Change in Blood Levels of Vitamin B-6 Derivatives in Pregnant and Lactating Rats

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Summary By using an HPLC method which we have developed, concentration changes of vitamin B-6 derivatives in blood of pregnant and lactating rats were studied. PLP and PL were the main derivatives in plasma and erythrocytes, and occasionally, PMP was found in the plasma of rats fed a normal solid diet which contained 8.3 mg PN·HCl/kg diet. Upon pregnancy, the plasma PLP concentration decreased significantly \( (p<0.01) \), whereas plasma PL tended to increase \( (p<0.05) \). PLP concentration in erythrocytes tended to increase upon pregnancy. These results suggest that metabolism or utilization of vitamin B-6 is altered upon pregnancy and that plasma PLP concentrations alone may not be a good indicator of nutritional status. Further confirmation was needed for the reliable determination of PMP because a large peak found at 7 min or so from erythrocyte samples under our analytical conditions was not converted to PIC-P via PLP, after the sample was treated with glyoxylic acid and then with KCN.

Key Words HPLC analysis of vitamin B-6 derivatives, changes of plasma vitamin B-6 levels upon pregnancy, vitamin B-6 contents of rat blood, nutritional evaluation of vitamin B-6

High-performance liquid chromatography (HPLC) offers a means separately to determine all the vitamin B-6 derivatives from a sample at one time. In contrast, microbiological method currently used for food analysis possesses such limits that only nonphosphorylated vitamin B-6 derivatives can be analyzed, and PIC, which is a very important urinary metabolite of vitamin B-6, if any, could not be determined at all \((1-3)\). By adopting the fluorescence HPLC as the means of analyzing vitamin B-6 derivatives in some food and biological specimens, the exact

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Abbreviations: PIC, pyridoxic acid; PLP, pyridoxal 5'-phosphate; PIC-P, pyridoxic acid 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PNP, pyridoxine 5'-phosphate.
content of each vitamer can be determined (4). Since blood and urine contain miscellaneous fluorescent materials quite similar to those of vitamin B-6 derivatives which interfere with the determination, the vitamin B-6 contents of some samples might be overestimated (5,6).

To know the exact content of vitamin B-6 in the blood, each vitamin B-6 derivative had been previously determined with independent methods. Previous researchers employed various methods to obtain exact data on each vitamin B-6 derivative. For instance tyrosine apo-decarboxylase method was used for PLP analysis (7,8). The same determination method was used for the resulting PLP after deamination of PMP by glyoxylic acid (9).

In view of vitamin B-6 analysis, recent interest has been focussed on whether the blood level of vitamin B-6 changes depending upon the specified physiological conditions, i.e., pregnancy, or senility etc., because serum PLP levels under pregnancy and lactating states were reported to decrease drastically, even with a normal diet was fed (10,11). To obtain such results, for the measurement of serum PLP, tyrosine apo-decarboxylase (10) or apo-tryptophanase (11) was used as an analytical tool.

We have recently developed an isocratic reversed-phase HPLC method with fluorescence detection which can be used to analyze all the vitamin B-6 derivatives with high sensitivity (4,12–14). The method is a time saving, inexpensive and accurate one. However, the sensitivity for PLP was poor and this important vitamer among the vitamin B-6 families could hardly be detected from the serum at normal levels in healthy human subjects. The KCN treatment of the sample by which PLP was converted to PIC-P was adopted as described previously (12,13). By using our developed method, it is now possible to analyze all of the vitamin B-6 derivatives in the blood.

The aim of this report is to visualize the concentration changes of vitamin B-6 in plasma and erythrocytes under some physiological conditions at a given time. HPLC analysis would provide very powerful evidence to explain why the level of serum PLP decreased depending upon the progress of pregnancy and lactation.

MATERIALS AND METHODS

1. Materials. PLP, PMP·HCl, PN·HCl, PL·HCl, and PM·2HCl were purchased from Nakarai Tesque Inc. (Kyoto, Japan). PNP was prepared from PLP as described earlier (15). PIC was a gift from Prof. M. Chikuma, Osaka Pharmaceutical University, and its molar absorption coefficient (5,970 M⁻¹ cm⁻¹) at 316 nm at pH 5.0 was used to calculate the concentration in the standard solution. All of these compounds were confirmed to yield a single peak by means of our HPLC system (14). These compounds, if necessary, were purified by being passed through a column of Amberlite IR-122 (Na⁺ form) (12). Acetonitrile, Na₂HPO₄·12H₂O, KH₂PO₄, and HClO₄ solution (70% saturation) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acid phosphatase from potato was
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 purchased from Sigma Chemical Co. (St. Louis, MO, USA; Type IV-S). Membrane filters (0.45 μm) were purchased from Advantec Toyo (Tokyo, Japan). Nembutal (pentobarbital sodium salt) was obtained from Dainippon Seiyaku (Osaka, Japan) and sodium heparin solution (1,000 U/ml) was obtained from Nakarai Tesque Inc.

