SEPARATION AND QUANTITATION OF THE SEVEN FORMS OF VITAMIN B-6 IN PLASMA AND 4-PYRIDOXIC ACID IN URINE OF ADOLESCENT GIRLS BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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(ABSTRACT)

The vitamin B-6 status of seemingly healthy adolescent girls was determined using several accepted and proposed parameters in an effort to establish guidelines for status evaluation. HPLC-derived plasma B-6 vitamer [pyridoxal phosphate (PLP), pyridoxine phosphate (PNP), pyridoxamine phosphate (PMP), pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM)] and 4-pyridoxic acid (4-PA) concentrations and urinary 4-PA levels of 28 white adolescent females, 12-15 years, having radiomonitored plasma PLP concentrations and coenzyme stimulation of erythrocyte alanine aminotransferase activities indicative of adequate status were determined. Mean daily vitamin B-6 and protein intakes of the subjects were 1.48 mg and 78.3 g, respectively. The ranges for plasma B-6 vitamer and 4-PA concentrations for these subjects which had seemingly adequate vitamin B-6 status were as follows: (nmol/L) PLP, 40.9-122.2; PNP, 0-16.1; PMP, 0-8.1; PL, 0-15.0; PN, 0-21.9; PM, 0-17.8; and 4-PA, 0-55.7. PLP was the predominant plasma B-6 vitamer as well as being the only

vitamer found in plasma of all subjects. Urinary 4-PA concentrations of the girls ranged from 0.11-2.50 µmol/mmol creatinine. The B-6 vitamer values of these white adolescent girls should be of use in the establishment of normal ranges for vitamin B-6 status parameters. HPLC methodologies seem to be advantageous for the rapid and accurate assessment of vitamin B-6 status.

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INTRODUCTION

In 1934 György recognized that vitamin B-6 existed as a distinct entity which prevented skin lesions in the rat. Vitamin B-6 was investigated by several laboratories about the same time. Kuhn and associates (1936) also isolated the "rat acrodynia factor". In 1938 Lepkovsky isolated the vitamin in pure crystalline form, and in the following year, Harris and Folkers (1939) elucidated the structure of vitamin B-6.

Snell and coworkers (1942) demonstrated that vitamin B-6 existed in 3 interconvertible forms: pyridoxine, also known as pyridoxol (PN), pyridoxal (PL), and pyridoxamine (PM), which are metabolically and functionally related. Each of the vitamers has a corresponding phosphorylated (P) form. The structure of the B-6 vitamers are given in Figure 1. The different vitamers must be phosphorylated at the number 5 carbon in order to have coenzyme activity. The major excretory metabolite of vitamin B-6 is 4-pyridoxic acid (4-PA).

Methodologies that have been utilized to determine vitamin B-6 status of humans include enzymatic procedures -- e.g., xanthurenic acid excretion, coenzyme stimulation of aspartate and alanine aminotransferase activities in whole blood or erythrocytes, and plasma PLP concentrations via the radioisotopically monitored $^{14}CO_2$ formation from L-tyrosine-^{14}C. Some researchers have utilized microbiological techniques to determine vitamin B-6 concentrations of plasma.

Pyridoxal (PL)

Pyridoxal 5'-phosphate (PLP)

Pyridoxine (PN)

Pyridoxine 5'-phosphate (PNP)

Pyridoxamine (PM)

Pyridoxamine 5'-phosphate (PMP)

4-Pyridoxic Acid (4-PA)

Fig. 1 Structures of the B-6 vitamers

Urinary 4-PA has been evaluated by both physical and chemical methodologies. Currently most of the vitamin B-6 researchers believe that the radiomonitored PLP method is the most acceptable procedure for the determination of status; however, these researchers also have indicated that more than one parameter should be used in the assessment of B-6 status.

Recent interest has focused on the use of high performance liquid chromatography (HPLC) as a means for determining vitamin B-6 levels in plasma. The physio-chemical and ionic properties of the B-6 vitamers facilitate their assay by HPLC methodologies. The B-6 vitamers have been successfully determined by HPLC techniques in various foods. HPLC methodologies have been extended to include research on biological materials from animals and humans. Proper extraction or hydrolysis procedures are necessary in order to separate the vitameric forms in the sample where vitamin B-6 is bound to protein. Recently Gregory and Kirk (1979) developed a relatively simple method for measuring 4-PA in urine by HPLC which may be useful as a means of determining vitamin B-6 status.

Although several studies have evaluated the vitamin B-6 status of various populations of both males and females, these have been conducted using various assessment methodologies, all of which have their limitations. Reynolds (1983) reported the results from an interlaboratory comparison study of vitamin B-6 methods which included

microbiological, radiometric, and HPLC for quantitating total vitamin B-6 or the various individual forms. There were large differences in the concentrations of the B-6 vitamers and 4-PA measured in pooled plasma samples as assessed by the various techniques. The enzymatic assay for plasma PLP which was analyzed in 6 labs yielded more uniform results than the other methods. Researchers are still seeking the best method for the assessment of vitamin B-6 status.

Few studies have examined the vitamin B-6 status of female adolescents (Kirksey et al., 1978; Sutker et al., 1985; Driskell et al., 1985; Driskell and Moak, 1986; and Driskell et al., 1987). These girls 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. Research is needed in order to establish "norms" for the evaluation of vitamin B-6 status of adolescent females using accepted as well as improved status indicators. A reproducible, standardized method by HPLC would be quite useful in determining vitamin B-6 status and the requirements of the vitamin in the body for normal function.

Therefore, the objectives of this research were:

1. To develop a sophisticated, reproducible, standardized HPLC method capable of separation and quantitation in nanogram ranges of B-6 vitamers and 4-PA in plasma.

2. To use the developed methodology to analyze the B-6 vitamers and 4-PA concentrations in the plasma of adolescent females, ages from 12 to 15 years, in an effort to establish "normal" values for this age group.
3. To determine 4-PA concentration in the urine of the subjects in an effort to establish "normal" values for urinary 4-PA for these girls.
4. To compare relationships of dietary intakes of the subjects to their radioisotopically monitored PLP concentrations as well as HPLC derived concentrations of the B-6 vitamers and 4-PA in plasma and 4-PA values in urine.

REVIEW OF LITERATURE

The interest in human vitamin B-6 nutrition continues and vitamin B-6 could easily be designated as the "vitamin" of the decade. The interest is due to the many roles that vitamin B-6 either plays in the body or is thought to play by researchers and the general public as well. Some of the roles of vitamin B-6 that warrant this interest include the ease of producing deficiency symptoms, the many vitamin B-6 dependent enzymes in a wide variety of metabolic pathways, relationship to brain metabolism, the increased need associated with pregnancy and various metabolic abnormalities such as coronary heart disease and asthma, losses in food processing, and the antagonistic effects of some drugs on the vitamin.

Methodology for the determination of vitamin B-6 status

Different methods exist for assessing vitamin B-6 status in man. These are mostly enzymatic, microbiological, chemical, or physical in nature. However, the "ideal" method for analyzing vitamin B-6 status has not been established.

Enzymatic methods

The tryptophan load test was one of the earlier determinants of vitamin B-6 status. Coursin (1964) provided recommendations for the standardization of the tryptophan

load test. After the ingestion of a test load of 2-5 g L-tryptophan, a vitamin B-6 deficiency was exhibited in subjects in that they had increased urinary excretions of several tryptophan metabolites which include 3-hydroxy-kynurenine, kynurenine, kynurenic acid, and xanthurenic acid. Xanthurenic acid was the major metabolite measured; investigators have quantitated some of the other metabolites (Hamfelt, 1967; Haskell, 1978).

Another method to determine vitamin B-6 status has been the coenzyme stimulation of aspartate and alanine aminotransferases as demonstrated by Cheney et al. (1967). Erythrocytes have higher aminotransferase levels than plasma and are used in the following reactions: L-Aspartate + 2 - oxoglutarate > oxaloacetate + L-glutamate involving the PLP dependent enzyme aspartate aminotransferase (EC 2.6.1.1., L-Aspartate: 2-oxoglutarate aminotransferase), EASAT, and L-Alanine + 2-oxoglutarate > pyruvate + L-glutamate involving the PLP dependent enzyme alanine aminotransferase (EC 2.6.1.2., L-Alanine: 2-oxoglutarate), EALAT (Tonhazy et al., 1950, as modified by Heddle et al., 1963). An indication of endogenous enzyme activity can be measured from the stimulated and basal values known as the activity co-efficient (AC) after extra PLP has been added to the reaction (Raica and Sauberlich, 1964). Erythrocyte aminotransferase activities are reflective of long-term dietary vitamin B-6 intakes; however, problems of erythrocyte turnover times and

relationships of enzymes to other measurements of vitamin B-6 status need to be fully evaluated. Therefore, this measurement cannot be a primary indicator of vitamin B-6 status.

Radiometric assays are used to determine the PLP concentrations in plasma. The most common one which seems to be preferred, at present, as a status indicator by most vitamin B-6 researchers is the assay of Chabner and Livington (1970) that monitors the radioactive $^{14}CO_2$ formed from the decarboxylation reaction of L-tyrosine-^{14}C->tyramine + $^{14}CO_2$ involving the PLP dependent enzyme tyrosine decarboxylase (EC 4.1.1.25, L-Tyrosine carboxy-lyase). The rate of formation of radioactive CO_2 is proportional to the vitamin B-6 activity of the sample. This enzymatic assay appears to be very reliable and at present, the most widely-used assay for the determination of PLP. The major disadvantage of this procedure is the fact that it measures only PLP and none of the other vitamers. There are conditions such as pregnancy, oral contraceptive use, coronary heart disease, and asthma during which PLP levels are reported to be lower (Reynolds and Leklem, 1985). However, it could be that the individual may still have an adequate status by having a greater concentration of vitamin B-6 in another form.

Microbiological method

Microbiological assays that have been used to determine the vitamin B-6 content in biological tissues are described by Horwitz (1975). *Saccharomyces uvarum* (ATCC 9080) has been one of the most widely used test organisms in the microbiological assay which gives growth responses to the specific forms of vitamin B-6. The vitamin B-6 standards concentrations are compared to the growth of the organism from turbidity responses. However, microbiological methodologies have the disadvantages of a lengthy procedure times and variability in growth responses.

Urinary 4-Pyridoxic acid

Chemical and physical methods have been used to determine 4-PA, the major excretory metabolite of vitamin B-6. Reddy (1958) developed a fluorometric method and more recently, Gregory and Kirk (1979) used HPLC techniques for the measurement of 4-PA in human urine. The measurement of the excretory metabolite in urine is more related to recent intakes of vitamin B-6. However, because of the significant interrelationships between urinary 4-PA, urinary vitamin B-6, plasma PLP levels, and vitamin B-6 intakes, individual blood and urinary measurements may be useful reflections of subjects' recent vitamin B-6 intakes and vitamin B-6 status. Shultz and Leklem (1981) reported upper and lower limits for

these parameter values which can be used in determining vitamin B-6 status of adult males and females.

Chromatography

Gas

Another method that has been used to quantitate vitamin B-6 has been gas chromatography. Relatively few successful applications of gas chromatography have been reported in the analysis of vitamin B-6 in food and biological materials. Because of their lack of volatility, vitamin B-6 polar groups have to be derivatized in order to utilize this methodology.

