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# DETERMINATION OF PYRIDOXAL, PYRIDOXAL 5'-PHOSPHATE AND 2-PYRIDOXIC ACID IN PLASMA OF SELECTED GROUPS OF PATIENTS WITH VIEW TO PROPER SAMPLE PREPARATION USING HPLC WITH FLUORESCENCE DETECTION

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*Vitamin $B_6$ is a global term for three structurally related biologically active forms, pyridoxol, pyridoxal and pyridoxamine. Their esters with phosphoric acid, mainly pyridoxal 5'-phosphate, act as a coenzyme in many enzymatic reactions. Important metabolite extracted in urine is biologically inactive 4-pyridoxic acid. The aim of the study was to develop a rapid and simple HPLC method for the measurement of pyridoxal, pyridoxal 5'-phosphate and 4-pyridoxic acid in human plasma. A method for the measurement of pyridoxal, pyridoxal 5'-phosphate and 4-pyridoxic acid using a high-performance liquid chromatography with fluorescence detection with regard to stability and recovery is described. For the separation, a reverse phase column LiChroCart 125-4, Purospher RP-18e, 5μm,*

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*was used. The mixture of ethanol and 25 mmol $l^{-1}$ $Na_2HPO_4$ (7:93, v/v), pH 6.0 was used as a mobile phase. The analytical performance of this method is satisfactory: the intra-assay and inter-assay coefficients of variation were below 10 %. Quantitative recoveries from spiked plasma samples were at intervals 93.0 - 101.8 %, except for pyridoxal (82.5 - 98.8 %). The limit of detection was 1.0 nmol $l^{-1}$ for pyridoxal 5'-phosphate, 0.7 nmol $l^{-1}$ for 4-pyridoxic acid and 1.1 nmol $l^{-1}$ for pyridoxal. The preliminary reference range of pyridoxal 5'-phosphate, pyridoxal and 4-pyridoxic acid in a group of blood donors are 42.0-75.6 nmol $l^{-1}$, 2.6-12.8 nmol $l^{-1}$ and 16.2-33.0 nmol $l^{-1}$, respectively. We have developed and evaluated a simple, relative rapid and selective HPLC method with fluorescence detection for the determination of pyridoxal 5'-phosphate, pyridoxal and 4-pyridoxic acid in human plasma. The presented method is inexpensive and suitable for clinical trials.*

## Introduction

Vitamin $B_6$ is a water-soluble compound that was discovered in the 1930s during nutrition studies on rats [1]. It exists in three forms (pyridoxol, pyridoxal and pyridoxamine) that are precursors of an activated compound known as pyridoxal 5'-phosphate (PLP). It acts as a coenzyme in all transamination reactions, and in some decarboxylation and deamination reactions of amino acids. It is also involved in other biological processes such as tryptophan-niacin conversion, heme synthesis, gluconeogenesis, and neurotransmitter synthesis [2,3]. 4-Pyridoxic acid (PA) is the major excreted catabolite found in urine. A deficiency of PLP has been implicated in several clinical disorders, including a seborrheic dermatitis-like eruption, atrophic glossitis with ulceration, angular cheilitis, conjunctivitis, intertrigo, and neurologic symptoms of somnolence, confusion, and neuropathy. Some clinical studies suggest that low plasma PLP concentrations are an independent risk factor for cardiovascular diseases [4-6].

Several methods for the determination of PLP and PL in plasma and whole blood have been developed. These include microbiological, enzymatic or chemical methods and, more recently, a high-performance liquid chromatography (HPLC) with ultraviolet, fluorescence and electrochemical detection [7-14]. A derivatization of PLP and PL is required for fluorescence detection; generally utilized methods for derivatization include bisulfite [10,11,14], cyanide [7] and semicarbazide methods [9,12] or chlorite oxidation technique [3,13] because PLP and PL have only low intrinsic fluorescence and plasma concentrations are typically in the range of 2-400 nmol $l^{-1}$. On the other hand, 4-PA features strong natural fluorescence.

