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QUANTITIES OF B_6 VITAMERS IN HUMAN MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

INFLUENCE OF MATERNAL VITAMIN B_6 STATUS*

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SUMMARY

A rapid, sensitive procedure is described for the analysis of the B_6 vitamers pyridoxal, pyridoxamine, and pyridoxine in human milk from women taking and not taking supplements containing the vitamin using high-performance liquid chromatography with fluorometric detection. Vitamer values represent the sum of their phosphorylated and unphosphorylated forms. Minimum detectable quantities were 1—3 ng. Excellent recoveries of these vitamers in milk were obtained. Similar B_6 vitamer concentrations of milk were obtained using the developed high-performance liquid chromatographic and the accepted microbiological techniques. Pyridoxal, actually consisting of pyridoxal plus pyridoxal phosphate, was the predominant B_6 vitamer in human milk. The concentration of B_6 vitamers in milk was reflective of the maternal vitamin B_6 status.

INTRODUCTION

In recent years there has been increasing concern regarding the nutritional status of lactating women and nutrient composition of human milk. Deleterious effects of low vitamin B_6 intakes in the young have been observed in humans as well as in rats. There has been some evidence that the dietary intakes of vitamin B_6 of lactating women influence the concentration of the vitamin in their milk [1, 2].

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Vitamin B_6 exists in three interconvertible forms: pyridoxine (PN, also known as pyridoxol), pyridoxal (PL), and pyridoxamine (PM), each of which has a corresponding 5′-phosphate (P). The physicochemical properties of the B_6 vitamers, in particular their ionic nature, facilitate their assay by high-performance liquid chromatography (HPLC). The B_6 vitamer content of human plasma, animal tissues, urine, and selected foods as measured using HPLC techniques has been reported [3—15]. The B_6 vitamers present in milk from mothers supplemented with pyridoxine hydrochloride were recently quantitated by Vanderslice et al. [16] and Hamaker et al. [17] using HPLC techniques; the mothers in the first study received 0.5 or 4 mg of the vitamin daily and those in the second study 2.5 or 15 mg. To our knowledge, the concentrations of the B_6 vitamers PL, PM, and PN, in their unphosphorylated and/or phosphorylated forms, in milk from mothers not taking vitamin B_6 supplements have not been reported.

The objectives of this research were to determine the influence of the vitamin B_6 status of lactating women on the B_6 vitamer content of their milk as measured by microbiological methods as well as by a reversed-phase ion-pair HPLC method with fluorometric detection which had previously been developed in our laboratory [15]. The relationships between vitamin B_6 intakes, vitamin B_6 status measurements, and B_6 vitamer concentrations in milk were also examined. The yeast employed in the microbiological assay of the vitamers is not able to utilize the phosphorylated derivatives of the B_6 vitamers [18]; hence, in order to compare vitamer values obtained using microbiological and HPLC procedures, the phosphorylated B_6 vitamers were dephosphorylated and quantitated in their respective unphosphorylated forms. Consequently, these individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated. Since the unphosphorylated derivatives constitute less than 10% of the total vitamin B_6 content of mammalian tissue [19], the non-phosphate values reported in this research are considered to represent primarily the tissue values of the phosphorylated vitamers.

EXPERIMENTAL

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system equipped with a fluorescence detector (300 nm excitation, 375 nm emission) and a μBondapak octadecylsilane column (30 cm × 3.9 mm I.D., 10 μm porous packing, C_{18}, Waters Assoc.) was utilized in this research. The mobile phase was a gradient of 85% methanol and PIC B-7 reagent (Waters Assoc.); the gradient conditions, vendors of standards, and instrumentation have previously been described in detail [15].

Deoxypyridoxine (DPN) was selected as the internal standard. Following chromatography of the individual vitamers PL, PN, and PM as well as DPN, a 250-μl aliquot of an aqueous combined standard solution (containing 50 ng of PL, 6.25 ng of PN, 6.25 ng of PM, and 118.75 ng of DPN per ml) was injected onto the column; satisfactory separation of the vitamers was achieved in about 15 min (Fig. 1). Identity was confirmed by standard addition (spiking) as well as by extra chromatographic spectrofluorometry on collected HPLC eluates.