2. Animals. Female Wistar rats, 4–5 months old, were used in this experiment. Rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were kept with 12-h light/dark cycles (07:00–19:00) and were allowed free access to a nonpurified solid diet (Type: MF, in which 8.3 mg PN·HCl/kg diet was contained. Oriental Yeast Co. Tokyo, Japan) and water.

Two experiments were planned: In Experiment 1, rats were mated in our laboratory. Pregnancy was ascertained by the observation of a vaginal plug, which was set at day zero of gestation. Mated females were separated from the males, and they were used for the experiment on day 19 or 20 of gestation (pregnant rat), and on day 5 or 6 after birth (lactating rat).

In Experiment 2, pregnant rats on day 14 were purchased from Japan SLC Inc., and then raised in our laboratory until the 18th day of gestation. For both experiments, control rats were age-matched virgin females.

3. Sample preparation for HPLC analysis. All procedures were carried out in a darkened room equipped with a dim light to minimize photo-degradation of vitamin B-6 derivatives. Rats were anesthetized with Nembutal. Blood was drawn from heart using a 10-ml plastic syringe with a disposable needle that contained 0.1 ml of sodium heparin solution (1,000 U/ml). Blood collected was held in an ice bath as much as possible during preparation. Blood was centrifuged at 1,500 × g for 15 min at 0–5°C. Plasma was drawn off with Pasteur pipettes and stored at −20°C until use. Erythrocytes were washed twice with equal volume of ice-cold phosphate-buffered saline, resuspended in saline and stored at −20°C until use. To disrupt blood cell membranes, 2 volumes of distilled water were added to one volume of the packed erythrocytes and stood for 5 min. The resulting lysate was centrifuged to remove precipitates.

The B-6 derivatives were extracted from plasma as well as erythrocyte lysate using 3 M cold perchloric acid (12). A 2-ml sample was mixed with 1 ml of 3 M perchloric acid under vigorous vortexing, followed by centrifugation at 15,000 × g for 10 min. The pH of extracted sample was adjusted to 3.5 with 50% KOH, then water was added to the sample solution to a volume of 5 ml. The resulting solution was centrifuged at 15,000 × g for 10 min, and the supernatant was divided into two parts: one part was subjected to HPLC analysis using a suitable volume (usually 200 μl); the other part, whose pH was adjusted to 7.5, was treated with an aliquot of KCN to bring it to a final concentration of 5 mM. The resulting solution was heated at 50°C for 3 h with vigorous shaking. The solution was readjusted to pH 3.5 with HCl, stirred well for 1 min, and then allowed to stand for 24 h at room temperature to convert PLP to PIC-P completely (13). A 200-μl aliquot of the resulting solution was usually injected.
Prior to injection into an HPLC system, all of the solutions were passed through a 0.45-μm membrane filter.

4. **Apparatus.** The chromatographic system was essentially the same as that used in previous report (12). The HPLC column for separation was a TSK-gel ODS 120A reversed-phase column (250 × 4.6 i.d. mm).

5. **Mobile phase.** The separation was usually performed at a flow rate of 0.5 ml/min at 30°C using isocratic solvent containing 1% acetonitrile, 0.1M sodium perchlorate, and 0.1M potassium phosphate buffer with pH 3.5. It was degassed before use and then necessitated volume of acetonitrile was carefully added to the degassed solution (12).

6. **Determination of vitamin B-6 derivatives.** For the measurement of vitamin B-6 derivatives other than PIC-P, the wavelength for fluorescence detection was 305nm for excitation and 390nm for emission (Condition I). For the analysis of PIC-P after KCN treatment, the wavelength for fluorescence detection was 320nm for excitation and 420nm for emission (Condition II). The wavelengths for fluorescence detection (13) were somewhat modified, because of the ease of adjustment and of virtual unchange in the sensitivity of analysis (<5%). The bandwidths of the excitation and emission monochrometers were set at 10nm (12). Identification of the peaks of PLP and PMP was carried out as follows: PLP was confirmed as PIC-P after the KCN treatment as described above. PMP was checked by the conversion to PLP after reacting the sample with glyoxylic acid (16), and was then converted to PIC-P with KCN treatment (13).

From the data printed off, the corresponding peak for each authentic vitamin B-6 derivative was determined on a chart paper and then, in the case where a base-line was flat, peak-area in μV·s was read on the data sheet. The amounts of vitamin B-6 derivatives were calculated from a calibration equation which was previously constructed. In the case where the base-line was rather rough, the peak height in mm was measured by a calliper, and then the data were fitted to an equation which was constructed by using the standard solution beforehand.