We here report a simple and robust HPLC with fluorescence detection using semicarbazide for pre-column derivatization that is suitable for the simultaneous measurement of PLP, PL and degradation product, 4-PA in human plasma.

## Materials and Methods

### Reagents and Chemicals

Pyridoxal 5'-phosphate, pyridoxal, 4-pyridoxic acid, EDTA, sodium hydroxide, sodium chloride, sodium hydrogenphosphate, sodium dihydrogenphosphate, sodium acetate, ammonium acetate, semicarbazide hydrochloride, glycine, perchloric acid, 5-sulfosalicylic acid, trichloroacetic acid, and hydrochloric acid were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC-gradient grade methanol, ethanol, and acetonitrile were from Merck KgaA (Darmstadt, Germany). All the other chemicals were of analytical grade. Since PLP is light sensitive, precautions were taken to minimize exposure of standard and plasma samples to daylight by using either amber plastic tubes or plastic tubes Vacuette protected with aluminium foil.

Pyridoxal 5'-phosphate, pyridoxal and 4-pyridoxic acid solutions were prepared daily in 1 mmol $l^{-1}$ hydrochloric acid and stored at 4 °C in dark.

### Instrumentation

The chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-10ADvp solvent delivery system, SIL-10ADvp autosampler, CTO-10ASvp column oven, RF-10Axl fluorescence detector and SCL-10Avp system controller. Data were collected digitally with Clarity chromatography software (DataApex, Prague, the Czech Republic).

### Subject and Samples

Samples of peripheral venous blood with EDTA as an anticoagulant were obtained from a group of healthy blood donors ($n = 69$, 34 women in the age of 28-61 years, mean age 46 years, and 35 men in the age of 27-59 years, mean age 47 years), patients with coronary artery disease ($n = 100$, 42 women in the age of 41-81 years, mean age 58 years, and 58 men in the age of 43-84 years, mean age 57 years), patients with non-insulin dependent diabetes mellitus ($n = 35$, 14 women in the age of 30-79 years, mean age 56 years, and 21 men in the age of 26-82 years, mean age 58 years) and obese adults ($n = 31$, 21 women in the age of 26-69 years, mean age 55 years, and 10 men in the age of 28-71 years, mean age 54 years). The group of obese adults was characterized by waist circumference (> 100 cm) and body mass index (> 30 kg $m^{-2}$). All the obese adults were recruited from trainees of the Healthy Lifestyle Courses arranged by the Health Institute, which provided guided energy intake reduction under medical supervision. All the

subjects recruited for the study were either abstainers or moderate alcohol consumers ($\leq$ 20 g $day^{-1}$). The patients who had any serious health complications were excluded. None of the studied subjects exhibited renal, hepatic, gastrointestinal, pulmonary or oncological diseases. A written informed consent was obtained from all participants before starting the protocol and the Hospital Committee on Human Research (Regional Hospital of Pardubice, the Czech Republic) approved the study. Plasma was separated from blood cells by centrifugation (1700 × g, 15 min, 8 °C) and immediately stored at –80 °C. Erythrocytes were washed four times with isotonic solution (0.9 % sodium chloride) and after their lysis with ice-cold deionized water and immediately stored at –80 °C.

## Sample Preparation

To 300 µl plasma, erythrocyte lysate or mixed standard solutions, 25 µl derivatization agent (85 mg semicarbazide hydrochloride and 75 mg glycine in 500 µl deionized water) was added. Well-capped 1.5-mL amber plastic tubes were vigorously mixed for 30 s on a Vortex mixer and incubated at room temperature for 30 min. After incubation, cold 1 mol $l^{-1}$ perchloric acid was carefully added (300 µl). After incubation (4 °C, 10 min) and centrifugation (22000 × g, 10 min, 4 °C), the supernatant (400 µl) was stabilized with 25 % sodium hydroxide (pH between 4 and 5) and filtered through a 0.20 µm nylon filter (4 mm diameter, Supelco, Bellefonte, PA, USA) and transferred into 0.2 ml cramped amber vial.

