VITAMIN B-6 STATUS OF A GROUP OF FEMALE ADOLESCENTS
E-ALAT, MICROBIOLOGICAL, AND HPLC METHODS

by

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INTRODUCTION

Vitamin B-6 was isolated in 1934 by György. The first function attributed to vitamin B-6 was that of preventing skin lesions in the rat (1). Vitamin B-6 in the form of pyridoxal phosphate is currently recognized as serving as a coenzyme for over 60 different enzyme systems (2).

Vitamin B-6 is a 3-hydroxy-2-methylpyridine base that exists in 6 different vitameric forms: an aldehyde, pyridoxal (PL); an alcohol, pyridoxine (PN), also called pyridoxol; an amine, pyridoxamine (PM); and their corresponding 5'-phosphorylated forms, pyridoxal phosphate (PLP), pyridoxine phosphate (PNP), and pyridoxamine phosphate (PMP)[3].

The 2 most widely used methodologies for assessing vitamin B-6 status in man are the determination of PLP concentrations in plasma via a radiometric assay (4-6) and the coenzyme stimulation of a PLP-dependent coenzyme, alanine aminotransferase (EC 2.6.1.2., L-Alanine: 2-oxoglutarate) in the erythrocytes, E-AIAT (7,8).

A limited number of studies has been conducted using a microbiological assay to determine plasma concentrations of PL, PN, and PM, but not enough data have been collected to establish normal ranges of vitameric forms in plasma (9,10). Recent interest has focused on the use of high performance liquid chromatography (HPLC), also called high pressure liquid chromatography, for determining vitamin B-6 levels in plasma (11,12).

While several studies have evaluated the vitamin B-6 status of
male and female adults (13), only a few studies have been conducted examining the vitamin B-6 status of female adolescents (14,15). Female adolescents are recognized as being at a high nutritional risk due to many factors including the stresses of growth and sexual maturation and the effects of peer influence on dietary habits.

This research project was an ancillary study of the S-150 Regional Project entitled Nutritional Health of Adolescent Females which was designed to assess dietary habits and a multitude of biochemical parameters of adolescent females (16). The specific objectives of this current research project were as follows:

1) To evaluate the vitamin B-6 status of a group of female adolescents by a commonly used method for assessment, the determination of coenzyme stimulation of E-ALAT activities;

2) To quantitate the PL, PN, and PM in plasma of these subjects by the standard microbiological assay and to compare the plasma PL, PN, and PM values to coenzyme stimulation of E-ALAT activities and to determine if a relationship between these 2 parameters exists;

3) To evaluate dietary vitamin B-6 intake of these subjects as compared to the Recommended Dietary Allowances (RDA) for adolescents; and

4) To attempt to develop a feasible HPLC method for the determination of PL, PN, and PM in plasma of female adolescents.
REVIEW OF LITERATURE

The interest in human vitamin B-6 nutrition is due to several reasons including the ease of producing deficiency symptoms, the role of vitamin B-6 dependent enzymes in a wide variety of metabolic pathways, and the relationship of vitamin B-6 to brain metabolism and development. Other areas of interest include the increased needs associated with pregnancy, the loss in content of vitamin B-6 during food processing, and the antagonistic action of several drugs with regard to vitamin B-6 nutrition (2).

Many different methods exist for assessing vitamin B-6 status in man. The ideal method of assessment would be sensitive in detecting subclinical deficiencies, would not be affected by age or sex, and would indicate long-term consumption instead of short-term consumption of the vitamin. The method would be sensitive, reproducible, easy to interpret, simple, and inexpensive to run, yet would also be reliable. Unfortunately, the ideal method for analyzing vitamin B-6 nutritional status in man has not been established.

The tryptophan load test was used as a determinant of vitamin B-6 status in some of the earlier survey research. Several vitamin B-6 dependent enzymes are involved in the catabolism of tryptophan. Following a test load of 2-5 g L-tryptophan, subjects with a vitamin B-6 deficiency exhibit increased urinary excretions of several tryptophan metabolites including 3-hydroxykynurenine, kynurenine, kynurenic acid, and xanthurenic acid (17); xanthurenic acid is the metabolite
most often measured. Problems encountered with interpreting the data from the tryptophan load test include the effects of protein intake, lean body mass, exercise, the amount of tryptophan used for loading, pregnancy, and oral contraceptive use on the data (2). However, Brown (17) reports that the tryptophan load test and the measurement of urinary metabolites is the most sensitive indicator of vitamin B-6 status in healthy individuals because it reflects functional adequacy of coenzyme levels, while other tests measure only coenzyme levels.

The excretion of urinary free vitamin B-6 measured microbiologically has been shown to be related to intake of vitamin B-6, but not to be a good measurement of vitamin B-6 status (2). The major urinary metabolite of vitamin B-6, 4-pyridoxic acid, has also been shown to be related to dietary intake (18). Reddy (19) developed a fluorometric method for the determination of 4-pyridoxic acid in human urine, and more recently, Gregory and Kirk (20) developed an HPLC method for measuring this metabolite.

Several radiometric assays have recently been used to determine the PLP concentration of plasma. The assay most often used monitors the radioactive $^{14}$CO$_2$ formed from the decarboxylation reaction of L-tyrosine-$^{14}$C $\rightarrow$ tyramine + $^{14}$CO$_2$ involving the PLP-dependent enzyme tyrosine decarboxylase (EC 4.1.1.25, L-Tyrosine carboxy-lyase) [4-6]. The rate of formation of radioactive CO$_2$ is proportional to the vitamin B-6 activity of the sample, primarily as PLP. The decarboxylation reactions of L-tryptophan $\rightarrow$ pyruvate (21) and of L-dopa $\rightarrow$ dopamine (22) both involve PLP-dependent enzymes and have been proposed for use as vitamin B-6 status methods in methods similar to the decarboxy-
laboration of tyrosine.

The determination of the activities of PLP-dependent aminotransferases is used widely for assessing vitamin B-6 status. Erythrocytes have been shown to have higher aminotransferase levels than plasma and are generally used in these status assays (2). The following two reactions are the most common ones employed in the assay; (1) L-alanine + 2-oxoglutarate → pyruvate + L-glutamate involving the PLP-dependent enzyme E-ALAT; and (2) L-aspartate + 2-oxoglutarate → oxaloacetate + L-glutamate involving the PLP-dependent enzyme aspartate aminotransferase (EC 2.6.1.1, L-Aspartate: 2-oxoglutarate aminotransferase) in the erythrocytes, E-ASAT (8). Extra PLP is added to the reaction and both stimulated and basal values are measured to give an indication of endogeneous enzyme levels.

The most widely used method for analyzing vitamin B-6 content in biological tissues is the microbiological assay. In principle, an organism is selected that has a growth response specific for vitamin B-6. A standard curve is established comparing vitamin B-6 concentration and growth of the organism. Growth can be determined by turbidometric, gravimetric, or radiometric responses (9,23-28).

Much of the earlier work with microbiological assays concentrated on maximizing the conditions of the analyses including media composition, choice of an organism, and determination of extraction methods for sample preparation. Several microorganisms have been used in assays for vitamin B-6 including Lactobacillus casei (ATCC 7469), Klockera brevis (ATCC 9774), and Saccharomyces uvarum (ATCC 9080) [9,25-27] . S. uvarum appears to be the most widely used organism and is the organism of choice recommended by the American Association of Analytical Chemists, AOAC (23).
S. uvarum, as well as most other test organisms, exhibits differential growth for the three vitamers PL, PN, and PM (9,28). A chromatographic procedure was developed where the individual vitamers, PL, PN, and PM were separated on a cation exchange column prior to analysis. Dowex 50W X-8, 100-200 mesh was determined to be the column packing of choice (29). The sample was placed on the column and the three vitamers were eluted singly with different buffers. Values for total vitamin B-6 content determined microbiologically in foods are generally found to be higher than values for unchromatographed samples (9,30). This is probably a result of the differential growth response of various organisms to PL, PN, and PM. Bioassay values correlate well with chromatographed values of vitamin B-6 in several foods including dry milk, whole wheat flour, and ground beef (9).

There is a wide range of values reported in the literature for microbiological assays of vitamin B-6. This probably is due to variations in sample preparation. The vitamers are generally found tightly bound to protein and may exist in either phosphorylated or non-phosphorylated forms. Prior to microbiological analysis, the sample must be deproteinated to separate the vitamin from the protein, and must also be dephosphorylated because the test organisms are not sensitive to the phosphorylated forms. A wide variety of hydrolytic steps have been proposed, most of them involving a combination of an acid hydrolysis, a boiling water bath incubation, autoclaving, or a series of enzyme digestions (9,23,24,27,28). Sample preparation steps need to be optimized for the particular sample being analyzed to obtain maximum recovery of vitamer components.
Recently, the use of HPLC to analyze B-6 vitamer content in biological samples has gained much attention (31-35). HPLC techniques offer the advantages of decreased analysis time, increased sensitivity, and the potential for automation. One of the major problems associated with the determination of B-6 vitamer content in plasma is the minimum detection limits that are necessary to detect the quantities present in plasma. Vanderslice and Maire (11) and Vanderslice et al. (12) have reported an HPLC technique to determine the B-6 vitamer content in human plasma, but their data were from 2 adult males. The use of HPLC analyzing the vitameric forms of vitamin B-6 in human plasma offers much potential for future research.