**RESULTS**

1. **Analytical patterns of vitamin B-6 derivatives in plasma**

Figure 1 illustrates the analytical patterns of plasma vitamin B-6 derivatives in pregnant and lactating rats as well as in nonpregnant rats (control). From the analytical chart in Condition I, the peaks corresponding to PL and PMP were apparently detected. On the other hand, it is difficult to see the PLP peak on the chart. The inset shows the charts after KCN treatment (analyzed under Condition II). A large peak corresponding to PIC-P appeared. As a result, PL, PMP, and PLP were recognized from the plasma, however PM, PNP, and PN were not recognized, at least not as a distinct peak. These derivatives, if any, existed under detection limit (<50 pg of each derivative except PLP per one injection). From the charts of Condition I, we can see some peaks between 10–14 min of retention time.
under our HPLC conditions. These peaks did not agree with those of the authentic samples, when authentic PM, PN, and PNP were spiked with a sample. The existence of PIC was recognized, but the exact amount of PIC was difficult to determine because of overlapping in the chart with other unknown peaks. Although the findings were qualitative, the contents of PIC tended to decrease during the pregnant and lactating periods.

2. **Analytical patterns of vitamin B-6 derivatives in erythrocytes**

Figure 2 illustrates the analytical patterns of vitamin B-6 derivatives in erythrocyte lysate. From the analytical pattern, peaks which seemingly corresponded to PMP, PL, and PIC were detected (Condition I). Especially, a peak corresponding to putative PMP, where the retention time was at 7.0 min, was very high and it was distinct from the analytical patterns of plasma. A distinct peak corresponding to PMP from the erythrocyte sample have already been pointed out by Shephard et al. (17) using a cation exchange HPLC method. When the authentic PMP was spiked, the peak completely coincided, but the compound did not produce a highly fluorescent species, PIC-P, when the sample was treated with glyoxylic acid (16), and subsequently with KCN. It was fairly confirmed that glyoxylic acid treatment of authentic PMP made a new peak corresponding to...
Fig. 2. HPLC analyses of vitamin B-6 derivatives in rat erythrocyte lysates. From left to right: HPLC patterns of nonpregnant, pregnant, and lactating rat sample obtained under Condition I (200 µl of sample solution was injected). All measurements were recorded at ATT 3. Insets show analytical patterns obtained at different ATT's under Condition II (200 µl of sample solution was injected).

PIC-P. Thus, a peak observed at 7.0 min or so, was found not to be PMP, as shown in Fig. 3.

As for PLP, it was also difficult to find the peak in the usual analytical patterns of erythrocyte lysate under Condition I. As described above, it was detected as a distinct peak of PIC-P after KCN treatment (Condition II).

3. Concentration changes of each vitamin B-6 derivative during pregnancy and lactation

The results of Experiment 1 are summarized in Table 1. The numbers of analyses were small, so statistical analysis was not carried out. In the plasma, the total amounts of vitamin B-6 did not change much during the specified physiological conditions. As for PLP, its erythrocyte level increased significantly with pregnancy and lactation. The plasma concentration of PL tends to vary, while PL was not detected in the erythrocytes. Although the amount of PIC was not quantitated through the experiment, PIC was found in plasma under normal physiological conditions. However, this compound was difficult to detect from erythrocyte samples of pregnant together with lactating rats.

Table 2 summarizes the data from Experiment 2, in which six rats on day 18 of gestation were used together with the same numbers of control rat. In this experiment, no peak corresponding to PMP was observed in any of the plasma.

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Fig. 3. Glyoxylic acid treatment of erythrocyte lysates and authentic PMP. From left to right: HPLC patterns of extracts by perchloric acid, those of glyoxylic acid and KCN-treatment of authentic PMP (upper row) and erythrocyte lysates (lower row). So as to fit within a chart paper each HPLC pattern was recorded at a different attenuation (ATT), as shown in each datum. To 1.5 ml of erythrocyte lysate extracted with 3 M perchloric acid, 0.1 ml of 0.25 M glyoxylic acid solution was added and incubated at 100°C for 30 min. Then a part of this solution was treated with KCN after the pH of the solution was adjusted to 7.5 as described by Tsuge et al. (13). Authentic PMP was also treated in almost the same manner as above.