## Chromatographic Analysis

Chromatography of PLP, PL and 4-PA was accomplished using an isocratic elution on a LiChroCART 125 × 4 mm i.d., Purospher STAR RP-18e, 5 µm analytical column fitted a LiChroCART 4 × 4 mm i.d., Purospher STAR RP-18e, 5 µm guard column (Merck KgaA, Darmstadt, Germany) at 37 °C. The mobile phase consisted of 7 % ethanol in 25 mmol $l^{-1}$ $Na_2HPO_4$ (v/v), pH 6.0. The flow rate was kept constant at 0.7 ml $min^{-1}$ in the intervals 0-7 min, at 1.2 ml $min^{-1}$ in the intervals 7-15 min and at 0.7 ml $min^{-1}$ in the intervals 15-20 min. The optimum response of PLP and PL fluorescent derivatives was observed when the excitation and emission wavelengths were set at 380 and 450 nm, while 4-PA was monitored at 320 and 420 nm. The amount of PLP, PL and 4-PA was quantified from the corresponding peak area using Clarity chromatography software (DataApex, Prague, the Czech Republic). The concentration of PLP, PL and 4-PA in the samples was determined from the calibration curve.

Statistical Analysis

The data are presented as mean values ± S.D. Differences between the control subjects and patients, as well as between women and men were analyzed with the use of the Student *t*-test, and analysis of correlation was carried out using Spearman Rank Order Correlation (software QCexpert, Trilobyte, Pardubice, the Czech Republic). A $p < 0.05$ value was considered statistically significant.

## Results and Discussion

Pre-Column Derivatization of PLP and PL

The derivatization procedure was performed by a slight modification of the method Talwar *et al.* [9]. The maximum recovery of derivatization procedure was achieved when the final concentration of semicarbazide and glycine in the reaction mixture was about 13.0 mg $ml^{-1}$ and 11.5 mg $ml^{-1}$, the pH of reaction mixture between 3.0 and 3.6 and incubation time about 30 min. The optimum incubation temperature was between 20 and 45 °C. Our results conform to those published previously [9].

The Effectiveness of Various Protein Precipitants and Stability

The effectiveness of various protein precipitants was tested: only perchloric and metaphosphoric acids as protein precipitants led to satisfactory recoveries of PLP, PL and 4-PA. We chose cheaper perchloric acid for all the analyses. Protein precipitation with trichloroacetic acid was insufficient and 5-sulfosalicylic acid interferes with HPLC determination.

The stability of PLP, PL and 4-PA was studied under various conditions during 12 hours. Figure 1 indicates that PLP was stable in standard solution, plasma and erythrocyte lysate for at least 12 h if the samples were kept in the dark at either 4 °C or room temperature; however, light exposure significantly accelerates degradation of PLP (Fig. 1C). On the other hand, PL and 4-PA were stable in standard solution, plasma and erythrocyte lysate for at least 12 h if the samples were kept in both the dark and daylight. Unlike other authors, we did not observe dramatic decline of PLP levels in the daylight. However samples were not exposed to a direct sunlight.

Fluorescent semicarbazone derivatives of PLP and PL, as well as 4-PA were stable on a cooled autosampler (4 °C) for at least 24 hours.