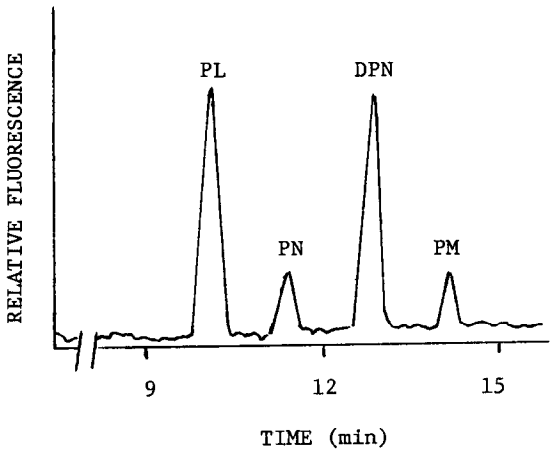

Fig. 1. Separation of B_6 standards by HPLC.

Linear calibration curves for PL, PM, PN, and DPN were obtained; minimum detectable quantities were 3 ng (18 ng/ml sample) for PL and 1 ng (6 ng/ml sample) for PM and PN.

Extraction of B_6 vitamers in milk samples

A known quantity of DPN was added to a 3-ml aliquot of pooled milk and the solution mixed. Next, 0.6 ml of a 2 U/ml potato acid phosphatase (EC 3.1.3.2; orthophosphoric monoester phosphohydrolase; Sigma, St. Louis, MO, U.S.A.) in 0.2 *M* potassium acetate, pH 4.5, was added to the sample in order to hydrolyze the phosphate esters of the B_6 vitamers [20]; samples were incubated for 1 h in a 37°C shaker water bath. The protein was precipitated by adding 0.25 ml of 100% trichloroacetic acid (TCA); the sample was mixed by vortex and incubated for 15 min in a 50°C water bath. Methylene chloride, 3 ml, was added to the samples followed by vigorous shaking to remove lipids; samples were then centrifuged for 15 min at 4°C and 4000 *g*. The resulting supernatants were adjusted to pH 5.2 with 33% sodium hydroxide and filtered through a 0.45-μm filter with a syringe attachment prior to injection into the HPLC system.

A typical chromatogram of milk extract is depicted in Fig. 2. Peak identity was confirmed by comparison of standard retention times with sample retention times, use of relative retention times, spiking, and extra chromatographic spectrofluorometry of eluates. Vitamer recoveries were determined by spiking the samples before extraction. The recoveries were as follows: PL, 86%; PM, 105%; and PN, 83%; the recovery of DPN was 95%. Phosphorylated vitamers were recovered as their respective unphosphorylated forms as follows: PLP → PL, 96%; PMP → PM, 70%; and PNP → PN, 83%. Hence, vitamer values represent the sum of both the phosphorylated and unphosphorylated forms of the vitamer. The data were not corrected for percent recoveries. The coefficients of variation for B_6 vitamer concentrations of milk samples that were extracted and analyzed on different days were around 5%; the same was true for the microbiological assay. The B_6 vitamer content of samples from three women was determined freshly expressed as well as frozen for 2 h and for

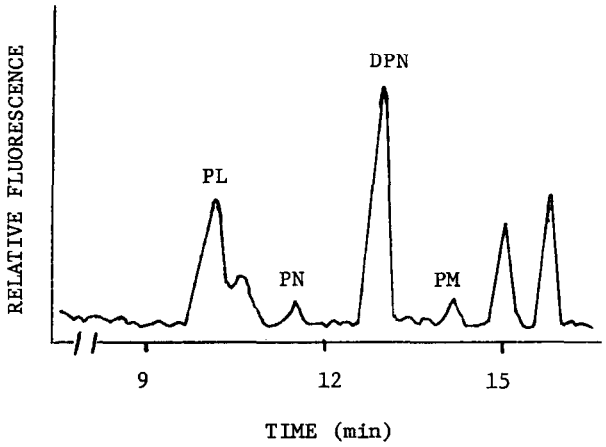

Fig. 2. Separation of B_6 vitamers in a representative milk extract by HPLC.

four months at −20°C; frozen and freshly expressed samples had similar vitamer contents thus indicating that human milk samples could be frozen for at least four months without loss of B_6 vitamers.