HPLC

In the last few years, HPLC has been widely employed in vitamin B-6 analysis because of the potential for high resolution and high sensitivity. Good quantitative analysis by HPLC methods require sufficient extraction of the B-6 vitamers because of the complex matrix and low concentrations of the vitamin in most foods and biological materials. Researchers have generally used anion or cation exchange, reverse phase, or paired ion reverse phase modes as a means to separate B-6 vitamers by HPLC. The natural chemical characteristics of the B-6 vitamers facilitate their assay by HPLC. Ionic silica and styrene divinybenzene polymeric packings have been used in columns for ion exchange separation of vitamin B-6 compounds. In reverse phase HPLC, sample compo-

nents are separated according to their affinity for a non-polar bonded stationary phase such as octadecylsilane and a polar mobile phase such as MeOH, H_2O, and acetonitrile where pH, ionic strength, and the percentages of the organic modifier may be controlled (Horvath et al., 1977). Paired ion reverse phase is a variation of reverse phase HPLC where an ionic surfactant such as heptane or octanesulfonic acid is present in a polar mobile phase in which the pH and proportion of the organic modifier are also controlled.

Anion exchange

Vanderslice et al. (1979, 1981, 1983) used Aminex A-25 anion exchange resin in conjunction with a glycine buffer at pH 10 to resolve the B-6 vitamers. Separation was accomplished by automated switching between 2 columns maintained at temperatures of 18° and 50° C. Fluorometric detection at various excitation and emission wavelengths was required in order to achieve the separation of the B-6 vitamers at this pH. This anionic method was successfully applied to the determination of vitamin B-6 in milk, animal tissues, meats, and fortified cereals. Because of chemical changes in the resin by the manufacturers, the Aminex A-25 column no longer provides the necessary chromatographic properties for separation of B-6 vitamers.

Cation exchange

HPLC separations by cation exchange HPLC have been reported (Williams and Cole, 1975; Yasumoto et al., 1975; Williams, 1979; Coburn and Mahuren, 1983). The separation of the nonphosphorylated forms of vitamin B-6 was reported by Williams and Cole (1975) utilizing Aminex A-5 resin. However, the methodology was not successfully applied to the analysis of foods and other biological materials. Yasumoto et al. (1975) also, using a Aminex A-5 cation exchange resin and postcolumn derivatization separated the nonphosphorylated B-6 vitamers in rice bran.

Other cationic systems include the development of a method by Coburn and Mahuren (1983) who used a silica gel based column. They included a post column reagent composed of 1.0 M potassium phosphate, pH 7.5 with 1 mg/mL sodium bisulfite. The postcolumn reagent neutralized the column effluent and enhanced the fluorescence of PLP. Coburn et al. (1983) successfully separated all the vitamin B-6 compounds in animal tissues including blood, plasma, and milk.

Reverse phase

Gregory and Kirk (1978) reported the separation of nonphosphorylated vitamin B-6 compounds on octadecylsilica columns. The mobile phase was 0.033 M potassium phosphate, pH 2.3, delivered on a μBondapak C_{18} column. Gregory and Kirk (1978) used fluorometric detection which enhanced the

specificity of the method which already provided good separation of the B-6 vitamers in cereals. Lim et al. (1980) reported a similar reverse phase separation; however, they used UV detection only, which was sufficient for the levels found in foods, but may not provide sufficient sensitivity for biological materials with vitamin B-6 levels in the nanogram ranges. Yoshida et al. (1978) and Morita and Mizuno (1984) employed reverse phase HPLC with fluorometric detection in the analysis of vitamin B-6 in blood; but the sensitivity of the methodologies was not sufficient for quantitation of the B-6 vitamers as reported by Gregory (1988).

Gregory et al. (1981) demonstrated that TCA added to sample extracts along with the acidic mobile phase, aided the separation of the phosphorylated and nonphosphorylated forms of vitamin B-6 by reverse phase HPLC. They used this methodology in addition to pre or postcolumn derivatization with semicarbazide. Hamaker et al. (1985) employed a TCA enhanced separation in a isocratic method for fluorometric quantitation of all vitamin B-6 compounds in milk extracts using reverse phase HPLC.

Several investigators have reported separation of nonphosphorylated vitamin B-6 compounds using alkylsulfonates as ion pairing agents (Trifiates and Sattsangi, 1982; Morrison and Driskell, 1985; Hefferan et al., 1986; and Pierotti et al., 1987). Morita and Mizuno (1984) separated PNP, PLP, PMP, and 4-PA using cetyltrimethyl ammonium bromide as the

ion pairing agent. The methods of Morrison and Driskell, 1985, Hefferan et al., 1986, and Pierotti et al., 1987, were successful in the determination of the nonphosphorylated forms of vitamin B-6 when milk, plasma, and rat tissues had been treated with potato acid phosphatase.

Tryfiates and Sattsangi (1982) developed a paired ion separation using octanesulfonic and heptanesulfonate and a step wise gradient to elute PLP, PNP, PMP, PL, PN, PM, and 4-PA. They used UV detection in the application of the method to separate the B-6 vitamers in rat tumors. Gregory and Feldstein (1985) modified the procedure of Trifiates et al. (1982) but utilized a phosphate bisulfite postcolumn reagent similar to the method of Coburn and Mahuren (1983) and also used sulfosalicylic acid (SSA) and anion exchange chromatography for sample preparation which was similar to the method of Vanderslice et al. (1980). Gregory and Feldstein (1985) successfully applied this methodology to the analysis of vitamin B-6 in milk, animal tissues, fruits, and vegetables; the method was later adapted to the measurement of pyridoxine-B-glucoside in foods of plant origin (Gregory and Ink, 1987). Separation of the vitamers were accomplished via an C_{18}column (Perkin-Elmer, 3.0x3.0 cm, 3-µm particle size, 4.6 mm i.d. x 3 cm). Newly obtained Perkin-Elmer 3.0 x 3.0 cm column did not sufficiently retain PLP; therefore, another separation was developed using Ultrex-5 C_{18}ion pair column (5-µm particle size, 4.6 mm i.d. x 25 cm; Phenemenex) which

provided better resolution going from 100% binary mobile phase of 0.033 M potassium phosphate, with 8 mM octanesulfonate, pH 2.2, to 100% of 0.033 M potassium phosphate with 17.5% (v/v) 2-propanol, pH 2.2 in 20 min at 1.7 mL/min. (Trumbo and Gregory, unpublished). Hollins and Henderson (1986) recently reported a similar procedure for the determination of B-6 vitamers in plasma.

Although HPLC has the potential for high resolution and high sensitivity in separation of B-6 vitamers; accuracy and precision of quanitative analysis are improved through the use of an internal standard. The internal standard should have chemical and chromatographic properties similar to that of the B-6 vitamers; however, it must not occur naturally in the matrix being analyzed. Various compounds have been reported to be suitable for use as an internal standard in HPLC for quantitation of vitamin B-6.

Internal standard

Laboratory groups of Gregory and Driskell have routinely used 4-deoxypyridoxine (DPN) as the internal standard in paired ion reverse phase HPLC. Coburn and Mahuren (1983) selected 2-amino-5-chlorobenzoic acid and Vanderslice and Maire (1980) employed 3-hydroxypyridine as internal standards in their cation exchange and anion exchange HPLC systems, respectively. Ubbink et al. (1985) use 6 methyl-2-pyridine carboxaldehyde in their reverse phase

semicarbazide. Shephard et al. (1987) employed 4-deoxypyridoxine 5'-phosphate (DPNP) as an internal standard in cation exchange HPLC; these researchers wanted to quantitate PLP, in particular; thus, they used the phosphorylated form of DPN.

Obviously, much vitamin B-6 research has been in the area of methodology. Recent advances in HPLC methodology have permitted the analysis of vitamin B-6 concentrations in foods and other biological materials with greater accuracy and precision. Researchers still have not established the "best method" for the determination of vitamin B-6 status in humans. The newly developed HPLC methodology needs to be applied to more human studies in order to determine if it is an appropriate method for the assessment of nutritional status of individuals in various population groups. Hence, status criteria for the B-6 vitamers have not been established by the HPLC methodology.

Vitamin B-6 status parameters

Presently, EALAT, EASAT-ACs and/or PLP concentrations have been utilized for the evaluation of vitamin B-6 status. Criteria have been adapted from the research of several investigators (Rose et al., 1976; Kirksey et al., 1978; Schultz and Leklem, 1981; Driskell and Moak, 1986; and Hunt et al., 1987). Rose et al. (1976) evaluated vitamin B-6 status of 414 adult males using EASAT-ACs values and plasma PLP levels. These researchers suggested that plasma values <8.5 ng/mL

(<34.4 mmol/L) are indicative of inadequacy based on percentages of subjects determined to have inadequacy when coenzyme stimulation of EASAT values were evaluated. Kirksey et al. (1978) evaluated the vitamin B-6 status of 127 females between the ages of 12 and 14 years by nutrient intakes calculated from 24-h dietary recalls, measurement of EALAT-ACs, and vitamin B-6 levels in erythrocytes. The daily intake of the vitamin was 1.24 mg ± 0.70 ($\bar{x} \pm$ SD) which represented 79% of the 1974 Recommended Dietary Allowance (RDA). Using >16% stimulation of the EALAT as the cutoff, 31% of the girls showed some inadequacy.

Schultz and Leklem (1981) proposed guidelines for the evaluation of marginal B-6 status for males and females. They correlated plasma PLP values with dietary vitamin B-6 intakes of 1.25 and 1.5 mg vitamin B-6 per day. The researchers suggested that plasma PLP concentration for males (9.2 to 10.2 ng/ML; <37.3 to 41.3 nmol/L) and females (<7.8 to 8.8 ng/mL; <31.6 to 35.6 nmol/L) to be indicative of marginal vitamin B-6 status. Driskell and Moak (1986) reported plasma PLP levels (8.5 to 10.0 ng/mL; 34.4 to 40.5 nmol/L) were suggestive of marginal vitamin B-6 status. These researchers determined the vitamin B-6 status of 96 white and 90 black female adolescents utilizing PLP concentrations, coenzyme stimulation of EALAT activities, and vitamin B-6 intakes. The mean coenzyme stimulation and PLP values of the subjects were 13.5% and 45.2 nmol/L. The esti-

mated daily vitamin B-6 intake of the 162 girls who did not take supplements was 1.25±0.04 mg ($\overline{x}$±SEM). The proposed guidelines for adequate and marginal status for plasma PLP value from these researchers (Rose et al., 1976; Schultz and Leklem, 1981; and Driskell and Moak, 1986) appear to be in more or less agreement. Hunt et al. (1987) reported finding even lower plasma PLP values in pregnant women in Mexico (4.05 ± 2.6 ng/mL; 16.6 ± 10.5 nmol/L, $\overline{x}$ ± SD); they suggested that plasma PLP values less than 19 nmol/L were unacceptable and represented a deficient status.

Since guidelines have been established for vitamin B-6 status in particular by EALAT assays, and have been suggested for plasma PLP concentration, criteria for status need to be extended to B-6 vitamer concentrations as determined by other methodologies such as HPLC for various populations. Adolescent females constitute a population that needs further research with regard to their nutrient needs. These girls are recognized as being at a high nutritional risk. Few studies have been published regarding the vitamin B-6 status and intakes of adolescent girls.