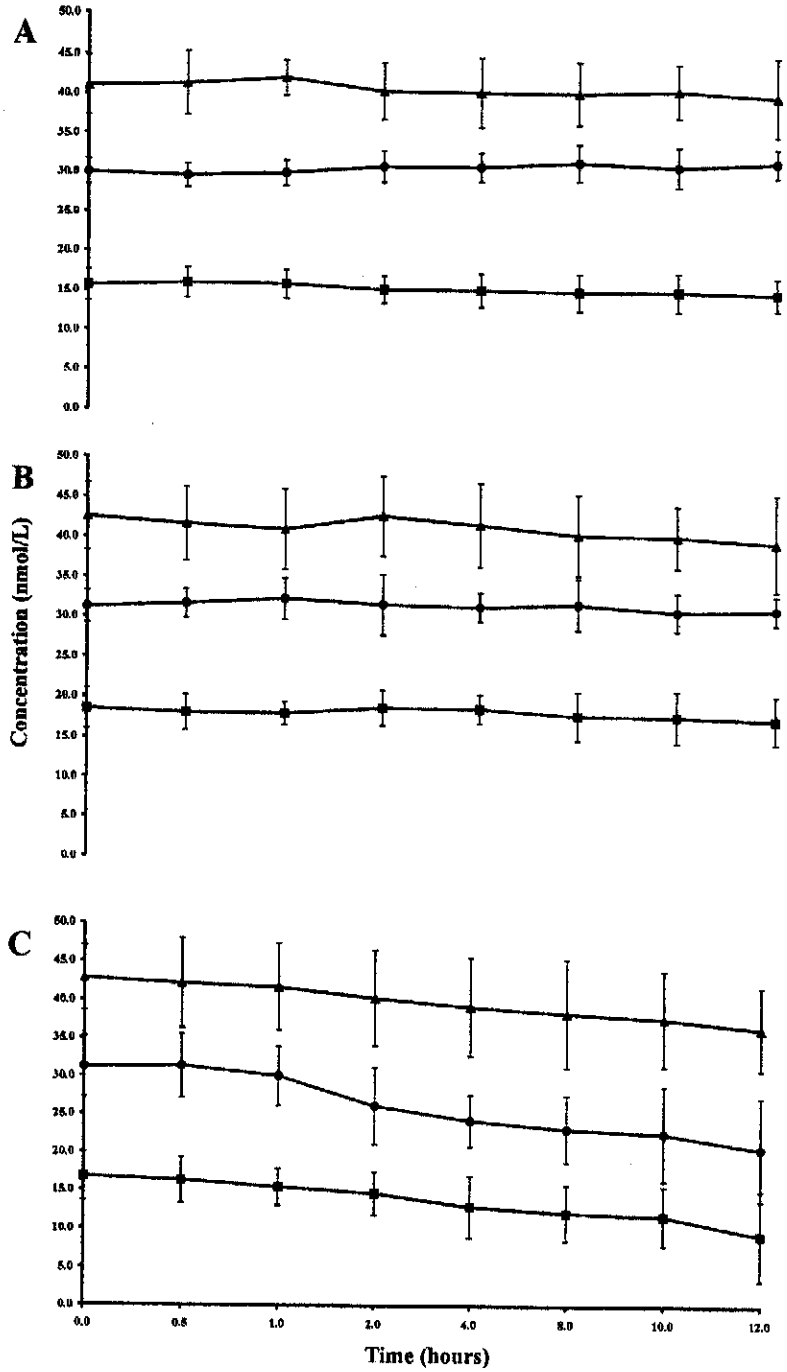


Fig. 1 The stability of PLP in EDTA plasma (■), erythrocyte lysate (▲) and standard solution (●), stored in the dark at 4 °C (A), in the dark at room temperature (B) and in the daylight at room temperature (C). Mean of triplicate assays is recorded

An HPLC chromatogram of PLP, PL and 4-PA in human plasma is shown in Fig. 2. The analytical parameters of PLP, PL and 4-PA analysis are shown in Tables I-III. To determine the within-day precision, the plasma samples were analyzed ten times on the same day under the same conditions. Similarly, the between-day precision was obtained on the same plasma samples analyzed on 12 different days. The coefficients of variation were below 10 %. The spike recoveries, obtained after the derivatization and protein precipitation with 1.0 mol $l^{-1}$ perchloric acid ranged between 94.0-101.5 % for PLP, 82.5-98.8 % for PL and