Subject description and dietary analysis

Twenty-one white lactating women in apparent good health, 21—35 years of age, and 3—7 months postpartum volunteered as subjects. The study was approved by the University's Human Volunteers Committee. Subjects were weighed and heights obtained. Information regarding food intake was obtained from each subject using one 24-h recall (with cross-checking and food models) and four days of food records; these dietary records were obtained and the protein and vitamin B_6 intakes were estimated as described previously [21]. These intakes were compared to the Recommended Dietary Allowances (RDA) for lactating women [22].

Milk sample collection and vitamin B_6 status analysis

Subjects manually expressed fore milk into vials on three consecutive mornings; the samples were frozen immediately by the subjects. West and Kirksey [1] found that the vitamin B_6 content in a fore sample of early morning milk was representative of milk expressed completely from the breast. Milk from the three vials was pooled for B_6 vitamer analysis.

The vitamin B_6 status of the subjects was determined via the two most commonly used parameters: coenzyme stimulation of erythrocyte alanine aminotransferase activity (EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase, E-ALAT) and radioisotopically measured plasma pyridoxal phosphate (PLP) concentration. Approximately 20 ml of non-fasting blood was obtained by a Registered Medical Technologist on the morning following the final morning of milk collection and the five days of food intake records. These two methods used for status assessment have been described previously [21].

B_6 Vitamers via microbiological analysis

A modification of the Association of Official Analytical Chemists (AOAC)

procedures [23] for analyzing B_6 vitamers in food materials was used to determine the PL, PM, and PN content of the milk samples. The assay inoculum was prepared by incubating *Saccharomyces uvarum* (ATCC 9080, American Type Culture Company, Rockville, MD, U.S.A.) on Pyridoxine Y media (Difco Labs., Detroit, MI, U.S.A.). The protein in the pooled milk samples was precipitated using sulfosalicylic acid (0.1 g per 2 ml milk) [24]. The sample was then centrifuged for 2 min at 3000 *g* and 4° C; the supernatant was filtered and 6 ml of 0.2 *M* hydrochloric acid were added and the mixture was placed into a boiling water bath for 1 h; this step enabled the phosphate groups to be cleaved from the B_6 vitamers. The pH of the sample was adjusted to 4.5 with 10% potassium hydroxide. A glass column (250 mm × 17 mm O.D., 14.5 mm I.D., 250 ml reservoir) was used in the separation of the vitamers, the column separation and subsequent analyses for B_6 content were as described in the AOAC procedure [23]. In order to check vitamer recoveries, milk samples were spiked with each vitamer prior to extraction. The recoveries were as follows: PL, 93%; PM, 87%; and PN, 84%. Milk samples were also spiked with phosphorylated forms of the vitamers; these vitamers were dephosphorylated and recovered as their respective unphosphorylated forms as follows: PLP → PL, 83%; PMP → PM, 85%; and PNP → PN, 82%. The data were not corrected for percent recoveries.

Statistical analysis

The subjects were classified into adequate and inadequate status groups based upon their coenzyme stimulation values. Coenzyme stimulation values $\geqslant$ 16% [25] or > 25% [26] are considered to be indicative of vitamin B_6 inadequacy. The criteria used for defining vitamin B_6 inadequacy in the current study is a stimulation value $\geqslant$ 16%. All data were evaluated using analysis of variance procedures [27]; means ($\bar{X}$) and standard deviations (S.D.) were also calculated. Pearson *r* correlation coefficients were determined between data obtained for the various parameters.