The RDA for vitamin B-6 is 1.8 mg/day for males and females ages 11-14. This allowance increases in adults and in pregnant and lactating women (36). In controlled metabolic studies, the requirement for vitamin B-6 has been shown to be more closely associated with protein intake than with caloric intake in that higher protein intake is associated with a higher requirement for vitamin B-6 (18,36).

In the National Food Consumption Survey, NFCS, involving over 37,000 people in 48 states taken from 1977-1978, vitamin B-6 was recognized as being a problem nutrient. Cross-sectionally, without regard to age, sex, or geographical location, 51% of those surveyed consumed less than 70% of the RDA for vitamin B-6 for their corresponding sex and age groupings. More children and teenaged males had intakes that met the RDA for vitamin B-6 than those that fell below 70% of the RDA while the opposite was true for teenaged and adult women (37). In a review article examining estimated dietary intakes of vitamin B-6, Driskell and Chrisley
(13) also found teenaged and adult women to consume < RDA more frequently than teenaged or adult males.

Many researchers generally use a combination of parameters to assess vitamin B-6 status. This helps compensate for any difficulties associated with interpreting the data obtained by only one technique. Ideally, the status parameter(s) will reflect long-term consumption of the vitamin.

One of the primary requirements of a method used for assessing status is that it is sensitive and responsive to deficiency states. Several studies have been conducted examining various biochemical parameters associated with a deficiency of vitamin B-6 (38-40). The general protocol of these studies was use of a high protein diet to help induce a vitamin B-6 deficiency and monitoring of the excretion of tryptophan metabolites, on an individual basis, as the criterion to define a deficiency state. The studies showed a wide individual variation in the amount of time it took to induce a deficiency. Urinary excretions of vitamin B-6 and 4-pyridoxic acid were found to be more indicative of recent intake than of long-term consumption (38,39). The researchers felt that erythrocyte aminotransferase levels were the most reliable of the methods tested for assessment of long-term vitamin B-6 status.

Using a fluorometric assay, Shane (10) found no PN or PTP in whole blood and approximate values of total vitamin B-6 representing both the phosphorylated and the non-phosphorylated forms of 40-60 nm/ml. In a review of several studies, Vanderslice et al. (12) showed a wide variation in reported values of vitamin concentration in plasma. Clearly,
more basic data is needed concerning the vitamer concentration of plasma. Much of the discrepancy between studies may be due to differences in protocol of sample collection, limitations and variations of the methodology, or simply a function of the use of small sample sizes. This researcher found no published articles comparing microbiological or HPLC levels of the B-6 vitamers in plasma to erythrocyte aminotransferase levels for potential use as status parameters.

Baker et al. (41) examined whole blood vitamin B-6 levels determined microbiologically in approximately 800 male and female subjects, aged 10-13 yr. Kirksey et al. (14) examined approximately 100 female subjects with respect to dietary intake, erythrocyte vitamin B-6 levels, and coenzyme stimulation of E-ALAT activities.

In this current research, dietary intakes, coenzyme stimulation of E-ALAT activities and microbiological plasma PL, PN, and PM levels will be determined for a group of female adolescents. The development of an HPLC method for the determination of plasma PL, PN, and PM levels will be attempted.
A. Selection of subjects

Eleven apparently healthy female Caucasian volunteers from the Blacksburg/Roanoke, Virginia area, aged 12 yr ± 6 mo participated in the study. The subjects were also participants in the S-150 Cooperative Regional Project entitled Nutritional Health of Adolescent Females (16). Subjects with a positive Sickledex test were excluded from the study. As a criterion for selection, none of the subjects had reached menarche. Family income was not used as a criterion for selection, but was obtained. Signed consent was obtained from the subject and from a parent or guardian prior to participation in the study.

B. Height, weight, and dietary measurements

Heights without shoes were obtained from the subjects. The subjects were weighed with light clothing on and clothing weights were estimated and subtracted.

A 24 h dietary recall fashioned after Christakis (42) was obtained from each subject by a trained interviewer. Food models were used to aid the subjects in estimating portion size. In the following month, another 24 h dietary recall was obtained from each subject. The subjects' protein, kilocalorie, and vitamin B-6 intakes were estimated (43,44) by averaging the intakes from the 2 dietary recalls and comparing the intakes to the RDA (36) for adolescent females.
C. Collection of blood samples and determination of the coenzyme stimulations of E-ALAT activities

A 12 h fasting blood sample of approximately 40 ml was obtained from each subject by a trained medical technologist. Approximately 3/4 of the blood taken was used for other S-150 procedures. The blood sample was collected by venipuncture into vacutainers containing EDTA as the anticoagulant. After collection, the samples were kept in ice and protected from the light to prevent vitamin degradation. The blood samples were centrifuged for 15 min at 5°C at 5000 x g and were frozen at -20°C prior to biochemical analyses.

The coenzyme stimulation of E-ALAT activity was determined by another member of our research group using blood from the subjects. The method of Tonhazy et al. (45) as modified by Heddle et al. (46), Raica and Sauberlich (47), and Driskell et al. (7) was used.

D. Microbiological analyses of B-6 vitamins in plasma

The vendors and their addresses for certain chemicals used in the microbiological analyses of B-6 vitamins are given in Appendix 1. The preparation of reagents for the microbiological assay of vitamin B-6 in plasma is given in Appendix 2.

1. Sample preparation

The frozen (-20°C) plasma samples were thawed overnight at 5°C. The samples, approximately 1-2 ml, were transferred into centrifuge tubes. If the samples were spiked with standards, a spike of 1 ml 100 ng/ml solution of PL, PN, PM, PLP, PNP, or PMP was added at this point. Sulfosalicylic acid was added to each sample at a concentration of 0.05 g sulfosalicylic/ml plasma. Sulfosalicylic acid was used to
precipitate the plasma proteins and to release the vitamin B-6 that is bound to these proteins (11,12). The tubes were covered with parafilm and vortexed intermittently at medium speed for 3 min. The samples were then centrifuged for 10 min at 5°C at 7000 x g. The supernatant was decanted into a syringe fitted with a 0.2 μm Acrodisc filter, the supernatant was filtered, and the precipitant was discarded. Three ml 0.2 N hydrochloric acid were added to each tube and the samples were placed in a boiling water bath for 1 h. The samples were allowed to come to room temperature and the pH was adjusted to 4.5 with 10% glacial acetic acid and 1 N potassium hydroxide. The plasma preparation for microbiological analyses is outlined in Figure 1.

2. Column preparation

Two hundred and fifty g Dowex 50-X-8, 100-200 mesh, ion-exchange resin (Biorad Laboratories) were weighed into a 2 l Erlenmeyer flask. While mechanically stirring, approximately 200 ml 6 N potassium hydroxide were added to the resin and a slurry was thus formed. The slurry was allowed to settle and the supernatant was discarded. Distilled water was used to rinse the resin by the following technique: approximately 600 ml water were added to the resin; the slurry was mixed by inverting; the resin was allowed to settle; then the supernatant was decanted. The resin was rinsed by this procedure until the supernatant was neutral when tested with litmus paper. Approximately 600 ml 3 N hydrochloric acid were added and the resin was heated in a boiling water bath with mechanical stirring for 30 min. The supernatant was discarded and the hydrochloric acid boiling water bath
1 ml plasma + 0.05 g SSA, vortex, centrifuge 20 min, 5°C, 7000 x g

\[ \text{↓} \]

remove top layer, filter through 0.2 μm filter, add 3 ml 0.2 N HCl, boil 1 h

\[ \text{↓} \]

cool, adjust to pH 4.5 and place into column

\[ \text{↓} \]

wash with pH 4.5, 0.01 M K_2Ac buffer
wash with pH 5.5, 0.02 M K_2Ac buffer

\[ \text{↓} \]

elute prewash

\[ \text{↓} \]

wash with pH 6.0, 0.04 M K_2Ac buffer

\[ \text{↓} \]

elute PL

\[ \text{↓} \]

wash with pH 7.0, 0.1 M K_2Ac buffer

\[ \text{↓} \]

elute PN

\[ \text{↓} \]

wash with pH 8.0 KCl and K_2HPO_4 buffer

\[ \text{↓} \]

elute PN

eluates ready for quantitation

**FIGURE 1**
Plasma preparation for microbiological analyses
treatment was repeated. The resin was then rinsed with distilled water until the supernatant was neutral when tested with litmus paper. The supernatant was discarded. Six N potassium hydroxide was added until the supernatant was alkali when tested with litmus paper, and the resin was stirred mechanically for 1 h. The resin slurry was then rinsed with distilled water until the supernatant was neutral when tested with litmus paper. The supernatant was discarded and the resin was then ready for use. The resin was suspended in 2 M potassium acetate and stored at 5°C until needed. Before use, the resin was rinsed with distilled water until the supernatant was neutral when tested with litmus paper.