Table I Precision and recovery of PLP for plasma samples analysis. *Mean of triplicate assays is recorded

| A) Precision (within-day) | | |
|---|---|---|
| *n* | Mean ± SD, nmol $l^{-1}$ | CV, % |
| 10 | 29.1 ± 1.8 | 6.2 |
| 10 | 62.4 ± 3.3 | 5.3 |
| 10 | 129.7 ± 5.2 | 4.0 |
| B) Precision (between-day) | | |
| *n* | Mean ± SD, nmol $l^{-1}$ | CV, % |
| 12 | 51.4 ± 3.8 | 7.4 |
| 12 | 109.4 ± 7.8 | 7.1 |
| C) Recovery | | |
| Added, nmol $l^{-1}$ | Observed, nmol $l^{-1}$ | Recovery, % |
| 0 | 43.3 ± 2.6 | - |
| 5 | 48.0 ± 3.6 | 94.0 |
| 10 | 52.8 ± 3.4 | 95.0 |
| 25 | 67.7 ± 4.1 | 97.6 |
| 50 | 92.6 ± 3.9 | 98.6 |
| 75 | 119.4 ± 5.0 | 101.5 |
| 100 | 142.5 ± 5.6 | 99.2 |
| | | mean 97.7 ± 2.8 |
| | | CV 2.8 |

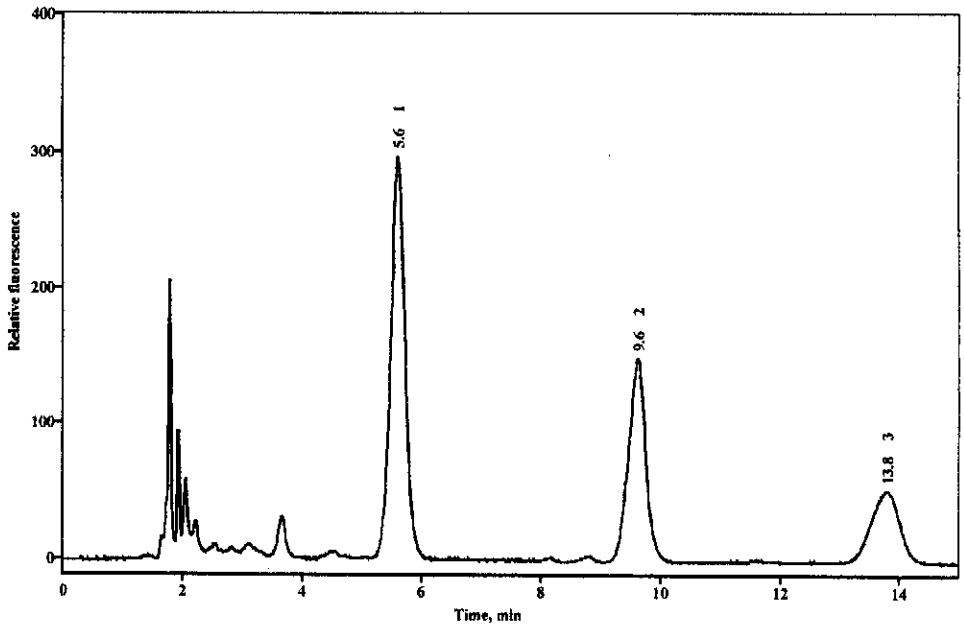


Fig. 2 An HPLC chromatogram of PLP (61.3 nmol $l^{-1}$), 4-PA (20.7 nmol $l^{-1}$) and PL (11.2 nmol $l^{-1}$) in human plasma. Peaks: (1) PLP, (2) 4-PA and (3) PL. HPLC conditions: an isocratic elution (mobile phase: 7 % ethanol in 25 mmol $l^{-1}$ $Na_2HPO_4$, pH 6.0). The stationary phase was an analytical column LiChroCart 125-4, Purospher RP-18e, 5 μm fitted a LiChroCart 4-4, Purospher RP-18e, 5 μm guard column (Merck KgaA, Darmstadt, Germany). The flow rate was kept constant at 0.7 ml $min^{-1}$ in the intervals 0-7 min, at 1.2 ml $min^{-1}$ in the intervals 7-15 min and at 0.7 ml $min^{-1}$ at intervals 15-20 min, separation ran at 37 °C and PLP along with PL were monitored at excitation and emission wavelengths of 380 nm and 450 nm, while 4-PA at 320 nm and 420 nm

93.0-101.8 % for 4-PA. The calibration curve was linear in the whole range tested: (3.0-300.0) nmol $l^{-1}$ of PLP, (3.0-300.0) nmol $l^{-1}$ of PL and (2.0-250.0) nmol $l^{-1}$ of 4-PA. The calibration curve values obtained from the combination of twelve standard curves are shown in Table IV. The lowest concentration that can be quantified with acceptable accuracy and precision was 3.0 nmol $l^{-1}$ for PLP, 3.0 nmol $l^{-1}$ for PL and 2.0 nmol $l^{-1}$ for 4-PA. Furthermore, the limit of detection for PLP, PL and 4-PA defined as signal-to-noise (S/N) ratio of 3:1 was 1.1, 1.0 and 0.7 nmol $l^{-1}$, respectively.