RESULTS AND DISCUSSION

On the basis of their coenzyme stimulation of E-ALAT activities, the subjects were classified as having either adequate or inadequate status; other status parameters were not utilized in that values indicative of inadequate status have not yet been established. Six subjects had coenzyme values > 25% and one female had a value of 22.2%; these seven subjects were classified as having inadequate vitamin B_6 status. Fourteen subjects who had coenzyme values <16% were classified as having adequate vitamin B_6 status.

Anthropometric and dietary assessment

The age, height, and weight measurements of the subjects classified as having inadequate and adequate vitamin B_6 status were similar. These values for all subjects combined were as follows: 27.1 ± 3.4 years, 164.4 ± 7.1 cm, and 58.6 ± 8.7 kg ($\bar{X}$ ± S.D.). The subjects were 4.6 ± 1.6 months ($\bar{X}$ ± S.D.) postpartum.

No significant differences were observed between intake data obtained by

the 24-h recalls and the four-day records. The average daily protein intake of subjects classified as having inadequate and adequate vitamin B_6 status were similar; the intake for all subjects combined was 89.6 ± 21.4 g ($\overline{X}$ ± S.D.). The vitamin B_6 intake of the seven subjects classed as being inadequate in status was 1.16 ± 0.24 mg ($\overline{X}$ ± S.D.) daily; none of the subjects in this status group reported taking supplements containing vitamin B_6. The fourteen subjects in the adequate status group reported consuming 1.52 ± 0.34 mg ($\overline{X}$ ± S.D.) of the vitamin from food sources; their intake from food and supplements combined was 11.23 ± 16.32 mg daily (median was 5.17 mg; one subject reported taking a supplement containing 65 mg of the vitamin). All of the women in the group with adequate status reported taking nutrient supplements that contained vitamin B_6. Women in the inadequate status group reported consuming significantly less ($p < 0.01$) vitamin B_6 from food, as well as from food and supplements combined, than subjects in the adequate group. All of the subjects classified as having inadequate status and five of the women classed as adequate reported consuming a daily intake of vitamin B_6 from food sources that was less than two-thirds of the RDA for lactating women; none of the subjects reported consuming as much as 2.5 mg daily (the RDA) of the vitamin from food sources.

Vitamin B_6 status assessment

The coenzyme stimulation of E-ALAT activities of subjects classified as having inadequate and adequate vitamin B_6 status was 34.9 ± 8.7% and 5.4 ± 6.4% ($\overline{X}$ ± S.D.), respectively. Stimulation values of the group classed as being inadequate were significantly higher ($p < 0.0001$) than those classed as adequate. The radioisotopically measured plasma PLP concentrations of subjects classed as being inadequate ($\overline{X}$ ± S.D., 15.3 ± 5.9 ng/ml) were significantly lower ($p < 0.003$) than those classed as being adequate (39.5 ± 18.1 ng/ml). Rose et al. [28] suggested that a plasma PLP level of < 8.5 ng/ml represented inadequate vitamin B_6 status; Russ et al. [29] suggested that a value < 13 ng/ml was representative of inadequate status. All plasma PLP values of subjects in this study exceeded 8.5 ng/ml but four subjects in the inadequate classification group had values below 13 ng/ml. A Pearson correlation coefficient of −0.59 ($p < 0.005$) was obtained in this study between coenzyme stimulation of E-ALAT values and plasma PLP concentrations. Russ et al. [29] reported finding a Pearson correlation coefficient of −0.60 between these two parameters.

B_6 Vitamers in milk by HPLC assay

The B_6 vitamer content of milk from the subjects as measured by HPLC assay are given in Table I. Phosphorylated B_6 vitamers were dephosphorylated and quantitated in their respective unphosphorylated forms. The PL concentrations of milk of women having inadequate classification were significantly lower ($p < 0.01$) than values of the adequate group. The PM values for milk from two subjects classed as inadequate were below minimum detectable levels. The PN values for milk from five women classed as adequate and two subjects classed as inadequate were below minimum detectable limits. The minimum detectable limits for PM and PN were 1 ng per injection which may be equivalent to as

TABLE I

B_6 VITAMER CONTENT OF MILK BY HPLC ASSAY

Individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated. Values represent $\bar{X}$ ± S.D. and are given in pmol/ml.

