Plasma Vitamin B$_6$ Vitamers before and after Oral Vitamin B$_6$ Treatment: A Randomized Placebo-controlled Study

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Background: Vitamin B$_6$ has attracted renewed interest because of its role in homocysteine metabolism and its possible relation to cardiovascular risk. We examined the plasma B$_6$ vitamers, pyridoxal 5′-phosphate (PLP), pyridoxal (PL), pyridoxine (PN), and 4-pyridoxic acid (4-PA) before and after vitamin B$_6$ supplementation.

Methods: Patients (n = 90; age range, 38–80 years) undergoing coronary angiography (part of the homocysteine-lowering Western Norway B-Vitamin Intervention Trial) were allocated to the following daily oral treatment groups: (A), vitamin B$_{12}$ (0.4 mg), folic acid (0.8 mg), and vitamin B$_6$ (40 mg); (B), vitamin B$_{12}$ and folic acid; (C), vitamin B$_6$; or (D), placebo. EDTA blood was obtained before treatment and 3, 14, 28, and 84 days thereafter.

Results: Before treatment, PLP (range, 5–111 nmol/L) and 4-PA (6–93 nmol/L) were the predominant B$_6$ vitamers identified in plasma. During the 84-day study period, the intraindividual variation (CV) in patients not treated with vitamin B$_6$ (groups B and D) was 45% for PLP and 67% for 4-PA. Three days after the start of treatment, the increases in concentration were ~10-, 50-, and 100-fold for PLP, 4-PA, and PL, respectively. No significant additional increase was observed at the later time points. The PLP concentration correlated to the concentrations of 4-PA and PL before treatment, but not after treatment. The PL concentration correlated with 4-PA before and after treatment.

Conclusions: Vitamin B$_6$ treatment has an immediate effect on the concentrations and the forms of B$_6$ vitamers present in plasma, and the changes remain the same during prolonged treatment. Our results suggest that the B$_6$ vitamers in plasma reflect vitamin B$_6$ intake.
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Vitamin B$_6$ functions as a coenzyme in >100 enzymatic reactions involved in the metabolism of amino acids, carbohydrates, neurotransmitters, and lipids (*1*). Pyridoxine (PN),[5] pyridoxal (PL), pyridoxamine (PM), their phosphorylated derivatives [pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP)], and the end product of vitamin B$_6$ metabolism, 4-pyridoxic acid (4-PA), are the major forms of vitamin B$_6$ found in mammalian tissues and body fluids (*1*).

In the diet, vitamin B$_6$ is predominantly present in three forms, PN, PM, and PL. After passive intestinal absorption, the major part of the vitamin is delivered to the liver and converted to PLP (*2–4*). PLP is available for other cells only after being hydrolyzed to PL by alkaline phosphatase, but most cells have pyridoxal kinase activity and are therefore able to rephosphorylate PL to PLP (*4, 5*). Most of the PL in excess of tissue requirements is oxidized to 4-PA by the liver and excreted in the urine (*4*).

The development of HPLC methods for the analysis of vitamin B$_6$ vitamers in plasma (*6*) has enabled analysis of vitamers in the low concentrations present in samples from healthy individuals. However, the data are sparse, and increased knowledge of physiologic concentrations may lead to a more complete understanding of the

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connection between plasma concentrations and nutritional status for this vitamin.

Primary dietary deficiency of vitamin B$_6$ is considered rare in developed countries because the vitamin is present in a wide variety of foods (7). Recently, low vitamin B$_6$ concentrations have been reported in elderly populations (8, 9), in individuals with traumatic femoral fractures (10), and in alcoholics (11, 12). Such low concentrations have been related to increased risk of cardiovascular diseases (9, 13–18). It is, however, uncertain whether the increased risk is mediated by increased homocysteine or whether low B$_6$ is an independent risk factor (19, 20).

PN therapy is frequently recommended for various conditions, including genetic disorders, such as cystathioninuria and homocystinuria, and a range of other disorders, such as premenstrual tension, seizures, carpal tunnel syndrome, and the nausea and vomiting that can occur during pregnancy (1, 3, 21–23). Because it is considered a safe compound, very high doses of B$_6$ [>2000-fold higher than the recommended dietary allowance (1.4–2.1 mg/day, depending on sex and age)] are used in some circumstances (7, 24). Such doses may be associated with peripheral neuropathy, but only sparse data are available on the mechanism and which B$_6$ vitamers are responsible (25). Indeed, little is known about the effect of PN supplementation on plasma vitamin B$_6$ concentrations and long-term response to this treatment.