Pyridoxal 5'-phosphate, pyridoxal and 4-pyridoxic acid were separated on a reverse-phase column using an isocratic system of ethanol and sodium hydrogenphosphate. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of PLP, PL and 4-PA as well as pooled plasma and whole blood were used for study of the mobile phase composition. Several mobile phases (namely buffers containing methanol,

Table II Precision and recovery of PL for plasma samples analysis. *Mean of triplicate assays is recorded

| A) Precision (within-day) | | |
|---|---|---|
| *n* | Mean ± SD, nmol $l^{-1}$ | CV, % |
| 10 | 3.10 ± 0.27 | 8.7 |
| 10 | 8.41 ± 0.66 | 7.8 |
| 10 | 29.44 ± 2.36 | 8.0 |
| B) Precision (between-day) | | |
| *n* | Mean ± SD, nmol $l^{-1}$ | CV, % |
| 12 | 3.42 ± 0.33 | 9.6 |
| 12 | 27.54 ± 2.47 | 9.0 |
| C) Recovery | | |
| Added, nmol $l^{-1}$ | Observed, nmol $l^{-1}$* | Recovery, % |
| 0 | 7.28 ± 0.69 | - |
| 2 | 8.93 ± 0.76 | 82.5 |
| 5 | 11.92 ± 1.06 | 92.8 |
| 10 | 16.95 ± 1.44 | 96.7 |
| 20 | 27.03 ± 2.10 | 98.8 |
| 30 | 35.87 ± 2.69 | 95.3 |
| 50 | 56.11 ± 3.47 | 97.7 |
| | | mean 94.0 ± 6.0 |
| | | CV 6.4 |

ethanol and acetonitrile) and several gradients were assayed. Optimization of the separation was obtained after studying the effect of the concentration of sodium hydrogenphosphate (from 5.0 to 100.0 mmol $l^{-1}$), the pH (from 4.0 to 7.0) and ethanol concentration. Column temperature was adjusted from 25 to 45 °C. The mobile phase conditions leading to the best separation were: 7 % ethanol in 25 mmol $l^{-1}$ sodium hydrogenphosphate, pH 6.0. The optimum temperature interval was from 35 to 40 °C. The criteria included the resolution, stability of the fluorescence intensity and the analysis speed. In reverse-phase HPLC, organic solvents such as acetonitrile and methanol are generally used to achieve the separation of analytes. However, both acetonitrile and methanol are considered as significant pollutants. Our method uses the eco-friendly ethanol in a mobile phase.

Table III Precision and recovery of 4-PA for plasma samples analysis. *Mean of triplicate assays is recorded

| A) Precision (within-day) | | |
|---|---|---|
| *n* | Mean ± SD, nmol $l^{-1}$ | CV, % |
| 10 | 10.2 ± 0.4 | 3.9 |
| 10 | 28.4 ± 0.9 | 3.2 |
| 10 | 52.3 ± 1.4 | 2.7 |
| B) Precision (between-day) | | |
| *n* | Mean ± SD, nmol $l^{-1}$ | CV, % |
| 12 | 22.6 ± 1.6 | 7.1 |
| 12 | 48.9 ± 3.1 | 6.3 |
| C) Recovery | | |
| Added, nmol $l^{-1}$ | Observed, nmol $l^{-1}$* | Recovery, % |
| 0 | 24.1 ± 0.8 | - |
| 5 | 28.8 ± 1.0 | 94.0 |
| 10 | 33.4 ± 1.1 | 93.0 |
| 25 | 48.3 ± 1.5 | 96.8 |
| 50 | 74.7 ± 2.20 | 101.2 |
| 75 | 98.2 ± 2.8 | 98.8 |
| 100 | 125.9 ± 3.5 | 101.8 |
| | | mean 97.6 ± 3.7 |
| | | CV 3.8 |