Status classification	PL	PM	PN	Total B_6 vitamers
Inadequate	226 ± 71*	42 ± 33**	51 ± 40	320 ± 103*
Adequate	627 ± 324	102 ± 63	42 ± 38	770 ± 341

*Significantly different from values of adequate group at $p < 0.01$.
**Significantly different from values of adequate group at $p < 0.05$.

much as 6 μg/l. Non-detectable levels were statistically evaluated as being zero. The PM concentrations of milk from women classed as inadequate were significantly lower ($p < 0.05$) than those of the group classed as adequate. The PN concentrations of milk from both classes of subjects were similar. The total B_6 vitamer (TL) concentration of milk from women classed as having adequate status as measured by HPLC assay was significantly lower ($p < 0.01$) than for those in the adequate classification. Two significant Pearson correlation coefficients were obtained between HPLC-derived data; these were as follows: PL and TL, $r = 0.98, p < 0.0001$; PM and TL, $r = 0.53, p < 0.02$.

The predominant B_6 vitamer in milk from subjects in both status classifications as measured via HPLC techniques was PL (representing both PL and PLP). The percent distributions of B_6 vitamers in milk from all subjects were as follows: PL, 76.8 ± 14.3%; PM, 13.8 ± 8.9%; and PN, 9.4 ± 8.6% ($\bar{X}$ ± S.D.). Vanderslice et al. [16] and Hamaker et al. [17] also found PL (PL + PLP) to be the major vitameric form.

B_6 Vitamers in milk by microbiological assay

The B_6 vitamer content of milk from the subjects as measured by microbiological assay is given in Table II; total B_6 vitamers were calculated as a sum of the vitamers. PL values for milk from the group classed as inadequate were

TABLE II

B_6 VITAMER CONTENT OF MILK BY MICROBIOLOGICAL ASSAY

Individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated. Values represent $\bar{X}$ ± S.D. and are given in pmol/ml.

Status classification	PL	PM	PN	Total B_6 vitamers
Inadequate	645 ± 57*	54 ± 11	56 ± 24	755 ± 66**
Adequate	820 ± 85	66 ± 15	68 ± 32	955 ± 98

*Significantly different from values of adequate group at $p < 0.0001$.
**Approached being significantly different from values of adequate group at $p < 0.10$.

significantly lower ($p < 0.0001$) than those of women in the adequate classification. The PM and PN values for milk from the inadequate classification group were rather similar to those of the adequate women. The total B_6 vitamer concentration of milk from women in the inadequate classification group was significantly lower ($p < 0.0001$) than for the adequate group. The following Pearson correlation coefficients were obtained between microbiologically derived data: PL and total B_6 vitamers (TL), $r = 0.96, p < 0.0001$; PM and TL, $r = 0.51, p < 0.02$; PN and TL, $r = 0.43, p < 0.05$.

The predominant B_6 vitamer in milk from both inadequate and adequate status groups as measured by microbiological techniques was PL (representing PL + PLP). Approximately equal quantities of both PM and PN were present in milk from both status groups. The percent distributions of B_6 vitamers in all subjects were as follows: PL, 85.7 ± 3.3%; PM, 7.0 ± 1.5%; and PN, 7.2 ± 2.8% ($\overline{X}$ ± S.D.).

Comparison of B_6 vitamer data obtained by HPLC and microbiological assays

Correlations were calculated between data obtained by HPLC and microbiological techniques. The PL and TL (total B_6 vitamer) concentrations of milk of all subjects as measured by HPLC and microbiological techniques were significantly and highly correlated with each other ($r = 0.63, p < 0.003$, and $r = 0.64, p < 0.002$, respectively). PM and PN values obtained by HPLC assay were not significantly correlated with those obtained using microbiological techniques.