3. Column chromatography of sample

A small quantity of glass wool was placed in the bottom of glass chromatography columns, 250 ml capacity, 17 mm OD, 14.5 mm ID (Montes Laboratories) to prevent the resin from leaking out of the columns. Twenty ml resin suspended in water were poured into each column and allowed to gravity drip until the water was evacuated. A glass wool plug was placed on top of the resin packing to prevent splashing of the resin by the buffers. Fifty ml boiling water were poured into each column followed by two, 50 ml washes potassium acetate buffer, pH 4.5. The sample was then placed on the column and allowed to gravity drip until the sample had passed through the column. Care was taken throughout the chromatography steps to avoid letting the columns run dry. The columns were then washed with 50 ml boiling potassium acetate buffer, pH 5.5, and the prewash fraction was collected in a 50 ml volumetric flask. The columns were then washed
with 50 ml boiling potassium acetate buffer, pH 6.0, and the pH fraction was collected in a 50 ml volumetric flask. Then was then eluted with 50 ml boiling potassium acetate buffer, pH 7.0, and then was eluted with 50 ml boiling potassium chloride and potassium phosphate buffer, pH 8.0, and the fractions were collected in 50 ml volumetric flasks. The pH of the fractions were adjusted to pH 4.5 with 10% glacial acetic acid and 1 N potassium hydroxide and brought volumetrically to 50 ml with distilled, ion-free water.

4. Maintenance of culture

Frozen, lyopholized _S. uvarum_ was obtained from the American Type Culture Company. The samples were rehydrated in Bacto™ broth (Difco Laboratories), 0.84 g Bacto™ broth/ 40 ml distilled, ion-free water. The broth was dispensed into 4 Pyrex culture tubes, 10 ml/tube, and each tube was capped. The tubes were autoclaved for 15 min at 15 psi and 121°C. Three tubes were used as stock broth tubes while the fourth tube was used to suspend the initial lyopholized sample. After the broth cooled, 1-2 ml broth from the fourth tube of Bacto™ broth was used to suspend the lyopholized sample of _S. uvarum_ using aseptic techniques. Two-three drops of this suspension were added to each of the other 3 sterile broth tubes to form a stock inoculum of _S. uvarum_. At this time, an agar slant was inoculated by a loop transfer from the original culture suspension. The 3 tubes of stock inoculum and the agar slant were incubated overnight in a shaker water bath at 28°C.

Agar slants were made in bulk, autoclaved, and stored at 5°C. Bacto™ agar (Difco Laboratories), 4.1 g/100 ml distilled, ion-free
water was dissolved over heat with constant stirring. Once dissolved, 5 ml agar were transferred into Pyrex screw cap tubes and the tubes were capped. The agar was then autoclaved for 15 min at 15 psi and 121°C and allowed to cool at an angle so that they would form agar slants. The slants were stored at 5°C until use.

Weekly transfers of _S. uvarum_ were made to 2 fresh agar slants and allowed to incubate overnight in a 28°C shaker water bath. One of the slants was used to innoculate the broth for the current test while the other slant was stored at 5°C for use for the following week's transfer.

5. Preparation of inoculum

Into 50 ml distilled, ion-free water, 1.3 g Pyridoxine Y media were dissolved. Ten ml media were measured into Pyrex culture tubes. The tubes were covered and the media was steamed for 5 min at 121°C and allowed to cool. The day prior to innoculating prepared samples and standards, a loop transfer was made from the fresh agar slant to the broth tubes using aseptic techniques and the broth was incubated overnight in a shaker water bath at 28°C. Three tubes were innoculated to insure that a culture suspension would be available for the test. After allowing the broth cultures to grow, the cultures were washed with 0.85% sodium chloride by the following method. The innoculated broth tubes were transferred aseptically to Pyrex screw cap tubes and spun at 2500 x g in a centrifuge for 3-4 min at room temperature. The culture formed a pellet at the bottom of the tube and the supernatant was decanted off and discarded. The pellet was then suspended in 10 ml sterile 0.85% sodium chloride and recentrifuged at 2500 x g for
3-4 min. The supernatant was decanted and discarded and the saline wash was repeated twice for a total of 3 saline washes. The final saline suspension was used to inoculate the test with.

6. Assay of B-6 vitamin standards

Two, 6 mm glass beads were placed in the bottom of each Pyrex culture tube. The individual working standards of 1, 2, 3, 4, and 5 ng/10 ml PL, PN, and PM were brought to room temperature. Each level of individual vitamin working standard was run in duplicate. Pyridoxine Y media was made according to the instructions given in Appendix 2 and 9 ml Pyridoxine Y media were added to the tubes containing the glass beads along with 1 ml appropriate working standard, resulting in individual standard curves of 1, 2, 3, 4, and 5 ng/tube of PL, PN, and PM.

The tubes were capped and steamed for 5 min at 121°C. Using aseptic techniques, each tube was inoculated with 1 drop of the saline-washed inoculum. The tubes were then incubated for 22 h in a shaker water bath at 28°C. After incubation, the tubes were autoclaved for 10 min at 15 psi and 121°C to stop growth of the organism, and the tubes were allowed to come to room temperature before being read on the spectrophotometer.

7. Assay of plasma samples

All samples were run in duplicate and 2 levels; in most cases with plasma, 2 and 5 ml sample eluate was used with 8 and 5 ml of media respectively to maintain 10 ml/tube. Two, 6 mm glass beads were placed in the bottom of Pyrex culture tubes. The media was made up so that it would be the same strength in both levels of the
sample. The sample eluates were allowed to come to room temperature. After the sample and the media were added to the tubes, the tubes were capped and steamed for 5 min at 121°C. Using aseptic techniques, each tube was inoculated with 1 drop of the saline-washed inoculum. The tubes were incubated for 22 h in a shaker water bath at 28°C. After incubation, the tubes were autoclaved for 10 min at 15 psi and 121°C to stop growth of the organism and the tubes were allowed to come to room temperature before being read on the spectrophotometer.

8. Calculation of vitamer concentration and % recoveries of spiked samples

The samples and standards were read at 550 nm on a Bausch and Lomb spectrophotometer and the absorbance was recorded. The PL, PN, and PM standards were plotted with vitamer concentration in ng/tube on the x-axis and absorbance on the y-axis. A typical calibration curve for B-6 vitamer concentrations is illustrated in Figure 2. The samples were calculated from the calibration curves of the appropriate vitamer taking dilution factors into account. Percent recoveries of vitamers in spiked plasma samples were calculated by the following formula:

\[
\frac{(\text{vitamer concentration in plasma} + \text{vitamer spike})}{(\text{vitamer concentration in plasma}) + (\text{known concentration of vitamer spike})} \times 100 = \% \text{ recovery of vitamer.}
\]

Recoveries of 80-100% were obtained from plasma samples spiked with 100 ng PL, PN, and PM. The recoveries for the phosphorylated forms of the vitamers PLP, PNP, and PMP calculated as % recovery of the corresponding non-phosphorylated forms were 55, 115, and 56%, respectively.
FIGURE 2
Typical microbiological calibration curves for PL, PN, and PM concentrations
E. **HPLC analyses of B-6 vitamins in plasma**

The vendors and their addresses for certain chemicals used in the HPLC analyses of B-6 vitamins are given in Appendix 1. The preparation of reagents for the HPLC assay of B-6 vitamins is given in Appendix 3.

1. **HPLC system**

In our laboratory, an HPLC method has been developed using paired-ion chromatography to separate PL, PN, and PM in standard solutions and in rat liver, kidney, and brain, and in human breast milk. All of the HPLC equipment used for these analyses was manufactured by Waters Associates. A Model 730 Data Module was used with a Model 720 Systems Controller. Two M-45 pumps were used for solvent delivery. A fixed wavelength fluorescence detector, Model 420-E, was fitted with a 300 nm excitation filter and a 375 nm emission filter. A Model U6-K loop valve injector was used. The column was a *Fondapak C* sub analytical column, 3.9 mm x 30 cm, with a 10 µ particle size. The guard column was manually packed with *Fondapak C* sub/Corasil. The solvent system used was 85% methanol/15% water in pump 1 and PIC B-7 solution (Waters Associates) paired-ion reagent in pump 2. The methanol and water were HPLC grade. A flow rate of 1.5 ml/min was used.