Currently, several clinical trials on B-vitamin treatment for cardiovascular disease are underway, many of them including vitamin B$_6$ (26–28). Hence, there is increased interest in this vitamin.

We recently developed a HPLC method for measuring the B$_6$ vitamers PLP, PL, PMP, PN, and PM and the degradation product 4-PA in plasma (29). In the current study, we used this method to assess plasma B$_6$ vitamer concentrations before and after 3, 14, 28, and 84 days of treatment with PN (40 mg/day) in patients undergoing coronary angiography. In addition, we calculated the inter- and intraindividual variation in B$_6$ vitamers from serial measurements of the analytes in the group not treated with PN.

Materials and Methods

PATIENTS AND BLOOD SAMPLES

This investigation is a substudy of the ongoing Western Norway B-Vitamin Intervention Trial (WENBIT), a prospective randomized double-blind study on the effects of homocysteine-lowering therapy on mortality and cardiovascular events. The participants of this substudy have recently been described (28). In brief, adult patients undergoing coronary angiography for suspected coronary artery disease or aortic valvular stenosis are eligible for WENBIT, independent of subsequent therapy. Exclusion criteria are malignant disease, alcohol abuse, mental illness, lack of availability for long-term follow-up, and participation in other trials.

A total of 90 consecutive patients (age range, 38–80 years; 21 females and 69 males) with suspected coronary artery disease recruited to the WENBIT study at Haukeland University Hospital were eligible for this study of plasma vitamin B$_6$ vitamers. Informed consent was obtained from all patients, and the regional ethics committee approved the study protocol.

In WENBIT, the possibility that vitamin B$_6$ has an independent effect on cardiovascular disease (30) has been taken into account by use of a factorial design where one of the groups includes vitamin B$_6$ alone. Hence, recruited patients were randomized in a 2 × 2 factorial design for daily oral treatment according the following protocol: A (n = 22), vitamin B$_{12}$ (0.4 mg), folic acid (0.8 mg), and vitamin B$_6$ (40 mg); B (n = 23), vitamin B$_{12}$ and folic acid; C (n = 21), vitamin B$_6$; or D (n = 24), placebo. For the first 2 weeks, the folic acid groups (A and B) received an additional loading dose of folic acid (5 mg/day). Packages of study tablets were prepared in random order and given serial numbers by Alpharma A/S (Copenhagen, Denmark).

In the present analyses, patients were grouped according to treatment with vitamin B$_6$ (A and C, treatment group; n = 43) or without vitamin B$_6$ (B and D, control group; n = 47). Nonfasting (basal) EDTA samples were collected before and, 3, 14, 28, and 84 days after the start of vitamin treatment. The blood samples were immediately placed on ice, centrifuged within 30 min, and stored at −80 °C until further analyses. Plasma vitamin B$_6$ vitamers were analyzed for the whole group in samples collected at days 0 and 84 and for approximately one-half of the participants on day 3 (n = 23), day 14 (n = 23), and day 28 (n = 24). The sets of samples analyzed on the intervening days were chosen randomly.

BIOCHEMICAL ANALYSIS

Vitamin B$_6$ vitamers were analyzed by ion-pair reversed-phase chromatography as described previously (29). The HPLC system was calibrated by injecting known quantities of vitamin B$_6$ vitamers and 4-PA and measuring the resulting peak areas. The working calibration solution was prepared daily and diluted to obtain 10 calibrators with the following concentrations: 4000, 2000, 1333, 667, 333, 167, 83, 42, 21, and 10 nmol/L (Sigma). All calibration curves within this concentration range showed a linear response (data not shown).

As assessed from control samples (n = 49) assayed over 3 months, the CVs (mean measured concentrations) were 13% (74 nmol/L) for PLP, 8% (40 nmol/L) for PA, 46% (11 nmol/L) for PL, and 16% (22 nmol/L) for PN.