The concentrations of PL, PM, PN, and total B_6 vitamers in human milk as determined by HPLC procedures were rather similar to values obtained by microbiological assay in the current study. These two methods are quite diverse and have different methods of vitamer extraction. Excellent correlations between HPLC and microbiologically derived data were observed in the current study for PL and total B_6 vitamers but not for PM and PN where several values were observed to be below minimum detectable limits. Vanderslice et al. [16] reported finding satisfactory agreement between microbiologically measured total vitamin B_6 and HPLC-derived total B_6 vitamer (calculated by addition) concentrations. Excellent recoveries from spikes were obtained in the present study for both microbiological and HPLC assays including extraction procedures.

B_6 Vitamers in milk versus status assessment

Significant correlations were obtained between the milk B_6 vitamer values and data obtained by the two status parameters (Table III). The B_6 vitamer content of the human milk as determined by both HPLC and microbiological techniques was definitely highly correlated with the vitamin B_6 status of the lactating women as measured by both coenzyme stimulation of E-ALAT activities and plasma PLP levels. Correlations approaching significance were observed between estimated vitamin B_6 intakes and coenzyme stimulation activities ($r = 0.39, p < 0.08$) as well as plasma PLP values ($r = 0.42, p < 0.06$). Significant or near significant correlations were also obtained between estimated vitamin B_6 intakes and the following milk B_6 vitamer values: microbiologically derived (M) PL, $r = 0.43$, $p < 0.06$; MPN, $r = 0.57$, $p < 0.008$;

TABLE III

SIGNIFICANT CORRELATIONS BETWEEN MILK B_6 VITAMER VALUES AND STATUS MEASUREMENTS

Individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated.

Parameters*	*r*	*p* values
E-ALAT:MPL	−0.65	<0.002
E-ALAT:MPM	−0.48	<0.03
E-ALAT:MPN	−0.40	<0.08**
E-ALAT:MTL	−0.71	<0.001
E-ALAT:HPL	−0.65	<0.002
E-ALAT:HPM	−0.44	<0.05
E-ALAT:HTL	−0.65	<0.002
RPLP:MPL	0.59	<0.006
RPLP:MTL	0.56	<0.009
RPLP:HPL	0.76	<0.0001
RPLP:HTL	0.74	<0.0001

*Abbreviations used: Coenzyme stimulation of erythrocyte alanine aminotransferase activities, E-ALAT; radioisotopically monitored plasma pyridoxal phosphate levels, RPLP; microbiological (M) and HPLC (H) measurement of pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), and total B_6 (TL) concentrations in milk.
**Approached significance.

MTL, $r = 0.52$, $p < 0.02$; HPLC-derived (H) PL, $r = 0.74$; $p < 0.0001$; HTL, $r = 0.73$, $p < 0.0002$. Large S.D. values were observed with relation to vitamin B_6 intakes, milk B_6 vitamer, and status parameter values. As reflected by the *r* values, subjects having lower vitamin B_6 intakes usually had higher coenzyme stimulation of E-ALAT activities, lower plasma PLP levels, and lower B_6 vitamer levels in their milk as quantitated by both HPLC and microbiological methods; the reverse was usually true for subjects with higher intakes. Other researchers have also found milk from humans to be quite variable in vitamin B_6 or B_6 vitamer content [1, 16] but they did not relate these findings to biochemical status measurements.

The B_6 vitamer concentration of the milk was affected by the vitamin B_6 status of the women in the current study. The B_6 vitamer concentration of the milk was also affected by supplementation and vitamin B_6 intakes in the current study. Subjects in the current study who had higher intakes of vitamin B_6 also had higher concentrations of B_6 vitamers in their milk. Similar findings obtained by microbiological assay for total vitamin B_6 have been reported by West and Kirksey [1].

Similar B_6 vitamer concentrations were obtained for human milk samples by the newly developed HPLC fluorometric technique as by the AOAC microbiological assay. Both the HPLC method and the microbiological procedures used in the current study seemed to be sensitive and accurate in qualitating and quantitating the B_6 vitamers found in human milk from women who took, as well as who did not take, supplements containing the vitamin. The vitamin B_6 status of lactating women could be determined by quantitating the B_6 vitamer content of their milk.

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