Vitamin B-6 status of adolescent females

Ritchey and Feeley (1966) reported that approximately 1.25 mg vitamin B-6 intake per day appeared to be adequate for preadolescent girls 7-9 years. Kirksey et al. (1978)

determined the vitamin B-6 status of 127 females between the ages of 12 and 14 y by coenzyme stimulation of EALAT activities and microbiologically derived vitamin B-6 levels in erythrocytes. Based on EALAT stimulation, 31% of the girls showed some inadequacy. Sutker et al. (1984) compared methodologies for assessing vitamin B-6 nutriture of 11 adolescent females who were 12 y of age. Three of the 11 girls had inadequate B-6 status as determined by EALAT-AC, plasma PLP, and microbiologically determined plasma PL, PM and total B-6 levels. The reported B-6 intake was 1.48 ± 0.56 ($\bar{x} \pm SD$) for the adequate and 1.43 ± 0.39 ($\bar{x} \pm SD$) for the inadequate group. The vitamin B-6 status of southern adolescent girls in a large, longitudinal regional project were determined by Driskell et al. (1985) using the parameter EALAT-AC; the vitamin B-6 status of 583 white and black adolescent girls living in Alabama, Arkansas, North Carolina, Oklahoma, and Virginia was assessed. The mean estimated daily vitamin B-6 intake of the girls from food sources was 1.20 mg. About half of the subjects reported consuming <66% of the 1980RDA for vitamin B-6. Approximately 20% of the girls had marginal (> 16 to 25%) vitamin B-6 status and 13% deficient status (>25%) as indicated by coenzyme stimulation values. Driskell and Moak (1986) assessed the vitamin B-6 status of 96 white and 90 black female adolescents utilizing plasma PLP concentrations, EALAT stimulations, and vitamin B-6 intakes. The mean EALAT stimulation and plasma PLP values of the subjects

were 13.5% and 45.2 nmol/L. Vitamin B-6 inadequacy was prevalent among these girls. EALAT stimulation values >25% were observed in 18% of the girls and values >16 and 25%, in 23%, suggestive of deficient and marginal vitamin B-6 status. Plasma PLP concentrations <34.4 nmol/L were observed in 26% of the girls and values from 34.4 to 40.5 nmol/L, in 14%. The method that was common to all the status determination studies was the EALAT stimulation. Driskell and coworkers (1987) evaluated the vitamin B-6 status of 62 black and 50 white adolescent girls living in Virginia and Alabama who had previously been assessed 2 y before when participating in a regional project. The mean daily vitamin B-6 intake of the girls from food was 1.25 mg. The vitamin B-6 status of 31% of the girls improved from y 1 to y 3; 71% of those girls increased their vitamin B-6 intake. Likewise, the status of 33% of the girls declined, in which case, their vitamin B-6 intake decreased from y 1 to y 3.

Reynolds and Leklem (1981) indicated that the coenzyme stimulation of the erythrocyte aminotransferases were reflective of longterm dietary vitamin B-6 intake; the problem of erythrocyte turnover time and the relationship of enzyme activity to other measures of vitamin B-6 status remains to be fully evaluated. Thus, EALAT and EASAT-ACs should not serve as primary indicator of B-6 status. At present, the most sensitive, widely used assay for PLP concentration is the radiomonitored method utilizing apotyrosine

decarboxylase. The major shortcoming with this method is that it measures only PLP and none of the other vitamins. PLP levels are also reported to be lower in pregnancy, coronary heart disease, asthma, and other metabolic disorders (Reynolds and Leklem, 1987). Knowledge as to the concentrations of the other B-6 vitamers are becoming more important in assessing status of individuals in healthy as well as diseased states.

Therefore, a reproducible, standardized method by HPLC would be quite useful in determining vitamin B-6 status and requirements for adolescent females. Guidelines for the evaluation of status are also needed. The plasma B-6 vitamers and plasma and urinary 4-PA concentrations of adolescent girls were determined in an effort to establish "normal" values for this age group.

MATERIALS AND METHODS

EXPERIMENT I

Development of HPLC method to quantitate plasma B-6 vitamers and 4-PA levels

The method developed in our laboratory (Hefferan et al., 1986; Morrison and Driskell, 1985; and Pierotti et al., 1984) for separation and quantitation of the B-6 vitamers in their nonphosphorylated forms found in various tissues of rats including plasma was modified for use in separating and quantitating the phosphorylated and nonphosphorylated B-6 vitamers and 4-PA found in human plasma. The various procedures utilized in accomplishing this task are detailed in the next few pages. The chemicals and vendors utilized in the HPLC analyses are listed in Appendix A.

Analytical system

A Waters Associates HPLC system (Milford, MA, USA) was available to facilitate the chromatographic separation which consisted of the following components:

1. Model 730 data module
2. Model 720 system controller
3. Two model 45 solvent delivery systems and one Milton Roy pump.
4. Model U6K universal injector
5. Temperature control system (TCS)

6. Model 440 UV absorbance detector equipped with:
   - A. Mercury lamp
   - B. 254 and 280 wavelength filters
7. Model 420E/420AC fluorescence detection equipped with:
   - A. Mercury lamp
   - B. Excitation filter
   - C. Emission filter
   - D. Wavelength kit which includes a range of filters from 280-530
8. Guard-Pak precolumn module equipped with:
   μBondapak C_{18} insert
9. μBondapak C_{18} column (3.9 mm x 30 cm, 10 μm porous packing, octadecylsilane)

Basic resolution equation

Chromatography theory is given, along with the attempted techniques for resolution of the B-6 vitamins, in the next several pages. Chromatographic resolution is related to several factors that are expressed in the following equation (McNair, 1984; Yost et al., 1980):

$$R = \left(\frac{K'}{1+K'}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\sqrt{N}}{4}\right)$$

capacity factor | selectivity factor | column efficiency factor

Regardless of the type of sample that a chromatographer is trying to resolve, one will be able to increase resolution by optimizing each term of the equation. Therefore, several

steps were performed to accomplish this goal.

1. Optimize K' or the capacity factor

   Adjust solvent polarity to produce

   A. K' = (capacity factor) = t'_R/t_o

   B. K' = 2 - 10

2. Maximize

   (selectivity) = $t'_{R(2)}/t'_{R(1)}$; $\alpha > 1.2$ is good

   A. Change solvents; if necessary from MeOH to acetonitrile

   B. Optimize packing; reactivate

   C. Change packing

3. Maximize

   (theoretical plates) = $16 \left(\frac{t_R}{W}\right)^2$

   A. Slower flow rate

   B. Longer column

   C. New column

   D. Smaller particle size

Reverse phase mode

Keeping in mind that the basic rule that "like dissolves like", the proper mode may be selected. Since the molecular weights of the compounds of interest (B-6 vitamers) were less than 2000, water-soluble, ionic and nonionic; reverse phase partition chromatography was selected.

Mobile phase

Since a reverse phase, nonpolar column (μBondapak C_{18}) was employed, a polar mobile phase was utilized. In liquid chromatography the composition of the solvent or mobile phase is one of the variables influencing the separation. Desirable properties which are common to mobile phases are (McNair, 1985; Yost et al., 1980):

A. Be pure, chromatography grade

B. Not react with the packing

C. Be compatible with the detector

D. Dissolve the sample, and

E. Have a low viscosity

In order to optimize α or the selectivity factor, several solvents were tried in order to acquire the most desirable mobile phase. Acetonitrile, Isopropanol, MeOH, and H_2O were tried. Isopropanol has a high viscosity and a low polarity index. Acetonitrile has a slightly lower viscosity than MeOH but has a lower polarity index. Thus, MeOH and H_2O combined well for a polar solvent. In addition several concentrations of this solvent were tried in order to optimize K' or the capacity factor. Concentrations such as the ratios 20:80; 40:60; 70:30, 80:20 and 85:15 v/v MeOH/H_2O were attempted in order to acquire the desired K' equal 2-10. The ratio MeOH/H_2O of 85:15 v/v was the best one and, hence, was utilized as solvent A in the mobile phase. Haptane sulfonic acid (PIC B-7) was used as a pair ion (PIC) reagent in the

mobile phase to facilitate resolution. Various pHs of the mobile phase were tried in order to optimize resolution. In addition, a Milton Roy postcolumn pump delivered a bisulfite phosphate buffer in attempt to raise the pH of the effluent before flowing through the detector. However, this solvent did not stay in solution and the pH of the effluent could not be controlled. Octanesulfonic acid (PIC B-8) was added to PIC B-7 to make a 50:50 solution; this combination seemed to help to control the pH of the system. Therefore, a binary mobile phase which consisted of a mixture of MeOH and H_2O (85:15 v/v; solvent A) and a combination of the 2 paired ion reagents PIC B-7 and B-8 (0.005 M heptane sulfonic acid, 0.005 M octane sulfonic acid and 1% acetic acid; solvent B) were delivered at a flow rate of 1.0 mL/min at ambient temperature. Several flow rates were tried; however, the flow rate of 1.0 mL/min was the best rate in order to retain the phosphorylated B-6 vitamers on the column. In addition, the pressure of the pumps was maintained at a desirable range which was compatible with the Waters HPLC system. The elution order of sample components are related to the increasing hydrophobic nature of the solute; the more soluble the solute is in water, the faster it will elute.

PIC B-7 and B-8 were used in the mobile phase to suppress ionization of the sample components. The ion suppression brought about by PIC B-7 and B-8 was a very advantageous process in which the pH of the system remained

acidic and the sample behaved as a neutral species. This facilitated the reverse phase system by expressing the resolution of the sample by its polarity. Another advantage in using PIC reagent is that it eliminates buffers and salt interferences that are common in cationic systems. The mobile phase solvents were degassed by vacuum filtration through a 0.3 mm glass fiber filter (Gelman, Ann Arbor, MI).

TCS

Various temperatures were tried in order to maximize the selectivity factor. Increasing the temperature of the column helps to reactivate the packing. It was found that maintaining a constant temperature of 37° C provided stable operating conditions of the columns and insulates the column from changes in ambient temperature. Another advantage of adding the TCS to the Waters HPLC system is that the heated column helped to keep PIC B-7 & 8 from precipitating as the mobile phase passes through the column. These constant conditions promoted a better means of reproducibility in resolution of samples.

Detectors

A fluorescence photometer as well as a UV detector were used in series in the HPLC system with the fluorescence cell being first. Fluorescence detection has been reported to have greater selectivity for the B-6 vitamers. Successful detection with fluorescence in a variety of sample matrices

have been reported by several investigators (Coburn and Mahuren, 1983; Gregory and Kirk, 1978; Driskell et al., 1985; Hefferan et al., 1986; Morrison and Driskell, 1985; Pierotti et al., 1987).

The B-6 vitamers have similar maximum excitation and emission values when buffered at pH 3.0 (Vanderslice et al., 1981). PLP and PNP peaks as well as 4-PA and PMP peaks eluted very quickly and close to each other. Confirmation and quantitation of these peaks can be made by the utilization of the UV detector as well as the fluorescence detector.

Various combination of wavelengths were used in order to detect all the forms of vitamin B-6. These included: (nm) 280, 300, 338, 360 and 365 excitation filters; 375, 380, 395, 420, 425, 455, 495, and 530 emission filters. However, due to the acidic conditions of the solvent system, a compromise was made in the choice of wavelength in order to detect all the vitamers. The wavelength which provided the best detection of the vitamers were 254 nm (UV), and 300/375 nm, excitation and emission filters, respectively (fluorometry).