The most successful gradient used for the separation of PL, PN, and PM in plasma is given in Table 1. Several gradients were used varying time, percent of solvent, and curve numbers, but this gradient was found to obtain the best separation of the B-6 vitamins in plasma.

2. **Development of a sample preparation**

The sample preparation used in the microbiological analyses of
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>85% ethanol: 15% water</th>
<th>PIC B-7</th>
<th>Curve+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>35</td>
<td>65</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>40</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>100</td>
<td>6</td>
</tr>
</tbody>
</table>

* A solvent flow rate of 1.5 ml/min was used

+ The curve numbers designate the rate of change in solvent composition in a given time period: curve 2 indicates a rapid, curvilinear change in solvent composition while curve 6 indicates a linear change in solvent composition
B-6 vitamins in human plasma was not suitable for HPLC analyses because of the dilution factor of the hydrochloric acid dephosphorylation step. Several other methods of sample preparation were attempted including isoelectric point precipitation and salting out techniques with 90% ammonium sulfate (48,49) but they were unsuccessful largely due to procedural steps that diluted the sample. Also, plasma PLP is tightly bound to albumin (10) and the preparation must be harsh enough to break this bond.

A modification of the methods of Gregory et al. (33) and Guilarte and McIntyre (24) was adopted, using a potato acid phosphatase, PAP (EC 3.1.3.2, Orthophosphoric-monoester phosphohydrolase) solution as a dephosphorylating agent. The optimum level of potato acid phosphatase to use to maximize dephosphorylation of PLP, PNP, and PMP was determined to be 0.1 g potato acid phosphatase/10 ml potassium acetate buffer, which was approximately the saturation point of the solution. The recoveries of the vitamins spiked in human plasma at 2 different levels of potato acid phosphatase solutions are given in Table 2.

Frozen plasma samples were thawed overnight at 5°C. The samples, approximately 3 ml, were measured into centrifuge tubes. If the samples were to be spiked with standards, they were spiked at this point with 300 μl of working stock standard 10,000 ng/ml of the appropriate standard. Potato acid phosphatase was added to the sample at a concentration of 0.6 ml potato acid phosphatase solution/3 ml plasma. The samples were incubated for 1 h in a shaker water bath at 37°C. The samples were cooled to room temperature and 0.25 ml
TABLE 2
Percent recoveries of B-6 vitamers in spiked plasma at 2 concentrations of potato acid phosphatase

<table>
<thead>
<tr>
<th>Vitamer</th>
<th>Grams potato acid phosphatase: ml potassium acetate buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>PL</td>
<td>93</td>
</tr>
<tr>
<td>PN</td>
<td>98</td>
</tr>
<tr>
<td>PM</td>
<td>97</td>
</tr>
<tr>
<td>PLP*</td>
<td>48</td>
</tr>
<tr>
<td>PNP</td>
<td>73</td>
</tr>
<tr>
<td>PMP</td>
<td>54</td>
</tr>
</tbody>
</table>

* Recoveries of phosphorylated vitamers were calculated as % recovery of corresponding non-phosphorylated vitamers.
100% trichloroacetic acid, TCA/ 3 ml plasma was added. The tubes were covered and vortexed at low speed for 30 s and incubated for 15 min in a water bath at 50°C. The samples were cooled to room temperature and 3 ml methylene chloride were added to each tube. The tubes were covered with plastic wrap and shaken vigorously by hand for 1 min. The samples were then centrifuged for 20 min at 5°C at 7000 x g. The top layer of the sample was pipetted off and the volume was recorded. The pH was adjusted to pH 5-6 with 10% glacial acetic acid and 1.0 N potassium hydroxide. The sample was filtered through a 0.2 μm Gelman Acrodisc filter and stored in amber vials at 5°C until ready to be injected into the HPLC. The HPLC plasma preparation is outlined in Figure 3.

With the sample preparation developed the sample was diluted approximately 40% compared to a dilution of approximately 400% using the microbiological sample preparation.

3. Separation of PL, PN, and PM standards

A typical chromatogram showing the separation of a PL, PN, and PM mixed standard, each at a concentration 250 ng/ml is illustrated in Figure 4. A 250 μl injection volume was used. With the HPLC method developed in this research, minimal detectable limits of approximately 4-5 ng for PL and PN and 3-4 ng for PM were obtained.

4. Identification of PL, PN, and PM peaks in human plasma-chromatographic attempts to improve resolution

A typical HPLC chromatogram of unspiked human plasma is given in Figure 5. A typical HPLC chromatogram of human plasma spiked with PL, PN, and PM is given in Figure 6. The vitamins PL, PN, and PM were
3 ml plasma + 0.6 ml PAP, vortex, incubate 1 h at 37°C

↓

add 0.25 ml 100% TCA, vortex, incubate 15 min at 50°C

↓

cool, add 3.0 ml MeCl, shake 1 min centrifuge 20 min, 5°C, 7000 x g

↓

remove top layer, adjust to pH 5-6
filter through 0.2 μm filter

↓

sample ready to inject

FIGURE 3
Plasma preparation for HPLC analyses
FIGURE 4
Typical HPLC chromatogram of PL, PN, and PM standards

Fluorescence (300 nm excitation, 375 nm emission)
FIGURE 5
Typical HPLC chromatogram of unspiked human plasma
FIGURE 6
Typical HPLC chromatogram of human plasma spiked with PL, PN, and PM.
identified in the human plasma samples by peak shape, retention times, and internal spiking, but were too low in concentration to be accurately quantitated. With the developed HPLC system, the sample would have to be concentrated a minimal of 4-5 times to obtain quantifiable vitamin peaks. Concentration of the plasma samples was not feasible for this research project in that we had a plasma volume of approximately 2-2.5 ml for each subject and a sample volume of 8-10 ml would be necessary in order to obtain quantifiable vitamin peaks. Because of the small n of this current project, pooling of the samples was not practical.

Chromatographically, several things were attempted to improve resolution and to make quantitation more feasible. An injection volume of 500 μl instead of 250 μl was tried, but this was found to cause excessive peak spreading. A solvent flow rate of 1.0 ml/min instead of 1.5 ml/min was tried to obtain better resolution, but this was also unsuccessful. Argon was bubbled through the solvents to displace any O₂ that may get into the system that would squelch the signal to the fluorometer and increase background noise; this made a positive, but minimal difference in background noise of standards.

5. Suggestions for future research

This researcher suggests that concentrating techniques for plasma samples be investigated in future research. Freeze-drying of the sample and concentrating over an inert gas offer two potential methods of concentrating the plasma samples. The plasma samples could be concentrated prior to the dephosphorylation step in the procedure or could be concentrated at the end of the procedure after being
dephosphorylated and deproteinated. The recoveries of the vitameric forms would need to be demonstrated in standard solutions before proceeding to plasma samples.

The HPLC column currently used is a 10 μ particle size C<sub>18</sub> column. If a 5 μ particle size column was used, the efficiency of the column would increase approximately 40% (50).

Gregory et al. (33) use the reagent semicarbazide in the solvent system to convert PL and PLP to semicarbazone forms that are more fluorescent. The use of this reagent in our current system should be investigated. Also, the use of post column derivitization procedures that would increase detection limits should be examined.

Vanderslice and Maire (11) and Vanderslice et al. (12) have successfully separated the 6 B-6 vitamers in human plasma. Their HPLC system used semicarbazide in the solvent system, 2 thermostated columns set up with reverse flow valves, and a variable wavelength detector which enables detection of each vitamer at their optimum excitation and emission wavelengths. The total vitamin B-6 concentration of 2 adult males on a regular diet was 63.6 ng/ml (mean). Theoretically, this concentration of total vitamin B-6 in human plasma would be minimally quantitatable in our system using the minimal detection limits found in standards of PL, PN, and PM, but in practice, minimal detection limits are not as low in samples as they are in standards. Also, minimal detection limits are affected by injection size of the sample, how degassed the solvents are, background noise inherent in a particular sample, and the age of the column. We were not able to obtain quantitatable B-6 vitamer peaks on the plasma samples tested.
The HPLC analysis of plasma B-6 vitamer levels offers much potential for use as a status indicator. HPLC techniques offer many advantages over currently used methods of vitamin B-6 status assessment.

F. Statistical analyses

The subjects were divided into 2 groups according to their coenzyme stimulation values. Subjects with coenzyme stimulation values <16% are hereby referred to as "adequate" in status, while subjects with coenzyme stimulation values ≥16% are hereby referred to as "inadequate" in status (14,51).