STATISTICAL ANALYSIS

The distributions of values for vitamin B$_6$ vitamers were normalized by logarithmic (base 10) transformation. Nonparametric testing was used for comparison of the control group with the B$_6$-treated group and for comparison of values obtained on the same individuals. To compare relationships between variables, nonparametric testing

(Spearman correlation) was used. Alterations in B_6 vitamers as a function of time were analyzed by comparing the control group (day 0) with the vitamin B_6-treated group for the day in question. Data were analyzed with use of QuatroPro and GraphPad (Prism2) software.

One participant not treated with vitamin B_6 had a plasma PN concentration at day 14 that was dramatically higher than at the other serial measurements. Thus, this outlying value (444 nmol/L) was omitted from the presentation of data in Fig. 1.

Results

Before intervention with vitamin or placebo, plasma concentrations of B_6 vitamers were similar in the four groups, A–D (data not shown). The vitamin B_6 vitamer concentrations (medians and interquartile ranges) for the whole study group before intervention are listed in Table 1. PLP and 4-PA were detected in all samples analyzed, whereas PL was present in 65% and PN and PM in only 3% of the control samples. PMP was not detected in any of the samples.

The age of participants ranged from 38 to 80 years. We found no significant correlation with age for PLP, PL, or 4-PA, but there was a tendency for a decrease in the PLP concentration and an increase in the 4-PA concentration (data not shown). There were no differences according to gender before treatment, whereas PL ($P = 0.011$) and 4-PA ($P = 0.009$), but not PLP ($P = 0.072$), were significantly higher in women than in men after 84 days of treatment with PN. When the concentrations of PL, 4-PA, and PLP were corrected for body weight (plasma concentration times body weight), the differences between males and females were the same after 84 days of treatment with PN [PL ($P = 0.036$), 4-PA ($P = 0.036$), PLP ($P = 0.45$)].

We found a significant correlation between plasma creatinine and plasma 4-PA ($r = 0.38$; $P = 0.001$), but not PL and PLP, before treatment. Plasma PLP was significantly correlated with plasma albumin both before ($r = 0.32$; $P = 0.002$) and after treatment ($r = 0.44$; $P = 0.006$). In contrast, PLP was not associated with C-reactive protein, suggesting that the association between PLP and albumin is not explained by concomitant changes in these two factors as the result of an acute-phase reaction.

The intraindividual variations as calculated from the serial measurement of the analytes in the control group were 45% for plasma PLP, 67% for 4-PA, and 92% for PL (Table 1).

After 3 days of supplementation with oral vitamin B_6, both qualitative and quantitative changes were observed in plasma B_6 vitamers (Fig. 1). The mean concentration increased >10-fold for PLP, ~50-fold for 4-PA, and >100-fold for PL and PN. These increases remained almost the same at 14, 28, and 84 days of treatment (Fig. 1). PM was detectable in only five patients, whereas PMP was not detected in any of the samples after PN treatment. The changes in plasma B_6 vitamers were not different between the two groups (A and C) receiving vitamin B_6, indicating no effect of vitamer B_6 distribution from folic acid or cobalamin.

The correlations of PLP to PL, PLP to 4-PA, and PL to 4-PA were evaluated at day 0 for all persons studied and at day 84 for the treatment group. PLP was correlated to PL ($r = 0.51$; $P <0.0001$) and 4-PA ($r = 0.525$; $P <0.0001$), but only before treatment. The correlation between PL and 4-PA was significant before treatment (day 0; $r = 0.52$; $P <0.0001$), and this correlation increased considerably after treatment (day 84; $r = 0.91$; $P <0.0001$).

Discussion

In this report, we have presented data on vitamin B_6 vitamers before and after treatment with a daily oral dose of PN (40 mg) for a period of 12 weeks in a trial including patients with confirmed coronary artery disease. Although vitamin B_6 has been included in this and several similar trials (26) with the intention to lower homocysteine, some studies suggest that low vitamin B_6 status is independently associated with cardiovascular risk (18, 30, 31). Notably, prospective studies indicate that increased homocysteine may be only a weak risk factor, if a risk factor at all (32, 33). Therefore, these trials should

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Table 1. Plasma concentrations and intraindividual and analytical variation of vitamin B_6 vitamers measured in patients (n = 90) participating in the WENBIT study.[a]

	Median, nmol/L	Interquartile range, nmol/L	95% confidence interval,[b] nmol/L	Intraindividual variation,[c] %	Analytical variation,[d] %
PLP	23	18–37	5–80	45	13
4-PA	20	16–29	6–75	67[e]	8
PL	3	0–5	0–20	92[f]	46

[a] Before supplementation with vitamin B_6 (day 0), the plasma B_6 vitamer concentrations in two groups (vitamin B_6-treated or not treated) were alike, and data are pooled.