Standards

Stock standard solutions of PLP, PNP, PMP, PL, PN, DPN, PM, and 4-PA were made on the basis of free base weight at a concentration of 1 mg/mL. PNP was synthesized by the method of Peterson and Sober (1953) and Tryfiates et al. (1982). The purity of the synthesis was determined by nuclear

magnetic resonance (NMR, IBM WP-200, Dansberry, Conn.), and is represented in Figure 2. Serial dilutions were performed to obtain working standard solutions. Instructions for making standards are given in Appendix B.

Internal standard

Quantitation was determined by the internal standard method. DPN (Figure 3) was the compound chosen because it was found to meet the necessary requirements of an internal standard which are (McNair, 1985; Yost et al., 1980):

A. Be well resolved from other peaks

B. Elute close to the peaks of interest

C. Have structural and behavioral similarities to the peaks of interest

D. Be stable, also unreactive with the sample column packing or the mobile phase

E. Not be found in the sample, and

F. Be available in high purity.

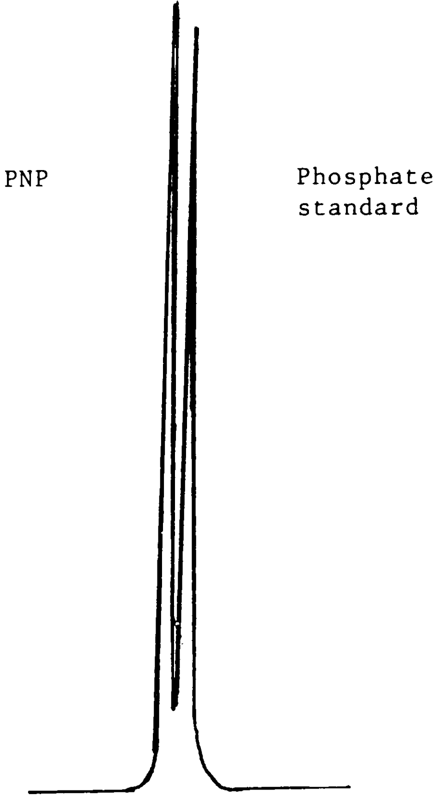

Fig. 2 Purity of synthesized PNP by NMR

CH_3

HO

CH_2OH

H_3C

N

Fig. 3 Structure of deoxypyridoxine (DPN)

Gradient

The best gradient program for this methodology is presented in Table 1. The gradient consisted of both MeOH and PIC B-7 and 8; the mobile phase was delivered linearly, convex, and concave, fashions at certain timed intervals. Most of the resolutions of standards were completed in 25 min and an additional 25 min was allowed for reequilibration before another injection. To ensure ample time for reequilibration, a blank injection was periodically performed (injection of H_2O or the mobile phase).

Separation of the standards

Separation of the standards was accomplished by the injection of the individual as well as the combined standards into the HPLC system using a gradient program. One mL of the combined standards contained 200 ng of each of the vitamers. This concentration resulted in the resolution of the vitamers and DPN as depicted in Appendix C. The injection volume was from 100 to 250 µL.

Injection volume

The injection volume was from 100-250 µL of the standards. A 250 µL injection placed a rather large volume on the column which created a column overload which was evidenced by band spreading and poor peak shape. The better

Table 1

Gradient elution conditions

Time min	Solvent A* %	Solvent B* %	Curve type[t]
0	0	100	
5	25	75	9
8	75	25	9
12	40	60	3
16	0	100	6
20	0	100	6

*Composition given in text.

[t]Waters Assoc. (1980) 9, convex; 3, concave; 6, linear.

volume was 100 μl which resulted in better proportioned and sharper peaks. The injections were delivered with a 100 or 250 μl. Hamilton syringes equipped with a blunt needle which was compatible with the U6K injector.

After satisfactory separation of the B-6 standards and internal standard (Appendix C) using a gradient elution program, peak identities were confirmed by standard addition (spiking) as well as by extrachromatographic spectrofluorometry on collected HPLC eluates. Peaks of interest were verified by mass spectrophotometer HPLC (LC Mass Spec, 770E, VG Analytical, Manchester, UK) for nonphosphorylated vitamers and phosphorylated vitamers by NMR.

Response factors

Response factors were calculated to accommodate any different responses by the detector to the varying fluorogenic properties of the pyridine ring substitute groups which make up the B-6 vitamers. The response factor formula as recommended by Waters Associates (1981) is presented.

$$\text{Response Factor} = \frac{\text{Amount of standard}}{\text{area response}} \times 1000$$

The response factor can be calculated from a single calibration standard. Single point calibrations are advantageous in that frequent recalibrations can be facilitated for any changing chromatographic conditions and detector response to the standards.

Linearity

Upper and lower limits of linearity were determined to be 1 to 50 ng, respectively. The lower limit of detector response was determined to be 2 times the background noise for quantitation of each vitamer, 4-PA, and the internal standard. The minimum detectable quantities (MDQ) are defined as being the quantities equivalent to detector responses twice the noise level (McNair, 1985; Synder and Kirkland, 1974; and Yost, 1980)

Precision

Precision measures reproducibility and depends on technique. After multiple injections, the coefficients of variation (Sokal et al., 1969), of the calibrating standards were determined to be better than 5% for all vitamers.

Plasma sample extraction

Plasma from adult volunteers was used to determine the methodology for sample extraction that resulted in the separation of the 6 vitameric forms of vitamin B-6 and 4-PA. These plasma samples were also used in the development of the chromatographic method.

The main objective of the sample extraction procedure is to solubilize the eluate for subsequent quantitation. Since vitamin B-6 exists in several forms, the extraction

method should release uniformly each vitamer. At the same time, the conditions of the extraction should not cause undesirable chemical or enzymatic reactions in the B-6 compounds.

Vitamin B-6 can be extracted by thermal or chemical methods. Protein can be denatured by elevated temperature and/or acidic conditions. Thermal conditions cause the hydrolysis of the phosphate esters. Consequently, this is not a feasible method in order to quantitate all forms of vitamin B-6. Other protein denaturants tried in this laboratory included perchloric acid (PCA), trichloroacetic acid (TCA), and sulfosalicylic acid (SSA). These procedures utilizing various acids are more ideal for methods that quantitate the phosphorylated, nonphosphorylated, and glycosylated forms of vitamin B-6.

Although SSA effectively deproteinates the sample, it is highly fluorescent. Perchloric acid also is an effective means of deproteinization and may be precipitated as a potassium salt by neutralization with KOH. However, it is not compatible with a reverse phase system when utilizing pair ion buffers. Therefore, TCA seems to be the best agent for denaturing the B-6 vitamers. It is compatible with the reverse phase chromatography and may be removed from the extract with diethyl ether. This laboratory uses freon amine already in the extraction method for radiomonitored PLP determination; thus, it also was utilized to serve the same

function in the HPLC plasma extraction method instead of diethyl either.

Not only was it necessary to get the sample "clean" enough for use in the HPLC, it was necessary to concentrate sufficient volume of the sample, in order to detect B-6 vitamers from individuals having inadequate status of vitamin B-6. Also, it would be advantageous to resolve the minimum detectable quantities of all the B-6 vitamers.

Freeze drying was first attempted to concentrate the samples. However, it was then, more difficult to "clean" the sample up because everything in the sample had also been concentrated. Drying the sample to dryness after the extraction methodology worked the best. Argon was tried. However, argon resulted in a big solvent front being present at the beginning of the chromatogram. Nitrogen worked the best as an inert gas for drying the sample. In addition, nitrogen created a much smaller solvent front on the chromatogram.

Perfected plasma extraction method

DPN, 5 ng, was added to 2 mL of human plasma (fasting) along with 0.2 mL 50% TCA; the mixture was incubated at 50° C for 15 minutes and cooled to 5°C. An equal volume of methylene chloride was added to the mixture followed by centrifugation at 7,000 x g for 20 min at 5 ^{o}C. The supernatant was removed and an equal volume of freon amine was added,

followed by recentrifugation, removal of the supernatant, and freon amine treatment again, with recentrifugation. The supernatant (at $50^{o}C$) was dried using nitrogen followed by reconstitution with 1 mL of solvent B; the sample was then adjusted to pH 2.9 and filtered through a 0.2 μm Acrodisc (Gelman) and then through a C_{18} Sep-pak (Waters Associates). The sample filtrate (100-250 μL) was then injected into the HPLC system. A representative chromatogram of the B-6 vitamers and 4-PA in a human plasma extract is depicted in Appendix C. Peak identities were confirmed as described for the B-6 vitamer standards.

Plasma samples were spiked before extraction with known quantities of each of the B-6 vitamers and 4-PA. The recoveries of the vitamers (Table 2) ranged from 88-97%. The plasma samples were also treated with potato acid phosphatase (orthophophoric-monoesterphosphohydrolase; EC 3.1.3.2) to convert the phosphorylated forms to the nonphosphorylated forms of the vitamin; 94 to 99% of the phosphorylated forms were recovered in this conversion as nonphosphorylated B-6 vitamers.

B-6 vitamins and 4-PA concentrations of plasma from freshly drawn blood and plasma which had been frozen for as long as 20 mo were compared. The concentrations of all B-6 vitamins and 4-PA in the frozen samples (Table 3) were within 5% of the values found in freshly drawn samples from these subjects. Therefore, freezing did not appear to affect the

Table 2

Recovery of B-6 vitamers from plasma

Vitamer	Recoveries* %
PLP	95.2
PNP	97.1
4-PA	94.9
PMP	88.0
PL	94.6
PN	93.5
PM	90.6

*Plasma was spiked with 10 ng of each vitamer/mL plasma; values represent recoveries from 3 samples.

Table 3

B-6 vitamer and 4-PA values from frozen plasma of adult females

Parameter	Subject # 1	2	3 nmol/L	4	5
RPLP*	194.2	60.7	59.1	49.8	99.1
FPLP[t]	178.2	64.7	61.9	42.9	90.6
UPLP[Ŧ]	202.3	73.6	64.7	60.7	93.1
PNP[t]	34.1	8.0	16.1	24.1	24.1
PMP[t]	16.9	nd[£]	nd	31.8	16.9
PL[t]	nd	nd	nd	nd	nd
PN[t]	208.9	17.7	35.5	98.2	20.7
PM[t]	27.3	34.9	87.3	nd	28.0

* Radiomonitored PLP.

[t] Fluorometric detector.

[Ŧ] UV detector.

[£] nd, nondectable.

separation and quantitation of the B-6 vitamer and 4-PA in human plasma.

B-6 vitamers and 4-PA analysis of plasma from males

Plasma samples were available for 22 young men (20 to 37 y of age, in good health, nonsmokers, and within normal weight ranges) who were subjects in a metabolic study. These men had been fed diets analyzed to contain 80.8-84.5 g of protein and 0.75-0.98 mg vitamin B-6 daily for 8 wk. The perfected HPLC method was utilized to determine the plasma B-6 vitamin and 4-PA levels of these men. A copy of this published study is in Appendix C.

EXPERIMENT II

Plasma B-6 vitamers and 4-PA and urinary 4-PA concentrations of healthy adolescent females

Subject recruitment procedures

Twenty-eight white adolescent females were recruited as subjects from Southwest Virginia following approval of the study by the Institutional Review Board (IRB) for Research Involving Human Subjects. Permission was obtained from the superintendent of Pulaski County Schools and the principals of 2 middle schools to place flyers (Appendix D) in their respective schools which described the study. Flyers were also placed in strategic locations such as churches and the

YMCA. An announcement was also published in the local newspaper, The Southwest Times (Appendix E).

