant EU legislation (with affix 0123 – for notified body: TÜV Süd Product Service GmbH)

Appendix VI Version history

Table 29: Version history

Version	Date of release (YYYY-MM-DD)	Description
1.0IVDR	2023-12-19	Initial creation IVDR