Table 1. Experiment 1: Concentration changes of blood vitamin B-6 derivatives in pregnant and lactating rats.

|                     | Plasma (nM) | Erythrocytes (nM) |
|---------------------|-------------|-------------------|
|                     | PMP         | PL               | PLP   | PMP               | PLP   |
| Nonpregnant (n=4)   | —           | 397(353, 384, 402, 451) | 362(315, 335, 384, 413) | —     | 221(220, 221)    | 221    |
| Pregnant (n=2)      | 46(37, 55)  | 361(333, 389)    | 297(113, 481) | —     | 512(489, 535)    |        |
| Lactating (n=2)     | 29(25, 35)  | 621(522, 720)    | 170(158, 182) | —     | 701(631, 771)    |        |

Mean value (each datum in parenthesis) obtained from peak height. —, under detection limit. Pregnant rats on day 19 and 20, and lactating rats on day 5 and 6 as well as age-matched control female rats were used.
samples. It was apparent that, upon pregnancy, plasma PLP concentration decreased markedly, on the contrary, the concentration of PL increased about twofold. From the erythrocytes, putative PMP and PLP were detected. After glyoxylic acid treatment, the putative PMP did not produce PLP. Thus, PLP was the only detectable vitamin B-6 derivative and the concentration of PLP tended to increase with pregnancy. The total amounts of vitamin B-6 found in plasma and erythrocytes of pregnant rats consistently increased more than 130%.

DISCUSSION

Through this experiment, we aimed to illustrate simultaneously the concentration changes of seven commonly found vitamin B-6 derivatives in rat blood under normal physiological conditions. However, it was difficult to show all the vitamin B-6 derivatives on one analysis. Our data on the plasma concentration of PLP in normal female rats collectively support the results previously reported by others (9, 18, 19). PLP and PL consistently have been shown to be the two major forms of vitamin B-6 occurring in the blood. It was difficult to recognize PMP from the erythrocytes. Erythrocytes have received relatively little attention. The analysis of vitamin B-6 in the blood has generally only involved the determination of the coenzyme form, PLP and occasionally of PL in plasma or in whole blood, with little work reported on erythrocytes alone (17). HPLC analysis is very useful if these procedures are to be used on a routine basis.

The plasma PLP level is generally considered to be the best indicator of vitamin B-6 nutritional status (18). In whole blood, PLP is typically distributed equally between plasma and erythrocytes, and typically plasma PLP concentrations correlated well with erythrocyte concentrations (20).

The present results suggest that metabolism or utilization of vitamin B-6 is altered in pregnancy and that plasma PLP concentrations alone may not be a good indicator of nutritional status in pregnancy (9, 19, 21, 22). The changes in PLP concentrations in blood reflect a striking shift in the distribution of PLP from plasma to erythrocytes during pregnancy. In the control rats, the distribution ratio of PLP between erythrocytes and plasma was approximately 0.6:1 in Experiment

Table 2. Experiment 2: Concentration changes of blood vitamin B-6 derivatives in pregnant and nonpregnant rats.

|                  | Plasma (nM) | Erythrocytes (nM) |
|------------------|-------------|-------------------|
|                  | PMP         | PL            | PLP       | PMP         | PLP       |
| Nonpregnant      | —           | 468±149 (n=5)  | 362±94 (n=6) | —           | 380±111 (n=4) |
| Pregnant         | —           | 821±187* (n=6) | 142±67** (n=6) | —           | 570±309 (n=6) |

M±SD, and n=numbers of available sample. —, under detection limit. *p<0.05, **p<0.01. Pregnant rats on day 18 as well as female rats of the same age as the control were used.
1 and 1:1 in Experiment 2. Those ratios are in agreement with other published ratios (6, 11). In the pregnant rat, however, the distribution ratio between erythrocytes and plasma was increased markedly to approximately 2:1 in Experiment 1 and approximately 4:1 in Experiment 2. This change in ratio was caused by both an increase in erythrocyte PLP and a decrease in plasma PLP. Although plasma PLP concentrations have been reported to decrease in pregnant mice, Leibman et al. (23) reported that pregnant mice had approximately a twofold increase in erythrocyte PLP concentration. These results suggest the possibility that the erythrocytes trap the available vitamin B-6 during pregnancy, thus contributing to the decrease in plasma PLP concentration. That is, upon pregnancy, some part of the vitamin B-6 moved from the plasma to the erythrocytes and accumulated there as PLP. It is known that PLP and PL bind to hemoglobin in erythrocytes, yet the physiological functions of PLP and PL in erythrocytes are unclear (24). Thus, the decrease in plasma PLP seems to be a characteristic consequence of pregnancy, and does not appear to reflect inadequate vitamin B-6 in the diet.

At the end of the discussion, it should be pointed out that, although HPLC analysis of the vitamin B-6 derivatives was a very promising method to determine the concentration of each derivative, experimental confirmation was needed to assign the peaks and to get reliable data because, as shown in Fig. 2, a false peak appeared near the PMP peak, which completely coincided with that of the authentic PMP spiked. To discriminate such false peaks under our HPLC conditions, we first tried to convert it to PM by using potato acid phosphatase (25). This method was not quantitative because authentic PMP added to serum sample was not completely hydrolyzed. Some part of PMP remained unchanged, especially, in the sample solution. On the other hand, nonenzymatic deamidation of PMP by glyoxylic acid (9, 16) proceeded almost completely even in the complex matrix. Thus, deamidation by glyoxylic acid was recommended.

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