The girls were within 6 mo of being 12, 14, or 16 y of age. The subjects and their parents were given a written explanation (Appendix F) of the study and asked to sign a consent of participation form (Appendix G). The subjects and their parents reported that the girls were in good health. Demographic characteristics of the subjects were obtained via use of 2 questionnaires (Appendix H and I) similar to that described in the Ten State Nutrition Survey (Christakis, 1973). The girls reported that they all had good appetites; also that their eating habits during the 3-d food record period were similar to what they had practiced throughout their lives. Most of them always ate in the cafeterias at schools. They frequently ate at restaurants, particularly at fast food establishments. Each female received a T-shirt (Appendix J) designed especially for the nutrition study for her participation in the research project. In addition the girls were informed via a letter of their B-6 status and any cases of anemia as determined from hemoglobin (Hb) and hematocrit (Hct) values.

Blood and urine collections

Approximately 15 mL of blood from the fasting subjects was obtained in vacutainers containing EDTA by a Registered

Medical Technologist between 7:30 and 10 a.m. The samples were kept in crushed ice and protected from light. Blood samples were centrifuged at 3000 x g and 5° C for 10 min using a refrigerator rotor. Plasma was frozen at -20° C for future radiomonitored plasma PLP and HPLC analysis. Erythrocytes were treated as described by Heddle et al. (1963) and frozen at -20° C for EALAT analysis.

Premeasured specimen cups were given to the girls to acquire a freshly voided urine sample. The specimen was then transferred into a brown bottle which contained 1 mL of toluene which was used as a preservative. Toluene had been shown earlier not to affect the results obtained using the developed HPLC methodology. The bottles of urine were frozen at -20° C for future 4-PA and creatinine analysis.

Dietary intake procedures

Food consumption records were obtained from the subjects. A 24 h recall (Appendix K) was obtained using the interview technique; food models and crosschecking were used. Consecutive 2-d food records (Appendices L and M) were obtained; thus 3 consecutive days of dietary records were available. The forms used in obtaining the dietary data were fashioned after those described in Nutritional Assessment in Health Programs (1973). The reported kilocalorie, protein, and vitamin B-6 intakes of the subjects over the consecutive days were estimated using handbook values. The estimated

values were compared with the 1980RDAs for the various age groups. Information concerning use of nutrient supplementation was obtained from the girls (Appendix H).

EALAT, plasma PLP, B-6 vitamers and 4-PA determinations

Plasma PLP concentrations were measured (Appendix N) enzymatically by stimulation of tyrosine decarboxylase apoenzyme, using the method of Chabner and Livingston (1970) and of Reinken (1972) as modified by Fries et al. (1981). Recoveries of 92-94% were obtained when plasma samples were spiked with PLP before analyses. EALAT activities were determined (Appendix O) according to the method of Heddle et al. (1963). The procedure of Raica and Sauberlich (1964) was used in measuring the coenzyme stimulation of EALAT activities. The B-6 vitamers and 4-PA levels were determined by the developed HPLC method (Chrisley, et al. 1988).

Urinary 4-PA and creatinine analyses

Urinary 4-PA analysis (Appendix P) were performed using the developed methodology and the extraction method of Gregory and Kirk (1978). The urinary creatinine was a modification of the Jaffe method utilizing a Stanbio Kit from Fisher Products (Appendix Q).

Statistical analyses

All data were evaluated by general linear models procedures (Sokal et al. (1969). Means ($\overline{x}$) and standard errors of the means (SEM) were calculated. An F test was run on data from the 24-h vs the 2-d food records. There were no significant differences found between the 24-h and 2-d intakes; thus, these records were combined. Also, after running a F test on the various values for girls which were premenarcheal vs postmenarcheal; no significant differences were found. These groups were combined. Pearson r correlation coefficients were determined between data obtained by the various assay parameters.

RESULTS AND DISCUSSION

Twenty eight white girls volunteered as subjects. These included 15-12 y; 3-13 y; 4-14 y; and 6-15 y olds.

Anthropometric measurements

The height and weight values of the girls in the 4 age groups are presented in Table 4. The 12 y olds were significantly shorter ($p < 0.05$) and lighter ($p < 0.05$) in weight than the 14 and 15 y olds. The height and weight measurements of the girls were significantly correlated with age ($r = 0.56$, $p < 0.002$; $r = 0.058$, $p < 0.001$, respectively) for the girls. Individual height and weight values for the subjects are given in Appendix R. The values were similar as reported by the National Center for Health Statistics survey (1977) and those of a survey conducted in the South involving adolescent girls (Driskell et al., 1981).

Demographic measurements

The girls were equally divided into 2 per capita income (PCI) groups (Table 5) which consisted of a medium (<$8605 annually) and a high group (> $8605 annually). Seventeen of the girls were classified as being in the medium PCI group and 11 girls were classified as the high PCI group. Individual PCI values for the girls are presented in Appendices S, along with the educational levels of the girls' parents. Parents of 22 of the 28 girls (Table 6) reported having at

Table 4

Ages, heights, and weights of adolescent girls

Age y	n	Height cm	Weight kg
12	15	157.4±1.2*t	49.3±2.1t
13	3	161.7±3.4	49.1±0.9
14	4	166.4±1.6	57.4±2.8
15	6	164.0±2.6	59.7±1.0

* Values represent $\overline{X}$ ± SEM.

t Significantly lower than values of 14 and 15 y girls, $p<0.05$.

Table 5

Per capita income levels of subjects

Per capita income* group	n	Per capita income $
Medium	17	5,911.8 ± 233.4[t]
High	11	12,768.2 ± 1016.8

*Per capita income groups: annually,

< $8,605 (medium) and > $8,605 (high).

[t]Values represent $\overline{X}$ ± SEM.

Table 6

Per capita income based on parents' education

Education*	n	Per capita income $
High school	6	6,203.3 ± 796.0[t]
College	22	9,238.6 ± 909.7

*Education groups: high school, 12 y; college, 13+ y.

[t]Values represent $\overline{X}$ ± SEM.

least 1 y of college education which may have contributed to the higher PCI. However, there were no significant differences between the 2 income groups for any of the parameters measured.

Seven of the 15-12y had experienced menarche. A t-test revealed no significant differences between the values for the 12 y olds who had experienced menarche and those who had not; thus, the 12 y girls were grouped together; all other girls were postmenarcheal. Only 1 of the subjects reported taking supplements; however, there was no significant difference between the data for this subject and the other subjects. The mean heights and weights of these girls were similar to those reported for the 50th percentile for girls of similar ages in the National Center for Health Statistics (1977).

Hemoglobin and hematocrit measurement

Hemoglobin (Hb) and hematocrit (Hct) levels of the girls were determined. Although these parameters were not pertinent to this study, the information was reported to the girls as an additional incentive get to them to participate in the study. Hb and hct values are presented in Table 7. Using levels recommended by the Health and Nutrition Examination Survey, 1971, to 1972 (Lowenstein et al., 1974) for classifying individuals into "low" categories (Hb < 115 g/L; hct < 0.36), all of these girls exhibited acceptable levels; hence, these girls were deemed to be generally

Table 7

Hemoglobin and hematocrit measurement of adolescent girls

Parameter	n	Values
Hemoglobin* (g/L)	28	136 ± 1.80ǂ
Hct (ratio)t	28	0.39 ± 0.01

* Hemoglobin expressed as g/L.

t Hematocrit expressed as ratio.

ǂ Values represent $\overline{X}$ ± SEM.

healthy. Individual values for Hb and hcts are listed in Appendix T.

Estimated kilocalorie, protein and vitamin B-6 intakes

Dietary intakes obtained from the 24-h recall were not significantly different from those obtained by the 2-d food records; therefore, these values were averaged and expressed on a daily basis. The estimated kilocalorie (kcal), protein, and vitamin B-6 intakes and vitamin B-6/protein ratios are given in Table 8. The individual values for the same are presented in Appendix U. There were no significant differences for kcal, protein, and vitamin B-6 intakes and the ratio of vitamin B-6 to protein intake for the girls of the different age groups.

Energy allowances for children through 18 y are based on medium energy intakes of children of these ages followed in longitudinal growth studies. According to the 1980RDA, females ages 11-14 require 2200 kcal with a range from 1500-3000 kcal (Food and Nutrition Board, 1980). Likewise, females, ages 15-18 y require 2100 kcal with a range from 1200-3000kcal. All the girls in this study had intakes which fell within these ranges. Kirksey et al. (1978) reported that 127 females, 11-14 y consumed 1921 ± 639 kcal, $\bar{x} \pm$ SD. Sutker et al. (1984) reported that the energy intakes of 11 white, 12 y girls was 2042 ± 614, $\bar{x} \pm$ SD, classified in having "adequate" status.

Table 8

Estimated daily kilocalorie, protein, and vitamin B-6 intakes and B-6 protein ratios of adolescent girls

Age y	n	Kcal	Protein g	Vitamin B-6 mg	Vitamin B-6 protein ratio
12-14	22	2094.3±175.4*	77.3±6.8	1.43±0.24	0.036±0.018
15	6	1984.3±200.2	71.8±6.9	1.10±0.12	0.017±0.002

* Values represent $\bar{X}$ ± SEM.

The 1980RDA for protein for girls 11 to 18 y is 46 g (Food and Nutrition Board, 1980). All the girls had protein intakes greater than the 1980RDA. Likewise, as in the present study (Table 8), investigators (Kirksey et al., 1978; Sutker et al, 1984; Driskell et al., 1987; and Driskell and Moak, 1986) reported intakes of protein of their subjects which were greater than the 1980RDA. Kirksey et al. (1978) reported the protein intake of 127 females was 71 $\pm$ 25 $\bar{x} \pm$ SD. Sutker et al (1984) reported that 8-12 y adolescent females consumed 77.9 $\pm$ 30.9 g $\bar{X} \pm$ SD, of protein. Driskell et al. (1987) found that 112 girls consumed 64.9 $\pm$ 30 g, $\bar{X} \pm$ SEM; 2 y later, the girls consumed 73.0 $\pm$ 3.0g, $\bar{X} \pm$ SEM, for protein. Driskell and Moak (1986) reported similar ranges for protein intakes for white and black adolescent females, 66.8 $\pm$ 4.7 and 68.1 $\pm$ 4.0 g, $\bar{X} \pm$ SEM, respectively.

The estimated vitamin B-6 intakes of the girls in the current study (Table 5) were similar to published values. Kirksey et al. (1978) reported the daily vitamin B-6 intakes of 127 girls between the ages of 12 and 14 y living in Indiana to be 1.24 $\pm$ 0.70 mg, $\bar{X} \pm$ SD. Sutker et al. (1984) reported the vitamin B-6 intakes of 11 girls, 12 y, living in Virginia to be 1.48 $\pm$ 0.56 mg, $\bar{X} \pm$ SD. The estimated daily vitamin B-6 intakes for 583 white and black adolescent girls ages 12-16 y in the southern region was reported by Driskell et al. (1985) to be 1.19 $\pm$ 0.03 mg, $\bar{X} \pm$ SEM. Driskell et al. (1987) reported the vitamin B-6 intakes for 62 black and 50

white adolescent girls living in Virginia and Alabama from 1981 and 1983 to be 1.25 ± 0.07 mg and 1.27± 0.06 mg, $\bar{X} \pm$ SEM, respectively. Driskell and Moak (1976) reported the vitamin B-6 intakes of 162 white and black girls in Virginia, who did not take supplements to be 1.25 ± 0.04 mg, $\bar{X} \pm$ SEM. The vitamin B-6 intakes for 3 consecutive days for 12 to 14 y and 15 to 18 y girls in the Nationwide Food Consumption Survey (Spring, 1977) were 1.31 ± 0.52 mg, $\bar{x} \pm$ SD, and 1.27 ± 0.58, respectively.