[b] Anti-log of log mean ± 2 SD.

[c] The intraindividual variation was calculated from serial measurements of the analytes in the control group on days 0, 3, 14, 28, and 84 (minimum two samples/person).

[d] The analytical variation was calculated from control samples (n = 49) assayed over 3 months. The concentrations of PLP, 4-PA, and PL used for calculation of analytical variation were 74, 8, and 46 nmol/L, respectively.

[e] One outlying value (553 nmol/L) has been excluded. If included, the result is 129%.

[f] One outlying value (215 nmol/L) has been excluded. If included, the result is 396%.
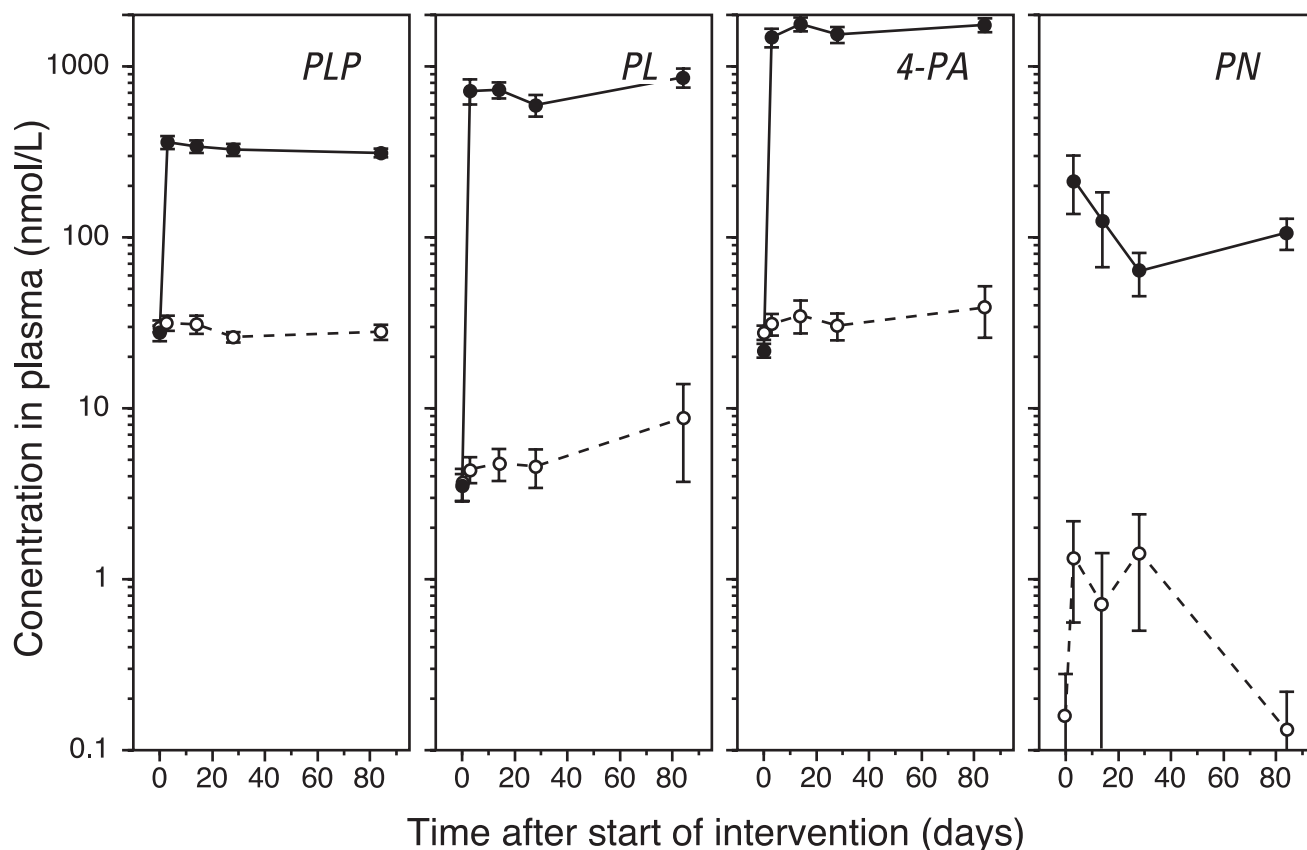
Fig. 1. Changes in plasma concentrations of PLP, PL, 4-PA, and PN in patients receiving vitamin supplementation with (●) or without (○) 40 mg/day vitamin B$_6$.