Significant differences between the parameters were obtained using the Students t-test. Means and standard deviation were also determined. Correlations between the values obtained for the various parameters were obtained using the Pearson r correlation coefficient (52).
RESULTS AND DISCUSSION

Subjects with coenzyme stimulations of E-ALAT < 16% were classified as "adequate" in status while the subjects with coenzyme stimulations of E-ALAT ≥ 16% were classified as "inadequate" in status. There were 8 subjects in the "adequate" group and 3 subjects in the "inadequate" group.

A. Height and weight measurements

Individual data including age, height, and weight of the subjects are given in Appendix 4. The height and weight values for the subjects classified by coenzyme stimulation activities of E-ALAT are given in Table 3. There were no significant differences in the height and weight values between the "adequate" and "inadequate" groups.

One subject, 104, was below the 50th percentile for height only, and 1 subject, 103, was below the 50th percentile for weight only, as compared to growth charts established by the National Center for Health Statistics (NCCHS) for female adolescents (53). Subject 109 was below the 50th percentile for both height and weight but was classified as "adequate" in status. Subject 134 was above the 95th percentile for both height and weight. Heights and weights of the subjects were significantly correlated (r=0.75, p < 0.01).

E. Dietary assessments

The individual nutrient intakes for kilocalories, protein, and vitamin E-6 are listed in Appendix 5. The dietary data from subject 111 were not included in the assessment of the subjects as a
<table>
<thead>
<tr>
<th>Variable</th>
<th>Coenzyme stimulation of E-AIAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;adequate&quot;</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>153.4 ± 6.8*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>44.7 ± 9.2</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>8</td>
</tr>
</tbody>
</table>

* Mean ± SD
group since only 1 dietary recall was obtained from the subject and according to the subject, this recall did not reflect her usual intake. Only 1 subject, 104, consumed vitamin B-6 in supplementary form and this intake was not included in her dietary estimate of vitamin B-6 because the subject indicated that supplements were not consistently taken.

Table 4 gives the values for kilocalories, protein, and vitamin B-6 intakes of the subjects divided into "adequate" and "inadequate" groups. There were no significant differences in intakes of kilocalories, protein, or vitamin B-6 between the groups. The overall intakes of kilocalorie, protein, and vitamin B-6 (mean ± SD) for all subjects were 2037 ± 508, 73.6 ± 26.8 g, and 1.47 ± 0.49 mg, respectively.

The individual dietary intakes of the subjects expressed as % recommended energy intake of kilocalories, and the % RDA of protein and vitamin B-6 are listed in Appendix 6. The % recommended energy intake of kilocalories, and the % RDA of protein and vitamin B-6 divided into "adequate" and "inadequate" groups is given in Table 5. There were no significant differences between the groups with respect to % recommended energy intake of kilocalories and % RDA of protein and vitamin B-6. The overall % recommended energy intakes of kilocalories, and the % RDA for protein and vitamin B-6 (mean ± SD) for all subjects were 92 ± 23, 160 ± 59, 81 ± 27.

A range of 1500-3000 kilocalories with a mean value of 2200 is recommended for daily energy intake by females aged 11-14 yr (36). Two subjects, 103 and 116, consumed <2/3 recommended energy intake for kilocalories, approximately 100 and 200 kilocalories less than
### TABLE 4
Nutrient intakes of subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coenzyme stimulation of F-ALAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;adequate&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot;inadequate&quot;</td>
</tr>
<tr>
<td>Kilocalories</td>
<td>2042 ± 614*</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>77.9 ± 30.9</td>
</tr>
<tr>
<td>Vitamin E-6 (mg)</td>
<td>1.48 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>2023 ± 173</td>
</tr>
<tr>
<td></td>
<td>63.5 ± 12.1</td>
</tr>
<tr>
<td></td>
<td>1.43 ± 0.39</td>
</tr>
</tbody>
</table>

* Mean ± SD
TABLE 5
Nutrient intakes of subjects expressed as % recommended energy intake for kilocalories and % RDA for protein and vitamin B-6

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coenzyme stimulation of E-AIAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;adequate&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot;inadequate&quot;</td>
</tr>
<tr>
<td>Kilocalorie (% recommended energy intake)</td>
<td>93 ± 28*</td>
</tr>
<tr>
<td>Protein (% RDA)</td>
<td>169 ± 67</td>
</tr>
<tr>
<td>Vitamin B-6 (% RDA)</td>
<td>82 ± 31</td>
</tr>
</tbody>
</table>

* Mean ± SD
the lower end of the range recommended. Both of these subjects were classified as "adequate" in status and were within normal ranges for height and weight according to NHHS growth charts (53).

The RDA for protein for females, aged 11-14 yr is 46 g/day (36). Only 1 subject, 116, consumed <100% RDA for protein, with an intake of 96% RDA, or 44 g protein. The mean % RDA consumed by the subjects was 160%. In the NFCS, the dietary habits of over 1,000 female adolescents, aged 12-14 yr, were evaluated (37). The dietary information was obtained from a 24 h dietary recall followed by a 48 h dietary record. The average amount of protein consumed by the female adolescents was 155% RDA or 71.3 g protein (36,37). Kirksey et al. (14) found the protein intake in g of 127 female adolescents, aged 12-14 yr, of 71 ± 25 (mean ± SD) or 154% RDA (36). Nutrient intake from the Kirksey et al. study was estimated via a 24 h dietary recall (14). The findings of this current research with respect to protein consumption are consistent with the findings of the NFCS (37) and Kirksey et al. (14).

The requirement for vitamin B-6 has been shown to be closely related to the consumption of protein with an increased vitamin B-6 requirement being associated with increased protein intake (18,36,38). The Dietary Standards for Canada (54) recommend a ratio of 0.02 mg vitamin B-6/ g protein ingested. Using an approximate protein consumption of 71-73 g/ day for female adolescents (14,37) a requirement of approximately 1.5 mg/ day vitamin B-6 can be derived from the ratio of 0.02 mg vitamin B-6/ g protein ingested (54).
Ritchey and Feeley (18) examined urinary excretion patterns as a means of assessing vitamin B-6 requirements in preadolescent females, aged 7-9 yr, and determined an intake of 1.3-1.7 mg/day vitamin B-6 to be adequate with protein levels of 20-40 g/day. Donald et al. (38) assessed the vitamin B-6 requirement of women, aged 21-30 yr, by examining urinary excretion patterns and several biochemical parameters and found an intake of 1.5 mg/day vitamin B-6 to be adequate when consuming 57 g/day protein. The protein intakes from the studies of Ritchey and Feeley (18) and Donald et al. (38) are below the mean protein intakes currently reported in the NFCS (37) study to be consumed in the United States for the corresponding age groups. Data on the specific requirement for vitamin B-6 in adolescents are not sufficient to make a satisfactory recommendation (49). The RDA for vitamin B-6 for adolescents is derived from requirements determined for children and adults (36).

In the NFCS (37), the mean vitamin B-6 intake for female adolescents, aged 12-14 yr, was 1.35 mg/day or 75% RDA (36). Kirksey et al. (14) found a daily intake of 1.24 ± 0.70 mg (mean ± SD) or 69% RDA. In this current study, the daily vitamin B-6 consumption was 1.47 ± 0.49 mg (mean ± SD) which is slightly more than the NFCS (37) and of the Kirksey et al. study (14). This slight difference may be a result of the small sample size of this current study, of regional differences in dietary consumption patterns, or of differences in methodology used for dietary assessment of the subjects.

The Pearson r correlation coefficients for kilocalories, protein, and vitamin B-6 intakes of subjects in the current study are given
in Table 6. A positive correlation \( r=0.69, p<0.03 \) was obtained between protein and vitamin B-6 intakes. In foods, vitamin B-6 is typically found bound to protein and consumption of vitamin B-6 is often associated with consumption of protein \((43,44)\). A positive correlation \( r=0.76, p<0.01 \) was found between kilocalorie and protein intakes. This can be explained by the high amount of protein consumed by the subjects on this study as compared to the amount of kilocalories consumed. A positive correlation was also found between kilocalorie and vitamin B-6 intakes \( r=0.89, p<0.01 \). Although it has been suggested \((55)\) that the requirement for vitamin B-6 be expressed as a ratio to the amount of kilocalories consumed, as the requirement is for thiamin and niacin, this concept has not been generally accepted \((15,36)\). Fries \((56)\) reported highly significant correlations of vitamin B-6 to kilocalories and protein \( r=0.62 \text{ and } r=0.57, \text{ respectively, } p<0.01 \) in a group of preschool children.