The 1980RDA for vitamin B-6 is 1.8 mg daily for 11 to 14 y and 2.0 mg for those 15 to 18 y (Food and Nutrition Board, 1980). Table 9 gives the percent of girls from the 12-14 y and 15 y age groups who reported consuming $<$ 2/3 1980RDA, $>$ 2/3 $<$ 1980RDA, and $>$ 1980RDA for vitamin B-6. Twenty-three percent of the 12-14 y and 83% of the 15 y girls consumed less than 2/3 1980RDA. Because the vitamin B-6 RDA for the 15 y group was 2.0 mg versus 1.8 mg for the younger girls, most of the 15 y girls consequently fell in the $<$ 2/3 1980RDA group even though their intakes were similar to those of the younger subjects. Kirksey et al. (1978) found that 46% of the 127 girls in their study had intakes of vitamin B-6 $<$ 2/3 1980RDA. Driskell et al. (1985) found similar results in 450 white and black 12-14 y girls in that they also consumed $<$ 2/3 1980RDA. Driskell and Moak (1986) reported of the 162 girls, 12-16 y, 48.9 percent consumed $<$ 2/3 1980RDA.

Table 9

Percentage of subjects consuming <2/3 1980RDA of vitamin B-6

Age y	n	<2/3RDA*	>2/3<RDA %	>RDA
11-14	22	23	50	27
15	6	83	17	0

* 1980RDA for vitaminB-6 for girls 11 to 14 y is 1.8 mg and for girls 15-18 y, 2.0 mg.

Hence, others have found that many of their adolescent female subjects reported consuming < 2/3 1980RDA for the vitamin.

As protein intake increases, the vitamin B-6 requirement has been shown to also increase. A ratio of 0.02 mg of vitamin B-6 per g of protein consumed has been suggested to be adequate in the Dietary Standard for Canada (1975) and has been adopted by the 1980 RDA (Food and Nutrition Board, 1980). Vitamin B-6 protein ratios are given for the girls in Table 8. Sixty one percent of the girls in the present study reported consuming less than 0.02 mg of the vitamin per g protein intake. Driskell et al. (1987) reported that 46% of the 112 girls in 1981 and 57% of the girls in 1983 consumed less than 0.02 mg of the vitamin per g protein. Also, Driskell and Moak (1986) reported that 58% of 186 female adolescents, white and black, consumed less that the suggested ratio for vitamin B-6 intake and protein.

Vitamin B-6 status determinations

Coenzyme stimulation of EALAT activities and plasma PLP concentrations are given in Table 10. Individual values for these parameters are presented in Appendix V. No differences in values obtained for the status parameters were observed between the age groups.

Coenzyme stimulation of EALAT activities >25% are indicative of vitamin B-6 deficiency (Sauberlich, 1972); values > 16% are indicative os some inadequacy of the vitamin

Table 10

Vitamin B-6 status of adolescent girls

Age y	n	EALAT %	RPLP* nmol/L	FPLP[t] nmol/L	UPLP[ŧ] nmol/L
12	15	9.9±1.1[£]	78.9±5.7	78.5±6.1	78.1±6.5
13	3	9.7±2.1	65.5±8.5	69.2±8.1	67.6±6.1
14	4	4.5±3.1	88.6±7.7	85.4±6.9	87.4±6.9
15	6	8.7±1.8	76.5±5.7	75.7±6.1	78.4±6.5

* Radiomonitored PLP.

t HPLC, PLP with fluorometric detection.

ŧ HPLC, PLP with UV detection.

£ Values represent $\bar{X}$ ± SEM.

(Kirksey, 1978). Thirty one percent of the 127 females, 12 14 y, living in northwestern Indiana (Kirksey et al., 1978) showed some inadequacy utilizing the criteria > 16% stimulation as a guideline for inadequate status. Utilizing the criteria of >25% stimulation as an indicator of vitamin B-6 inadequacy, 13% of these girls had inadequate vitamin B-6 status. Driskell et al. (1985) used the following status classification based upon coenzyme stimulation of EALAT values: adequate, < 16%; marginal, 16% to 25%; and deficient > 25%. Overall, 67% of all subjects (n = 583, white and black adolescents) were classified as having adequate status, 20% as marginal, and 13% as deficient. In contrast, all the girls in the present study exhibited adequate vitamin B-6 status in that their percent stimulation of EALAT activities were <16%.

The girls' vitamin B-6 status was also determined by the widely used radiometric assay for PLP concentration, the radiomonitored method (RPLP) utilizing apotyrosine decarboxylase; this method is currently considered to be the most acceptable for status determination (Leklem and Reynolds, 1980). The values obtained using this method were compared with those obtained via the newly developed HPLC method. Plasma PLP levels of the subjects as determined using the radiometric method, RPLP, as well those obtained by HPLC, UV and fluorometric, analyses are given in Table 11. Values for each subject are given in Appendix V. Values for plasma

Table 11

Plasma B-6 vitamer and 4-PA levels and EALAT-AC of adolescent girls

Congener	n*	Concentration[t] nmol/L
RPLP	28	78.4±3.6[‡]
FPLP	28	78.1±3.6
UPLP	28	78.4±3.8
FPNP	10	2.2±0.7
UPNP	5	1.1±0.6
PMP	5	0.8±0.4
PL	3	1.1±0.7
PN	3	1.2±0.8
PM	16	4.2±1.0
4-PA	12	7.8±2.4
		%[§]
EALAT-AC	28	8.8±0.9

* Subjects with quantifiable levels.

t Nondetectable and nonquantifiable levels, calculated as zeros.

‡ Values represent $\bar{X}$ ± SEM.

§ Values represent % stimulation.

PLP concentrations obtained by RPLP and HPLC, both UV and fluorometric, analyses were highly correlated (r = 0.98, p<0.0001, r = 0.96, p<0.0001, respectively); high correlation (r = 0.97, p<0.0001) was also observed between values obtained using the 2 HPLC detectors. Thus, similar data were obtained utilizing all 3 of these techniques.

Several researchers (Rose et al., 1976: Shultz and Leklem, 1981; and Driskell and Moak, 1986) have proposed guidelines for plasma PLP levels indicative of marginal vitamin B-6 status and Hunt et al. (1987) suggested values that were unacceptable or deficient. Rose et al. (1976) evaluated vitamin B-6 status of 414 adult males. These researchers suggested that plasma values <8.5 ng/mL (<34.4 nmol/L) are indicative of inadequacy based upon percentages of the subjects determined to have inadequacy based upon percentages of the subjects determined to have inadequacy by EALAT activities. Shultz and Leklem (1981) proposed guidelines for the evaluation of marginal vitamin B-6 status for adult males and females. They correlated plasma PLP values with dietary intakes. These researchers suggested that plasma PLP concentrations for males were <9.2 to 10.2 ng/mL (<37.3 to 41.3 nmol/L) and females, <7.8 to 8.8 ng/mL (<31.6 to 35.6 nmol/L) to be indicative of marginal vitamin B-6 status. Driskell and Moak (1986) reported that plasma PLP levels of 8.5 to 10.0 ng/mL (34.4 to 40.5 nmol/L) were suggestive of marginal vitamin B-6 status. These researchers

determined the vitamin B-6 status of 96 white and 90 black female adolescents utilizing PLP concentrations, coenzyme stimulation of EALAT activities, and vitamin B-6 intakes. Plasma PLP concentrations <34.4 nmol/L were observed in 26% of the girls and values from 34.4 to 40.5 nmol/L in 14%. Sutker et al. (1984) reported plasma PLP concentrations of 9-12 y adolescent females were 69.6 $\pm$ 15 nMol/L, $\bar{x} \pm$ SD. The mean plasma PLP for the girls in the study was 78.1 $\pm$ 3.6 nmol/L, $\bar{x} \pm$ SEM. Plasma PLP values for all the adolescent females were indicative of an adequate vitamin B-6 status no matter which set of guidelines were utilized.

The HPLC derived PLP concentrations were also in the ranges which were reported by others who used values acquired by HPLC methodologies, Table 12. The PLP values for the girls (Table 11) were somewhat lower than the PLP values for males in a study conducted by Chrisley et al. (1988) utilizing the same HPLC methodology. However, as shown in Table 12, the PLP values of the girls were in the ranges reported by several researchers (Shephard et al., 1987; Hollins et al., 1986; Coburn and Mahuren, 1983; Chauhan and Dakshinamuriti, et al., 1981; and Vanderslice et al., 1981).

Plasma PNP, PMP, PL, PN, PM, and 4-PA measurements

Plasma B-6 vitamer and 4-PA levels of the subjects are given in Table 11; individual levels are listed in Appendix V. Quantifiable peaks were calculated when peak heights were

Table 12

Concentrations of B-6 vitamers in human plasma

nmol/L

Congener	Present	Study	Chrisley et al.	Shepherd et al.	Hollins et al.	Coburn et al.	Lumeng et al.	Lui et al.	Chauhan et al.	Vanderslice et al.
PLP	78.1±3.6	40.9-122.2*†	88.0±03.9*	50.6±25.0‡	61.5±33.6‡	57.0±26.0‡	60.0±10.0‡	29.5±17.0‡	72±11‡	74±35‡
PNP	2.2±0.7	0- 16.1	6.4±01.6	nd£	7.5±01.5‖	nd	nd	nd	nd	nd
PMP	0.8±0.4	0- 8.1	12.1±05.7	0.8±00.8	nd	8.0± 8.0	2.0± 1.0	0.4± 0.4	15± 3	31±10
PL	1.1±0.7	0- 15.0	38.4±05.0	9.0±07.2	5.4±09.0	23.0±10.0	15.0± 5.0	12.6± 4.8	251±51	nd
PN	1.2±0.8	0- 21.9	41.4±07.5	0.6±01.8	nd	29.0±33.0	30.0±10.0	2.4± 1.7	nd	180±51
PM	4.2±1.0	0- 17.8	17.6±02.0	0.6±00.6	nd	2.0± 2.0	8.0+ 3.0	0.6±10.1	164±38	6±8
4-PA	7.8±2.4	0- 55.7	39.3±11.4	29.5±20.7	25.2±06.5	49.0±19.0	40.0± 8.0	57.3±19.7	nd	nd
n	28		22	27	10	38	6	9	27	2
Subjects	Adolescent girls		Adult men	Adults	Adults	Adults	Adults	Adults	Adults	Adults

* Values represent $\overline{X} \pm$ SEM.
† Values represent ranges.
‡ Values represent $\overline{X} \pm$ SD.
£ Nondectable
‖ Value of one individual; nonquantifiable in others.