For the series without vitamin B$_6$, the numbers of participants included were 47 (day 0), 23 (day 3), 23 (day 14), 24 (day 28), and 42 (day 84). The corresponding numbers for the series with vitamin B$_6$ were 43, 21, 18, 28, and 39, respectively. Note that the *y axis* is logarithmic scale. Data are presented as the mean ± SE (*error bars*). Because PN was not detected on day 0 in any of the patients receiving the vitamin supplement, the data point at zero time could not be shown on the logarithmic scale.

focus not only on increased homocysteine as the possible cause of disease, but should also include detailed investigations on all relevant vitamins, including vitamin B$_6$.

In human plasma, vitamin B$_6$ vitamer concentrations are low unless the person takes supplements containing vitamin B$_6$ (*6*). Therefore, it is essential to have a method that can detect low concentrations of vitamin B$_6$ compounds in plasma. We recently developed a HPLC-based method that separates and detects nanomolar quantities of five B$_6$ vitamers and the degradation product 4-PA (*29*). In the present study, we were able to identify PLP, 4-PA, and to a lesser degree PL, as well as occasionally PN and PM in plasma of patients not treated with PN. PMP was not detected in any of the samples either before or after PN treatment. This finding is in agreement with most published studies (*24, 34–37*). Previously, PLP has been shown to comprise 70–90% of the total vitamin B$_6$, whereas PL was reported to account for ~8–30% of the total plasma vitamin B$_6$ (*38*). It is important to note that there is wide variability in the measurement of PL in the literature. The concentration of PL has been reported to vary between 1.1 and 90 nmol/L in the general population (*35–37, 39–46*). Although the plasma PL concentra-

tions found in the current study are comparable to these values, we cannot rule out the possibility that our method may underestimate PL. At present, there are no commercial samples with assigned values or external quality assessment schemes available; it thus remains difficult to establish generally accepted reference values for vitamin B$_6$ vitamers and 4-PA. Therefore, there is a pressing need for biological quality-control samples with B$_6$ values assigned by reference procedures to clarify the accuracy of the existing methods.

Protein binding plays a dominant role in the distribution and elimination of B$_6$ metabolites. PLP is almost completely bound to protein in plasma (*47*), whereas PL is only partly bound and PN and the end product 4-PA are completely free (*47*). In humans, 4-PA represents >90% of the B$_6$ compounds that are excreted in the urine (*48*).

In the present study, the B$_6$ vitamers PLP, PL, and PN and the metabolite 4-PA were measured before treatment and after 3, 14, 28, and 84 days of treatment. We thus obtained serial measurements (two to five measurements per patient) of the analytes. This allowed us to calculate the intraindividual variation in B$_6$ vitamers based on the

findings in the group not treated with PN. Very little is known concerning the intraindividual variation for blood tests reflecting vitamin B_6 status. Previous studies have focused only on circadian variations. In these studies, the plasma concentration profiles of PLP and PL were constant within the observation period of 24 h *(49, 50)*, whereas urinary excretion of 4-PA was lower during the night compared with the time after lunch *(24)*. These results suggest that the timing of phlebotomy is not critical, at least for the PLP and PL concentrations, and that circadian variations may not interfere with assessment of intraindividual variation. Our data show that over a 3-month period, the intraindividual variation is considerable: 45% for plasma PLP, 67% for 4-PA, and ~92% for PL. We do not have any clear explanation for the observed high intraindividual variation in the present study. Because the concentrations of PLP, PL, and 4-PA are related to dietary variables *(51)*, variation in dietary intake may account for part of the variability; thus, the intraindividual variations observed reflect what one would expect in the routine setting. Notably, the intraindividual variation of PLP is lower than the values observed for the other B_6 vitamers, and this may be related to the marked protein binding of this species. We speculate that protein binding may antagonize degradation by alkaline phosphatase and cellular uptake and thereby ensure a more stable PLP concentration in the circulation.