C. **Coenzyme stimulation of E-ALAT activities**

The E-ALAT coenzyme stimulation activity has been shown to be a sensitive and reliable indicator of normal vitamin B-6 status and to respond to both deficiency and supplemented states \((40,47,52,57,58)\). Adequate status has been defined as having a coenzyme stimulation of E-ALAT of \(<16\%\) by Kirksey et al. \((14)\) and Woodringe and Storvick \((52)\) and as having a coenzyme stimulation of F-ALAT \(<25\%\) by Sauberlich et al. \((2)\). In our laboratory, we have used the coenzyme stimulation of E-ALAT between 16-25% to indicate a marginal status. Because of the small sample size involved in this current research, the use of 3 status classifications; adequate, marginal, and inadequate, was not used.
<table>
<thead>
<tr>
<th>Variables</th>
<th>Pearson r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilocalorie : protein</td>
<td>0.76</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Kilocalorie : vitamin B-6</td>
<td>0.89</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Protein : vitamin B-6</td>
<td>0.69</td>
<td>&lt; 0.03</td>
</tr>
</tbody>
</table>
The more stringent cutoff value of <16% E-AIAT coenzyme stimulation was used as the criterion for classification of the subjects as adequate in vitamin B-6 status.

The individual E-AIAT coenzyme stimulation activities of the subjects are given in Appendix 5. The coenzyme stimulation of E-AIAT for the subjects classified as "adequate" was 5.4 ± 5.3% (mean ± SD) and 23.5 ± 5.1% (mean ± SD) for the subjects classified as "inadequate". The coenzyme stimulation of E-AIAT for all subjects was 10.4 ± 9.8% (mean ± SD). Three out of 11 subjects, or 27.3%, were classified as "inadequate" in status. Using the same criterion of ≥16% F-AIAT coenzyme stimulation to indicate an inadequate status, Kirksey et al. found 31% of their adolescent females were classified as inadequate in status (14).

Kirksey et al. found subjects with high coenzyme stimulation of E-AIAT activities to have significantly lower dietary intakes of kilocalories, protein, and vitamin B-6 than subjects with lower E-AIAT coenzyme stimulation activities (14). This relationship between coenzyme stimulation of E-AIAT activities and dietary intakes of kilocalories, protein, and vitamin B-6 was not found in this current research project, possibly as a result of the small n of this project, differences in methodologies of estimating nutrient intakes, or of the use of different people as subjects.

The subjects with coenzyme stimulation of E-AIAT activities near 0.0% showed a wide range of vitamin B-6 intakes suggesting that there may be a large individual variation in the requirement for vitamin B-6 (2,36). A range of vitamin B-6 intakes in subjects
with E-ALAT coenzyme stimulation activities near 0.0% was observed by Hampton et al. (59) in the elderly and by Driskell et al. (60) in young men and women and women on oral contraceptives.

D. Microbiological determinations of plasma PL, PN, and PM concentrations

The individual plasma B-6 vitamer concentrations of PL, PN, and PM determined microbiologically are given in Appendix 7. The plasma vitamer concentrations of PL, PN, and PM for the subjects divided into "adequate" and "inadequate" groups are given in Table 7. The total plasma vitamin B-6 concentration for the "adequate" group was 56.8 ± 7.2 ng/ml (mean ± SD). The total plasma vitamin B-6 concentration for the "inadequate" group was 13.1 ± 1.9 ng/ml (mean ± SD). The overall total plasma vitamin B-6 concentration for the subjects was 44.9 ± 21.3 ng/ml. There was a significant difference between the "adequate" and "inadequate" groups with respect to plasma PN concentrations (p < 0.001), plasma PM concentrations (p < 0.002) and total plasma vitamin B-6 concentrations (p < 0.001).

Plasma has been reported to have higher total vitamin B-6 levels determined microbiologically than erythrocytes, serum, or whole blood (61). Baker et al. (61) reported total vitamin B-6 levels for whole blood, erythrocytes, and plasma (mean ± SD) of normal subjects of 37 ± 6, 20 ± 3, and 59 ± 13 ng/ml. Plasma vitamin B-6 levels are responsive to deficiency states and to supplemented states (32, 39, 62). The only reported vitamin B-6 levels determined microbiologically in adolescents found by this investigator were those of Baker et al. (14) with a mean serum total vitamin B-6 concentration of 36 ng/ml in approximately 600 male and female adolescents, aged 10-13 yr.
TABLE 7
Microbiological determination of plasma B-6 vitamer concentrations of subjects

<table>
<thead>
<tr>
<th>Vitamer</th>
<th>Coenzyme stimulation of F-ALAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;adequate&quot;</td>
</tr>
<tr>
<td>ng/ml</td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>12.1 ± 4.4*</td>
</tr>
<tr>
<td>PN</td>
<td>30.6 ± 7.0+</td>
</tr>
<tr>
<td>PM</td>
<td>14.1 ± 3.6#</td>
</tr>
<tr>
<td>Total</td>
<td>56.8 ± 7.2+</td>
</tr>
</tbody>
</table>

* Mean ± SD
+ Significantly different from each other at p < 0.001
# Significantly different from each other at p < 0.002
This is slightly lower than the overall total plasma vitamin B-6 level found in this current study of 44.9 ± 21.3 ng/ml (mean ± SD), but as previously noted, plasma has been found to have higher total vitamin B-6 levels than serum, erythrocytes, or whole blood (61).

Sauberlich et al. (2) have reported tentative guidelines for evaluating vitamin B-6 nutritional status in the adult as ≤25 ng/ml plasma total vitamin B-6 indicating a marginal or inadequate status and ≥50 ng/ml plasma total vitamin B-6 indicating an acceptable status (2). The mean plasma total vitamin B-6 concentrations of the subjects separated into "adequate" and "inadequate" groups corresponds well with the tentative guidelines for evaluating vitamin B-6 status.

Three of the 11 subjects, or 27.3% had plasma total vitamin B-6 levels ≤25 ng/ml, corresponding to marginal or inadequate status by the suggested guidelines of Sauberlich et al. (2). Seven of the 11 subjects, or 63.6% had plasma total vitamin B-6 levels of ≥50 ng/ml corresponding to adequate in status by the suggested guidelines of Sauberlich et al. (2). These same 3 and 7 subjects were classified as "inadequate" and "adequate" in status by the coenzyme stimulation activities of E-ALAT. One subject, 109, had a plasma total vitamin B-6 concentration between 25-50 of 49.1 and was classified as adequate in status by the coenzyme stimulation of E-ALAT activities.

The individual distributions of PL, PN, and PM in the plasma expressed as a percentage of the total vitamin B-6 in the plasma for the subjects are given in Appendix 8. The distributions of PL, PN, and PM in the plasma of the subjects classified as "adequate" or "inadequate" are given in Table 8. In the "adequate" group, the order of mean
TABLE 8
Distribution of plasma B-6 vitamers of subjects measured by microbiological assay

<table>
<thead>
<tr>
<th>Vitamer</th>
<th>Coenzyme stimulation of F-ALAT</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;adequate&quot;</td>
<td>&quot;inadequate&quot;</td>
</tr>
<tr>
<td>PL</td>
<td>21.2 ± 7.7*</td>
<td>46.8 ± 17.6</td>
</tr>
<tr>
<td>PN</td>
<td>53.9 ± 11.4</td>
<td>19.1 ± 1.5</td>
</tr>
<tr>
<td>PM</td>
<td>24.9 ± 5.6</td>
<td>34.1 ± 19.2</td>
</tr>
</tbody>
</table>

* Mean ± SD
% distribution of vitamers from largest to smallest is PN, PM, and PL. The same order of the vitamers was also shown in the "adequate" group when examining mean vitamer concentrations in plasma. Vanderslice et al. (12) also showed PN as the vitamer with the largest concentration in plasma of normal adult males determined via an HPLC assay. In the "inadequate" group, the order of mean percent distribution of vitamers from largest to smallest is PL, PM, and PN. The same order of the vitamers was also shown in the "inadequate" group when examining mean vitamer concentrations in plasma. The difference in order of the content and distribution of the B-6 vitamers in the "adequate" and "inadequate" groups should be investigated with a larger sample size. The differences in the order of the content and distribution of the B-6 vitamers in this current study may be a function of the small sample size or of the effects of poor recoveries of PLP and PMP in the microbiological assay.