Table IV Calibration curve values for the method. *Thirteen-point for a determination of analytical parameters and seven-point for a routine analysis

| Standard | Regression equation | Mean slope 95 % confidence interval | Intercept 95 % confidence interval | Correlation coefficient |
|---|---|---|---|---|
| PLP* | $y = 9.9521x - 6.9512$ | 9.9521 (9.9214-9.9825) | 0.7 (0.4-1.1) | 0.9994 |
| PL* | $y = 9.4924x - 4.7414$ | 9.4924 (9.1125-9.8210) | 0.5 (0.4-0.6) | 0.9996 |
| 4-PA* | $y = 9.6498x - 10.76116$ | 9.6498 (9.2147-10.0415) | 1.1 (0.6-1.7) | 0.9997 |

Comparison of the patients and the control group is presented in Table V. Reference ranges for PLP, PL and 4-PA are 42.0-75.6 nmol $l^{-1}$, 2.6-12.8 nmol $l^{-1}$ and 16.2-33.0 nmol $l^{-1}$, respectively. The normal values for PLP, PL and 4-PA in plasma differ between laboratories [15-20]. Our reference ranges are based on those published previously and range in a wide interval. We found no significant differences in PLP, PL and 4-PA concentration between women and men in any of the groups of patients and blood donors. We observed, in all follow-up groups, no significant correlation vitamers B6 concentrations and age, either.

The aim of this study was to develop and validate an HPLC method with fluorescence detection for the routine determination of vitamers B6 in human plasma. Within two years we have carried out more than 1000 analyses. We esti-

Table V A comparison of plasma PLP, PL, and 4-PA between the patients and the control group. Data are expressed as mean ± SD. Differences between the control subjects and patients were analyzed with the use of the Student *t*-test. A $p < 0.05$ value is considered statistically significant

| | Patients with coronary artery disease A, $n = 100$ | *p*-Value A vs. D | Patients with non-insulin dependent diabetes mellitus B, $n = 35$ | *p*-Value B vs. D |
|---|---|---|---|---|
| Age (*y*) | 41-48 | - | 26-82 | - |
| Female/male | 42/58 | - | 14/21 | - |
| PLP, nmol $l^{-1}$ | 43.4 ± 33.8 | 0.053 | 54.5 ± 40.0 | 0.612 |
| PL, nmol $l^{-1}$ | 8.6 ± 8.8 | 0.840 | 12.3 ± 6.6 | 0.025 |
| 4-PA, nmol $l^{-1}$ | 27.3 ± 20.4 | 0.322 | 0.322 | 0.372 |

| | Obese adults | *p*-Value | Control group |
|---|---|---|---|
| | C, $n = 31$ | C vs. D | D, $n = 69$ |
| Age (*y*) | 26-71 | - | 27-61 |
| Female/male | 40106 | - | 34/35 |
| PLP, nmol $l^{-1}$ | 57.1 ± 42.7 | 0.802 | 59.8 ± 74.0 |
| PL, nmol $l^{-1}$ | 8.6 ± 7.5 | 0.670 | 7.7 ± 21.0 |
| 4-PA, nmol $l^{-1}$ | 31.3 ± 20.6 | 0.185 | 24.6 ± 35.0 |

mate that lifetime of an analytical column is at least 1000 injects. We can establish that the presented method is inexpensive and suitable for clinical trials.

Abbreviations used: PL — pyridoxal, PLP — pyridoxal 5'-phosphate, 4-PA — 4-pyridoxic acid

## Conclusion

We have developed a relative rapid, simple, and very sensitive HPLC method with fluorescence detection for the determination of PLP, PL and 4-PA in human plasma. By virtue of this method it is possible to determine PLP, PL and 4-PA in erythrocytes and urine, too.

## Acknowledgements

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