We found no significant correlation between age and PLP or 4-PA, but there was a tendency for a decrease in PLP and an increase in 4-PA with increasing age. Previous studies have shown that plasma PLP concentrations tend to decrease with age and increasing frailty, whereas plasma 4-PA increases with age *(8, 52–54)*. The suggested explanations for this age effect are lower vitamin B_6 intake in older individuals, less efficient retention of the vitamin, increased B_6 catabolism, and variations in kidney function *(8, 52, 53)*.

Plasma 4-PA, but not PL and PLP, was significantly correlated with plasma creatinine. This finding is in agreement with previous studies *(8, 51)* and indicates that 4-PA is more sensitive to variations in kidney function than other vitamin B_6 compounds. Supporting this, Coburn et al. *(55)* recently reported high plasma concentrations of 4-PA during renal insufficiency.

We found no differences according to gender before treatment, whereas PL and 4-PA, but not PLP, were significantly higher in women than in men after 84 days of treatment with PN. The larger increment in women could partly be explained by a smaller body dilution pool. We reached this conclusion because the difference remained after the concentrations of the vitamin B_6 vitamers were adjusted according to body weight. Data from animal studies have suggested pronounced sex differences in tissue concentrations of enzymes of vitamin B_6 metabolism *(56)*. However, controlled clinical studies on sex differences in human vitamin B_6 metabolism have not been performed.

After supplementation with a relatively high dose of PN (40 mg/day), a new steady state was reached by 3 days. At this time, PLP had increased ~10-fold, whereas the increases in both PL and 4-PA were considerably higher. Our results confirm *(35, 42, 48, 57, 58)* and expand earlier studies on the pharmacokinetics of vitamin B_6.

Speitling et al. *(57)* investigated the metabolism of 600 mg of PN given orally to 10 healthy young males. The maximum plasma concentration of PN was reached after 1.3 h, and it took ~9 h until PN was totally eliminated from the plasma. Plasma PL and 4-PA showed essentially the same time courses. Compared with the other B_6 compounds, PLP increased only moderately. Our study extends these observations and shows that the chronic administration of oral PN produces steady-state concentrations of the B_6 vitamers after 3 days and that further increases in plasma PLP, PL, and 4-PA are not obtained by prolonged vitamin B_6 treatment.

The concentration of PLP in plasma does not increase in a dose-dependent manner after intake of supraphysiologic PN doses. This was shown by Edwards et al. *(59)* in healthy volunteers who received PN doses of 10–800 mg/day for 1 week. The concentrations of PL, PN, and 4-PA in plasma, on the other hand, increased as a function of the PN dose administered. Similar results were obtained by Ubbink et al. *(60)* for healthy females who received doses of 10–100 mg of PN. In the present study, the mean PLP concentration increased ~10-fold, whereas 4-PA increased ~50-fold and PL and PN increased >100-fold after vitamin B_6 treatment. The increases that we observed are comparable to those observed by Edwards et al. *(59)*. We speculate that only a limited amount of PLP can be bound by the albumin in plasma and that surplus PLP is metabolized by alkaline phosphatase to PL and eventually 4-PA. In accordance with this view, we found that PLP correlated with albumin.

Currently, there is no agreement concerning which vitamin B_6 vitamer should be used for routine assessment of vitamin B_6 status. Furthermore, there is not always a good correlation between different analytes, and it is difficult at this stage to recommend a definitive single status measurement *(61)*. In the present study, we demonstrated the lack of correlation between different vitamin B_6 vitamers except between PL and 4-PA. The correlation of these two vitamers became more pronounced after treatment with vitamin B_6, possibly because of an increase in the metabolism/excretion rate after supplementation. Our results warrant further studies to assess whether measurement of one or a few of the vitamin B_6 vitamers will suffice for the assessment of vitamin B_6 status.

In conclusion, we found that PLP, 4-PA, and to a lesser degree PL were the predominant B_6 metabolites in plasma. After treatment with vitamin B_6, PN was also detectable, and PN and PL showed the largest increases in concentration after treatment. Vitamin B_6 treatment had an immediate effect on the concentrations and the forms

of B_6 vitamers in plasma, whereas no further changes were observed during a more prolonged treatment.

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