2 times the noise level. A high correlation ($r = 0.88$, $p<0.0001$) was observed between the subjects' plasma PNP concentrations as measured utilizing the 2 HPLC detectors. Ten of the subjects in the current study had detectable plasma levels utilizing the HPLC-fluorometric detector and 5 of these subjects had detectable levels utilizing the HPLC-UV detector (Table 11). Chrisley et al. (1988) recently reported (Table 12) higher values for plasma PNP in men using the fluorometric and UV HPLC method. Hollins et al. (1987) reported (Table 12) PNP plasma values from a healthy volunteer but not in 10 other adults which was similar to the PNP concentration of men reported by Chrisley et al.(1980). However, the girls in this study exhibited (Table 12) lower plasma PNP concentrations than the adult males in the studies conducted by Chrisley et al. (1988) and Hollins et al. (1987), respectively. Other researchers did not report finding detectable plasma PNP values.

Plasma PMP was detected in 5 of the girls in the current study; the values were similar to those reported by Shepherd et al., 1987; Lumeng et al., 1980; and Lui et al., 1985. The girls exhibited lower plasma PMP values than those reported in adults (Chrisley et al., 1988; Coburn and Mehuren, 1983; Chauhan et al., 1981; and Vanderslice et al., 1981). The mean plasma PL values were lower than any of the reported values. Mean plasma PN concentrations were similar to the PL concentrations. Mean PL and PN values were similar

to those reported by Shepherd et al. (1987) and Lee et al. (1985). The mean plasma PM values detected in the girls were in agreement with reported values of Coburn and Mahuren (1983) and Lumeng et al. (1980). The 4-PA values, detected in 12 girls were lower than the values reported by others for adults (Chrisley et al., 1988; Shepherd et al., 1987; Hollins et al., 1986; Coburn and Mahuren, 1983; Lumeng et al., 1980; Liu et al., 1985; Chauhan et al., 1981; and Vanderslice et al., 1981).

The predominant plasma B-6 vitamer was PLP which constituted approximately 89% of the distribution of the vitamers in these girls. This is in agreement with findings of other researchers (Chrisley et al., 1988; Shepherd et al., 1987; Hollins et al., 1986; Liu et al., 1985; Lumeng et al, 1980; Chauhan et al., 1981; and Vanderslice et al.,1981). Subjects in the current study had practically equal distributions of PL and PN; Coburn and Mahuren (1983) reported finding nearly equal distributions of PL and PN in plasma from 38 subjects although the values were higher.

Urinary 4-PA and creatinine measurements

Urinary 4-PA levels and urinary 4-PA/creatinine ratios are presented in Table 13, along with the dietary intakes and the % of the intake excreted as 4-PA of the girls; individual values are given in Appendix W. Shultz and Leklem (1981) suggested guidelines for evaluating the vitamin B-6 status

Table 13

Dietary vitamin B-6 intakes, urinary 4-PA excretion values, 4-PA/creatinine ratio and % intake excreted.

Study	n	Vitamin B-6 intake µmol	4-PA excreted µmol	4-PA creatinine ratio µmol/mmol/L	Intake excreted %
Present study	28	8.80±0.73*	5.35±0.78[†]	0.76±0.11	56.8
Schuster et al.	19	10.97±6.12[‡]	7.86±3.78[†]	0.89±4.90	69.2
Schuster et al.	19	9.19±3.05[‡]	6.98±3.99[†]	0.78±4.20	76.0
Schuster et al.	19	8.33±2.52[‡]	6.35±2.18[†]	0.72±0.25	76.2
Schuster et al.	19	10.97±6.12[‡]	9.53±4.32[£]	1.08±0.49	86.9
Schultz and Leklem	41	9.50±3.0[‡]	5.57±3.0[£]	0.63±0.34	59.0
Donald et al.	8	8.9	3.40[£ ‖]	--	28.0

* Values represent $\bar{X}$ ± SEM.
† Values from random urine sample from adolescent girls.
‡ Values represent $\bar{X}$ ± SD; urine sample from adults.
£ Values represent 24-h sample.
‖ Mean calculated from data in figure.

for women and suggested that 4-PA value < 4.6-5.2 µmol/24-h would be indicative of marginal B-6 status. All of the girls in this study exhibited urinary 4-PA values indicative of adequate vitamin B-6 status utilizing these guidelines. Donald et al. (1971) found that repletion of 8 young women with 8.9 µmol pyndoxine satisfied their vitamin B-6 requirement; the girls in the study had a vitamin B-6 intake of 8.8 µmol/pyridoxine. Random morning urine samples seem to be indicative of 24-h excretion and urinary data can be expressed per g of creatinine (Driskell, 1984). Schuster et al. (1984) compared random voided samples to 24-h urine samples (Table 13) of the same subjects to evaluate the validity of determining the 4-PA/creatinine ratio in random urine samples as an alternative to total 24-h 4-PA excretion in assessing vitamin B-6 nutritional status. No differences in 4-PA/creatinine between the 24-h samples and either morning or afternoon random samples taken the next day were found. Thus, the use of the 4-PA/creatinine ratio in random urine samples appear to support an alternative to 24-h urinary 4-PA excretion. Sauberlich et al. (1972) suggested that excretion of <20 µg 4-PA per g of creatinine is indicative of marginal vitamin B-6 intakes. All of the girls exhibited values > 20 µg 4-PA per g of creatinine. The normal range for creatinine is 0.8 - 2.0 g/24-h (Stanbio Laboratory). Eighty two percent of the girls in this study had creatinine values within this range. It is reported that

20-50% of the ingested vitamin B-6 is converted to 4-PA. These girls excreted 56.8% of the vitamin B-6 consumed as 4-PA which is in agreement with the study by Shultz and Leklem (Table 13) in that females excreted 59% of their intake of vitamin B-6 as 4-PA. The adults in the study of Schuster et al. (1984) excreted somewhat higher % of their vitamin B-6 intake as 4-PA which included a range from 69.2 to 86.9. The young women in the study of Donald et al. (1971) excreted approximately 30% of the 8.9 μmol of pyridoxine intakes as 4-PA when repleted.

It would appear that all of the 28 adolescent females who volunteered as subjects in this study exhibited adequate vitamin B-6 status as indicated by accepted as well as what is known regarding the proposed parameters. The girls had radiomonitored plasma PLP levels and EALAT-AC activities indicative of adequate status. The ranges for plasma B-6 vitamer and 4-PA concentrations for these subject which had seemingly adequate vitamin B-6 status were as follows: (nmol/L) PLP, 40.9-122.2; PNP, 0-16.1; PMP 0-8.1; PL, 0-8.1; PL, 0-15.0; PN, 0-21.9; PM, 0-17.8; and 4-PA, 0-55.7. Urinary 4-PA concentrations of the girls ranged from 0.11-2.50 μmol/mmol creatinine. The B-6 vitamer values of these white adolescent girls should be of use in the establishment of normal ranges for vitamin B-6 status parameters.

SUMMARY AND CONCLUSIONS

A sophisticated, reproducible, and standardized HPLC method was developed which was capable of separation and quantitation in the nanogram ranges of B-6 vitamers and 4-PA in plasma. Also, the developed methodology was used to analyze the B-6 vitamers and 4-PA concentrations in the plasma of 28 adolescent females, ages 12 to 15 y. This methodology was also used to determine urinary 4-PA values of the subjects.

The mean heights and weights of the girls were similar to those reported for the 50th percentile for girls of similar ages.

Seventeen of the girls were in the medium PCI group (<$8605) and 11 of the girls were in the high PCI group (>$8605). However, there were no significant differences in parameter values of the 2 income groups. All of the girls exhibited acceptable ranges for Hb and Hct values.

There were no significant differences for kcal, protein, and vitamin B-6 intakes and the ratio of vitamin B-6 to protein intakes for the girls of different age groups. The mean vitamin B-6 and protein intakes of the subjects were 1.48 mg and 78.3 g. Twentythree percent of the 12-14 y and 83% of the 15 y girls reported consuming < 2/3 of the 1980RDA for vitamin B-6.

Because the vitamin B-6 RDA for 15 y was 2.0 mg versus 1.8 mg for the younger girls, most of the 15 y girls

consequently fell in the <2/3 1980 RDA group even though their intakes were similar to those of the younger subjects. The girls had radiomonitored plasma PLP and coenzyme stimulation of EALAT-AC activities indicative of adequate status; hence, these girls were deemed to be generally healthy.

The B-6 status values of the girls as determined from the accepted parameters were compared to the developed HPLC methodology. The ranges for the plasma B-6 vitamer and 4-PA concentrations were as follows: (nmol/L) PLP, 40.9-122.2; PNP, 0-16.1; PMP, 0-8.1; PL, 0-15.0; PN, 0-21.9; PM, 0-17.8; and 4-PA, 0-55.7. There was high correlation between the radiomonitored PLP and the HPLC derived PLP, both fluorometric and UV values ($r = 0.98$, $p<0.0001$; $r = 0.96$, $p<0.0001$ respectively). PLP was the predominant plasma B-6 vitamer as well as being the only vitamer found in plasma of all subjects. Urinary 4-PA concentration of the girls ranged from 0.11-2.50 μmol/mmol creatinine. The plasma B-6 vitamer and urinary 4-PA values of these white adolescent females should be useful in the establishment of "normal" ranges for vitamin B-6 status parameters for this age group. Also, the developed HPLC methodology for the determination of the B-6 vitamers in plasma and 4-PA in plasma and urine should be advantageous for the assessment of vitamin B-6 status.

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APPENDIX A

Vendors of Chemicals for HPLC Methods

1. Fisher Scientific Co., Fairlawn, NJ
   - 0.2 μm gelman acrodisc filters, 09 730 218
   - 0.3 μm type A/E glass fibers, 48 mm 0973078
   - HPLC grade water W-5
   - HPLC grade methanol A-452
   - HPLC grade methylene chloride D-143
   - HPLC grade iso-propyl alcohol A451-1
   - HPLC grade acetonitrile A998-1

2. J. T. Baker Products, Phillipsburg, NJ
   - HPLC grade acetic acid 9515-3

3. Sigma Chemical Co., St. Louis, MO
   - Potato acid phosphatase 2 u/mg type IV-S acid phosphatase P1146
   - Pyridoxal hydrochloride P9130
   - Pyridoxine hydrochloride P9755
   - Pyridoxamine hydrochloride P9380
   - Pyridoxal 5'-phosphate P9255
   - Pyridoxamine 5'-phosphate P9505
   - 4-Pyridoxic Acid P9630
   - 4-Deoxypyridoxine P0501

4. Waters Associates, Inc., Milford, MA
   - PIC B-7 85103
   - PIC B-8 85142
   - μBondapak C_{18} 27324
   - Sep-Pak C_{18} 51910

APPENDIX B

Preparation Instructions for Standards

B-6 vitamers and DPN

1. Stock standard 1,000,000 ng/mL

   a. Weight out into separate 100 mL volumetric flask:

	9
PL	0.1218
PN	0.1216
PM	0.1434
PLP	0.1478
PNP	0.1457
PMP	0.1283
DPN	0.1238

   b. 4-PA
   Weigh out 10 mg of 4-PA into 10 mL flask
   Add 20 µL of 1-N NaoH and bring to volume
   10 mL with HPLC grade water

2. Serial dilution - working standard 200 ng/mL
   PLP, PNP, PMP, PL,PN, PM and DPN

   a. Volumetrically measure 0.5 mL of appropriate stock standard into 100mL volumetric flask. Bring to volume with HPLC grade water. Makes 5000 ng/mL solution.

   b. Volumetrically measure 4 mL of appropriate 5000 ng/mL solution into 100 mL volumetric flask. Bring to volume with PIC B-7 and B-8. Makes 200 ng/mL working standard.

   c. Serial dilution 4-PA working standard 50 ng/mL Volumetrically measure 0.5 mL of stock standard into 100 mL volumetric flask. Bring to volume with HPLC grade water. Makes 5,000 ng/mL solution.

   d. Volumetrically meassure 1 mL of working standard into 100 mL volumetric flask and make to volume with PIC B7 and 8. Makes 50 ng/mL solution.