Pearson r correlation coefficients of plasma PL, PN, PM and total vitamer concentrations and coenzyme stimulation of E-ALAT activities are given in Table 9. A significant correlation (r=0.82, p<0.01) between E-ALAT coenzyme stimulation values and plasma total vitamin B-6 concentrations was obtained. The individual vitamers PM (r=0.882, p<0.01) and PN (r=0.69, p<0.02) were also significantly correlated to coenzyme stimulation of E-ALAT activities. The vitamer PL and E-ALAT coenzyme stimulation activities approached significance (r=0.57, p<0.07).
### TABLE 9
Pearson r correlation coefficients of biochemical data of subjects*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Pearson r</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL : PM</td>
<td>0.65</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>PL : total B-6</td>
<td>0.66</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>PM : total B-6</td>
<td>0.85</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>PN : PM</td>
<td>0.66</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>PN : total B-6</td>
<td>0.94</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>PN : E-ALAT</td>
<td>0.69</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>PM : E-ALAT</td>
<td>0.88</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>E-ALAT : total B-6</td>
<td>0.82</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

* All others not significant
E. **Family income levels of the subjects**

The individual income levels of the subject's family is given in Appendix 4. The dietary or biochemical parameters did not seem to be affected by the income level of the subject's family. This may be a result of the low n of this current study. The income levels were approximately evenly distributed among the "adequate" and "inadequate" groups.
The heights and weights of the subjects (n=11) were generally within the normal ranges established by the NCHS (53). The dietary intake of protein was $73.6 \pm 26.8 \text{ g}$ (mean $\pm$ SD), or 160% of the RDA (36). The dietary intake of vitamin B-6 was $1.47 \pm 0.49 \text{ mg}$ (mean $\pm$ SD), or 81% of the RDA (36). There were no significant differences in dietary intakes of kilocalories, protein, or vitamin B-6 between the "adequate" and "inadequate" groups.

Three subjects, or 27.3%, were classified as inadequate in status using a coenzyme stimulation of E-ALAT activity of $<16\%$ as the criterion. Subjects with coenzyme stimulation values near 0.0% reported a wide range in consumption of vitamin B-6 which may indicate a wide individual variation in the requirement for vitamin B-6.

Plasma total vitamin B-6 levels determined microbiologically were $44.9 \pm 21.3 \text{ ng/ml}$ (mean $\pm$ SD). Using $\leq 25 \text{ ng/ml}$ as the criterion for status classification (2), 3 subjects, or 27.3% were classified as inadequate in status; the same 3 subjects which were classified as inadequate in status by the coenzyme stimulation activity parameter. There was a significant difference ($p<0.001$) between the "adequate" and "inadequate" groups with respect to plasma total vitamin B-6 levels determined microbiologically. There was a significant correlation ($r=0.624$, $p<0.01$) between coenzyme stimulation activities of E-ALAT and plasma total vitamin B-6 levels determined microbiologically. The order of vitamin concentration as well as % distribution from
largest to smallest of the B-6 vitamins, in the plasma for the
"adequate" group was PN, PM, and PL, while the order of distribution
for the "inadequate" group was PL, PM, and PN. The differences in
the distributions of the B-6 vitamins between subjects classified
as "adequate" or "inadequate" in status warrants future research with
larger sample sizes.

An HPLC method was developed that separated PL, PN, and PM in
standard solutions. The B-6 vitamins were detectable in human plasma
samples, but were not of large enough concentrations to be quantitated.
The samples must be concentrated 4-5 times in order to obtain quanti-
tatable vitamin peaks with the HPLC method described in this current
research. Freeze-drying and concentrating over an inert gas were
suggested as 2 methods of sample concentrating techniques to investi-
gate in future research. The HPLC method as described in this current
research offers much potential for development for use in quantitating
plasma B-6 vitamins.

The classification of subjects into adequate and inadequate status
by plasma total vitamin B-6 levels determined microbiologically and
coenzyme stimulation of E-ALAT activities agreed well with each other
in this group of adolescent females. Dietary intakes of the subjects
were not correlated to any of the biochemical parameters examined.


16. Minutes of the S-150 Technical Committee Meeting, November 7-8, 1979, Atlanta, Georgia.


APPENDIX 1
Vendors for certain chemicals*

Waters Associates  (Milford, Massachusetts)

- PIC B-7, 1-heptane sulfonic acid - 85103
- Bondapak C18 Corasil - 27248

Sigma Chemical Company  (Saint Louis, Missouri)

- Potato acid phosphatase, 2 units/mg solid - P 1146
- 5-sulfosalicylic acid - S 2130
- Pyridoxal - P 9130
- Pyridoxine - P 9755
- Pyridoxamine - P 9380
- Pyridoxal 5-phosphate - P 9255
- Pyridoxamine 5-phosphate - P 9505

Difco Laboratories  (Detroit, Michigan)

- Bacto YM broth - 0712
- Pyridoxine Y media - 0951

American Type Culture Company  (Rockville, Maryland)

- Saccharomyces uvarum stock culture - ATCC 9080

Kontes Laboratories  (Vineland, New Jersey)

- Chromatography glass column, 250 ml, 17 mm OD, 14.5 mm ID - K 420280 222

Fisher Scientific Company  (Raleigh, North Carolina)

- 0.2 u Gelman Acrodisc filters - 09 730 218

Bio Rad Laboratories  (Rockville Center, New York)

- Dowex 50-W-X8, 100-200 mesh resin - 745 6441

ICN Nutritional Biochemicals  (Cleveland, Ohio)

- Pyridoxine phosphate - 102778

* Order numbers for the corresponding companies follow item listing.
APPENDIX 2
Preparation of reagents for the microbiological assay of vitamin B-6

1. Potassium acetate buffers

a) 0.01 M, pH 4.5

Potassium acetate

In a 2 l volumetric flask, dissolve 1.962 g potassium acetate in approximately 1600 ml distilled, ion-free water. Adjust to pH 4.5 with 10% acetic acid and 1.0 N potassium hydroxide. Bring to volume with distilled, ion-free water.

b) 0.02 M, pH 5.5

Potassium acetate

In a 2 l volumetric flask, dissolve 3.924 g potassium acetate in approximately 1600 ml distilled, ion-free water. Adjust to pH 5.5 with 10% acetic acid and 1.0 N potassium hydroxide. Bring to volume with distilled, ion-free water.

c) 0.04 M, pH 6.0

Potassium acetate

In a 2 l volumetric flask, dissolve 7.848 g potassium acetate in approximately 1600 ml distilled, ion-free water. Adjust to pH 6.0 with 10% acetic acid and 1.0 N potassium hydroxide. Bring to volume with distilled, ion-free water.

d) 0.1 M, pH 7.0

Potassium acetate

In a 2 l volumetric flask, dissolve 19.630 g potassium acetate in approximately 1600 ml distilled, ion-free water. Adjust
to pH 7.0 with 10% acetic acid and 1.0 N potassium hydroxide. Bring to volume with distilled, ion-free water.

2. Potassium chloride-phosphate buffer, pH 8.0

Potassium chloride
Potassium phosphate
In a 2 l volumetric flask, dissolve 74.600 g potassium chloride and 17.400 g potassium phosphate in approximately 1600 ml distilled, ion-free water. Adjust to pH 8.0 with 10% acetic acid and 1.0 N potassium hydroxide. Bring to volume with distilled, ion-free water.

3. Stock standards - 50 µg/ml PL, PN, and PM

Pyridoxal HCl
Pyridoxine HCl
Pyridoxamine di-HCl
To make individual stock standards, 50 µg/ml PL, PN, and PM, weigh into 3, separate 500 ml volumetric flasks, 0.0305 g pyridoxal HCl, 0.0304 g pyridoxine HCl, 0.0358 g pyridoxamine di-HCl. Bring each to volume with 25% ethanol. Store in amber bottles at 5°C. Stock standards are stable for 2-3 months.

4. Working stock standards - 100 ng/ml PL, PN, and PM

Stock standard pyridoxal HCl
Stock standard pyridoxine HCl
Stock standard pyridoxamine di-HCl
To make individual working stock standards, 100 ng/ml PL, PN, and
PM, volumetrically measure 2 ml appropriate stock standard into a 1 l volumetric flask. Bring to volume with distilled, ion-free water. Store in amber bottles at 5°C. Working stock standards are stable for 2-3 weeks.

5. Working standards - 1, 2, 3, 4, and 5 ng/10 ml PL, PN, and PM
   
   Working stock standard pyridoxal HCl
   
   Working stock standard pyridoxine HCl
   
   Working stock standard pyridoxamine di-HCl
   
   To make individual working standards, 1, 2, 3, 4, and 5 ng/10 ml PL, PN, and PM volumetrically measure 1, 2, 3, 4, and 5 ml respectively of appropriate working stock standard into 100 ml volumetric flasks. Bring to volume with distilled, ion-free water. Working standards are stable for 2-3 weeks.

6. Media preparation
   
   Pyridoxine Y media
   
   To make enough media for approximately 100 tubes, add 26.5 g Pyridoxine Y media to 500 ml distilled, ion-free water and stir mechanically until dissolved. Make fresh as needed.
APPENDIX 3
Preparation of reagents for HPLC assay of vitamin B-6

1. PIC B-7 solution - 0.005 M

PIC B-7
Measure 1000 ml HPLC grade water into a 2 l Erlenmeyer flask. Add 1 bottle PIC B-7 and stir mechanically for at least 5 min. Under vacuum, filter through a Gelman glass fiber filter, 0.1 - 40 μ pore size, and sonicate for 1-3 min. PIC B-7 solution is stable at 5°C for 1 week.

2. Potassium acetate buffer - 4.0 M, pH 4.5

Potassium acetate
In a 1 l volumetric flask, dissolve 392 g potassium acetate in approximately 800 ml distilled, ion-free water. Adjust to pH 4.5 with 10% acetic acid and 1.0 N potassium hydroxide. Bring to volume with distilled, ion-free water. Store at 5°C.

3. Potato acid phosphatase enzyme solution

Potato acid phosphatase
In a 10 ml volumetric flask, dissolve 1.0 g PAP in pH 4.5, 4.0 M potassium acetate buffer, and stir mechanically until dissolved. Enzyme solution is made fresh daily.

4. Stock standards - 1,000,000 ng/ml PL, PN, PM, PLP, PNP, and PMP

Pyridoxal HCl
Pyridoxine HCl
Pyridoxamine di-HCl
Pyridoxal phosphate
Pyridoxine phosphate
Pyridoxamine phosphate

To make individual stock standards, 1,000,000 ng/ml PL, PN, PM, PLP, PNP, and PMP, weigh into 6 separate 100 ml volumetric flasks, 0.1218 g pyridoxal HCl, 0.1216 g pyridoxine HCl, 0.1434 g pyridoxamine di-HCl, 0.1478 g pyridoxal phosphate, 0.1473 g pyridoxine phosphate, and 0.1692 g pyridoxamine phosphate. Bring each to volume with distilled, ion-free water. Store in amber bottles at 5°C. Stock standards are stable for 1 week.

5. Working stock standards - 10,000 ng/ml PL, PN, PM, PLP, PNP, and PMP

Stock standard pyridoxal HCl
Stock standard pyridoxine HCl
Stock standard pyridoxamine di-HCl
Stock standard pyridoxal phosphate
Stock standard pyridoxine phosphate
Stock standard pyridoxamine phosphate

To make individual working stock standards, 10,000 ng/ml PL, PN, PM, PLP, PNP, and PMP, volumetrically measure 1 ml appropriate stock standard into a 100 ml volumetric flask. Bring to volume with distilled, ion-free water. Store in amber bottles at 5°C. Working stock standards are stable for 1 week.

6. Working standards - 200 ng/ml PL, PN, PM, PLP, PNP, and PMP

Working stock standard pyridoxal HCl
Working stock standard pyridoxine HCl
Working stock standard pyridoxamine di-HCl
Working stock standard pyridoxal phosphate
Working stock standard pyridoxine phosphate
Working stock standard pyridoxamine phosphate

To make individual working standards 200 ng/ml PL, PN, PM, PLP, PNP, and PMP, volumetrically measure 2 ml appropriate working stock standard into a 100 ml volumetric flask. Bring to volume with distilled, ion-free water. Store in amber bottles at 5°C. Working standards are stable for 1 week.

Stock standards, working stock standards, and working standards can be made up as combined standards or as individual standards.
APPENDIX 4
Age, height, weight, and income levels of subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Height</th>
<th>Weight</th>
<th>Age</th>
<th>Income level*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm</td>
<td>kg</td>
<td>mo</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>157</td>
<td>48.5</td>
<td>147</td>
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<td>103</td>
<td>148</td>
<td>37.8</td>
<td>138</td>
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<td>148</td>
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<td>53.0</td>
<td>141</td>
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<td>33.3</td>
<td>143</td>
<td>3</td>
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<td>50.5</td>
<td>140</td>
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<td>44.7</td>
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<tr>
<td>116</td>
<td>150</td>
<td>45.9</td>
<td>152</td>
<td>3</td>
</tr>
<tr>
<td>134</td>
<td>168</td>
<td>61.3</td>
<td>148</td>
<td>3</td>
</tr>
<tr>
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<td>148</td>
<td>35.5</td>
<td>149</td>
<td>1</td>
</tr>
<tr>
<td>139</td>
<td>156</td>
<td>48.9</td>
<td>146</td>
<td>2</td>
</tr>
</tbody>
</table>

*Income level per capita: 1 ≤ $2700, 2 = $2701-5700, 3 ≥ $5701
APPENDIX 5
Nutrient intakes of subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Kilocalories</th>
<th>Protein</th>
<th>Vitamin B-6</th>
</tr>
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<td>2036</td>
<td>53.0</td>
<td>1.81</td>
</tr>
<tr>
<td>103</td>
<td>1414</td>
<td>49.1</td>
<td>0.97</td>
</tr>
<tr>
<td>104</td>
<td>1845</td>
<td>60.7</td>
<td>1.03 (2.03)*</td>
</tr>
<tr>
<td>108</td>
<td>2190</td>
<td>76.8</td>
<td>1.44</td>
</tr>
<tr>
<td>109</td>
<td>2429</td>
<td>66.4</td>
<td>1.46</td>
</tr>
<tr>
<td>110</td>
<td>2772</td>
<td>93.6</td>
<td>2.40</td>
</tr>
<tr>
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<td>428</td>
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<td>2729</td>
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<tr>
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<td>2031</td>
<td>80.2</td>
<td>1.45</td>
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</table>

*Value in parentheses includes dietary supplement

+Values from 111 did not reflect typical nutrient intakes for the subject and were not included in the dietary evaluations of the subjects as a group.
APPENDIX 6
Nutrient intakes of subjects expressed as % recommended energy intake for kilocalories and %RDA for protein and vitamin B-6

<table>
<thead>
<tr>
<th>Subject</th>
<th>Kilocalories</th>
<th>Protein</th>
<th>Vitamin B-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% recommended energy intake</td>
<td>%RDA</td>
<td>%RDA</td>
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<td>93</td>
<td>115</td>
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<td>107</td>
<td>54</td>
</tr>
<tr>
<td>104</td>
<td>84</td>
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<td>57 (113)*</td>
</tr>
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<td>96</td>
<td>167</td>
<td>80</td>
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<tr>
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<td>81</td>
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<tr>
<td>139</td>
<td>92</td>
<td>174</td>
<td>81</td>
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</table>

*Value in parentheses includes dietary supplement
+Values from 111 did not reflect typical nutrient intakes for the subject and were not included in the dietary evaluations of the subjects as a group
APPENDIX 7
Plasma B-6 vitamin concentrations measured microbiologically and coenzyme stimulation activities of E-ALAT

<table>
<thead>
<tr>
<th>Subject</th>
<th>PL (ng/ml)</th>
<th>PN (ng/ml)</th>
<th>PM (ng/ml)</th>
<th>Total (ng/ml)</th>
<th>Coenzyme stimulation of E-ALAT (%)</th>
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<tr>
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<td>30.5</td>
<td>12.5</td>
<td>50.0</td>
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APPENDIX 8
Distribution of B-6 vitamins in plasma of subjects measured by microbiological assay

<table>
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<tr>
<th>Subject</th>
<th>PL</th>
<th>PN</th>
<th>PM</th>
</tr>
</thead>
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<tr>
<td>101</td>
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</tr>
<tr>
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<td>19.4</td>
<td>48.5</td>
<td>31.7</td>
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<td>60.4</td>
<td>24.8</td>
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The vita has been removed from the scanned document
VITAMIN B-6 STATUS OF A GROUP OF FEMALE ADOLESCENTS

by

Libby Ruth Sutker

(ABSTRACT)

The vitamin B-6 status of 11 white adolescent females living in the Blacksburg/Roanoke, Virginia area was evaluated by dietary intakes, coenzyme stimulation of erythrocyte alanine aminotransferase (F-ALAT) activities, and plasma total vitamin B-6 levels measured microbiologically. Heights and weights of the group were generally within normal ranges. The vitamin B-6 intakes of the adolescents were $1.47 \pm 0.49$ mg/day (mean $\pm$ SD). Three subjects, or 27.3%, the same subjects classified as inadequate in status by coenzyme stimulation of E-ALAT, were classified as marginal or inadequate in status using $\leq 25$ ng/ml total plasma vitamin B-6 levels as the criterion. The plasma pyridoxal, pyridoxine, pyridoxamine and total B-6 vitamer concentrations of subjects having inadequate E-ALAT values were lower, generally significantly, than those of girls with adequate levels. In the subjects with adequate status, the vitamer found in the largest plasma concentration was pyridoxine. A significant correlation ($r=0.82, p < 0.01$) was observed between total plasma vitamin B-6 levels determined microbiologically and coenzyme stimulation values.