All solutions are stored in amber bottles at 5° C. Stock standards and working standards were made as individual standards or combined standards.

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PLASMA B_6 VITAMER AND 4-PYRIDOXIC ACID CONCENTRATIONS OF MEN FED CONTROLLED DIETS

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SUMMARY

A rapid and sensitive procedure is described for the analysis of all the B_6 vitamers and 4-pyridoxic acid in human plasma utilizing reversed-phase high-performance liquid chromatography with ultraviolet and fluorometric detection. Pyridoxal phosphate values obtained by radiometric and chromatographic, ultraviolet and fluorometric, assays were highly correlated as were pyridoxine phosphate values determined using both detectors. Plasma B_6 vitamer and 4-pyridoxic acid concentrations of 22 men fed diets containing 0.75–0.98 mg of vitamin B_6 daily for eight weeks were in the range of reported values; pyridoxal phosphate was their predominant plasma B_6 vitamer. This methodology should be useful in the assessment of vitamin B_6 requirements.

INTRODUCTION

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). 4-Pyridoxic acid (4-PA) is the major excretory catabolite. Microbiological assays, enzymatic assays and open-column cation-exchange (OCC) chromatography are some of the methods developed for the analysis of vitamin B_6 in human plasma. However, a recent inter-laboratory

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comparison study revealed large differences in the concentrations of B_6 vitamers and 4-PA measured by these techniques in a pooled plasma sample [1]. The physicochemical properties of the B_6 vitamers facilitate their assay by high-performance liquid chromatographic (HPLC) methodologies. The B_6 vitamer content of plasma from humans has been quantitated by HPLC techniques [2–10]. It would be quite advantageous to be able to detect nanogram quantities of each metabolite, thus providing a better understanding of the metabolism of vitamin B_6. Coburn and Mahuren [6] utilized a cation-exchange HPLC procedure for detecting nanogram quantities of all seven common vitamin B_6 metabolites in a variety of biological samples. Lui et al. [7] compared the cation-exchange and the OCC HPLC methods and the L-tyrosine apodecarboxylase (LTD; L-tyrosine carboxy-lyase; EC 4.1.1.25) assay in which case the coefficients of correlations for two of the three methods measuring plasma PLP were greater than 0.93 and between the two chromatographic methods quantifying PL and 4-PA, 0.82 and 0.63, respectively. In the present study a reversed-phase HPLC method utilizing UV and fluorometric detectors has been developed to detect nanogram quantities of the phosphorylated and non-phosphorylated forms of vitamin B_6 and its excretory catabolite 4-PA in plasma from young men having radiomonitored plasma PLP levels indicative of adequate vitamin B_6 status.

EXPERIMENTAL

Analytical instrumentation

The chromatographic separation was accomplished using a Waters Assoc. (Milford, MA, U.S.A.) HPLC system which consisted of the following components: a Model 730 data module, a Model 720 system controller, two Model 45 solvent delivery systems, a Model U6K universal injector, a column temperature control system, a Model 440 UV absorbance (254 nm) and a Model 420 E/AC fluorescence detector equipped with a mercury lamp (300-nm excitation and 375-nm emission filters). The analytical column was a μBondapak octadecylsilane (ODS) column (30 cm×3.9 mm I.D., 10 μm porous packing, Waters Assoc.) preceded by a guard column packed with μBondapak ODS.

TABLE I

GRADIENT-ELUTION CONDITIONS

Time (min)	Solvent A* (%)	Solvent B* (%)	Curve type**
0	0	100	Convex
5	25	75	Convex
8	75	25	Concave
12	40	60	Linear
16	0	100	Linear
20	0	100	

*Composition given in text.
**Ref. 11.

Mobile phase

The mobile phase for gradient elution consisted of methanol–water (85:15, v/v; solvent A) and a combination of the two paired-ion reagents PIC B-7 and B-8 (0.005 *M* heptane sulfonic acid and octane sulfonic acid in 1% acetic acid; solvent B). The flow-rate was 1.0 ml/min at ambient temperature. The mobile phase solvents were degassed by vacuum filtration through a 0.3-mm glass fiber filter (Gelman Science, Ann Arbor, MI, U.S.A.). The programmed gradient-elution conditions are given in Table I.

Reagents

The following reagents were obtained from Sigma (St. Louis, MO, U.S.A.): pyridoxal hydrochloride, pyridoxal 5′-phosphate, pyridoxamine dihydrochloride, pyridoxamine 5′-phosphate, pyridoxamine hydrochloride, 4-pyridoxic acid, 4-deoxypyridoxine (DPN) and potato acid phosphatase. Pyridoxine 5′-phosphate was synthesized according to the method of Peterson and Sober [12]. The identity of PNP was confirmed by nuclear magnetic resonance (NMR) as well as by HPLC.

Resolution of standards

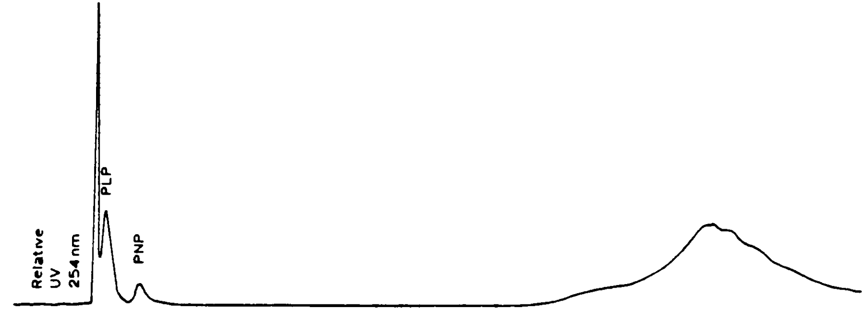
After resolution of the individual standards (PLP, PNP, PMP, PL, PN, PM, 4-PA and the internal standard, DPN), a 100-μl aliquot of combined standards (containing 5–200 ng/ml) was injected onto the column which was maintained at 37°C. Peak identities were confirmed by retention times, comparison of the ratio of retention times of the vitamers to that of the internal standard, by spiking with standards, as well as off-line chromatographic spectrofluorometry on collected HPLC eluates. Using the gradient elution program, these standards were satisfactorily separated in 25–35 min; a representative chromatogram (standards) is given in Fig. 1. Minimum detectable quantities were 2–5 ng; calibration curves were linear to 50 ng. Theoretical plates (N) were approximately 3000, even after column use of over a year; whereas the α values ranged from 1.1 to 2.6. Likewise, the K values were 0.2, 0.3, 0.8, 1.0, 3.0, 3.0 and 4.0.

Blood sampling

Approximately 15 ml of blood was obtained between 7:00 and 8:30 a.m. in vacutainers containing EDTA from each fasting subject by a Registered Medical Technologist. The samples were kept in crushed ice and protected from light. Blood samples were centrifuged in a refrigerated rotor at 3000 *g* and 5°C for 10 min. Plasma samples were then frozen at −20°C.

Extraction of B_6 vitamers and 4-PA from human plasma

DPN (5 ng) was added to 2 ml of human plasma (fasting) along with 0.2 ml of 50% trichloroacetic acid; the mixture was incubated at 50°C for 15 min and cooled to 5°C. An equal volume of methylene chloride was added to the mixture followed by centrifugation at 7000 *g* for 20 min at 5°C. The supernatant was removed and an equal volume of freon amine was added, followed by recentrifugation, removal of the supernatant and freon amine treatment again, with recentrifugation. The



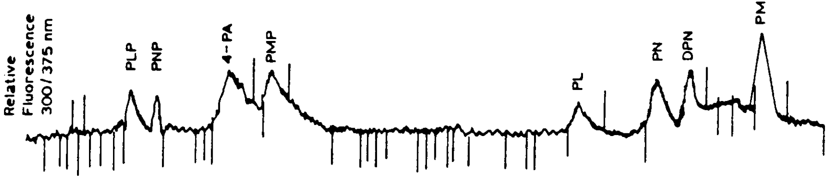


Fig. 1. Representative chromatogram of B_6 vitamers and 4-PA in combined standard solution containing 5 ng of each. The retention times were as follows: 1.0, 1.6, 4.2, 5.4, 14.0, 16.4, 17.3 and 19.4 min.

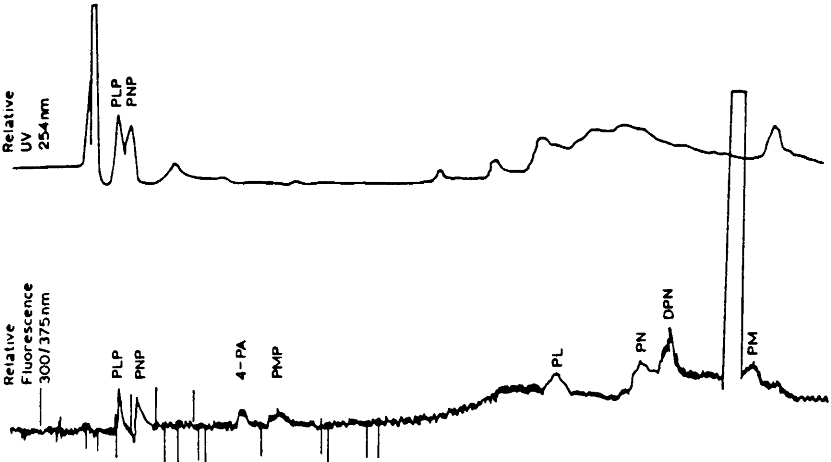


Fig. 2. Representative chromatogram of B_6 vitamers and 4-PA in human plasma. The retention times were as follows: 1.0, 1.6, 4.4, 5.4, 13.8, 16.2, 17.2 and 19.5 min.

supernatant (at 50°C) was dried using nitrogen followed by reconstitution with 1 ml of solvent B; the sample was then adjusted to pH 2.9 and filtered through a 0.2-μm Acrodisc (Gelman) and then through a C_{18} Sep-Pak (Waters Assoc.). The sample filtrate (100–250 μl) was then injected into the HPLC system. A representative chromatogram of the B_6 vitamers and 4-PA in a human plasma

TABLE II

RECOVERY OF B_6 VITAMERS FROM PLASMA

Plasma was spiked with 10 ng of each vitamer per ml plasma ($n = 3$).

Vitamer	Recovery (%)
PLP	95.2
PNP	97.1
4-PA	94.9
PMP	88.0
PL	94.6
PN	93.5
PM	90.6

extract is depicted in Fig. 2. Peak identities were confirmed as described for the B_6 standards.

Plasma samples were spiked before extraction with known quantities of each of the B_6 vitamers and 4-PA. The recoveries of the vitamers (Table II) ranged from 88 to 97%. The data were not corrected for percent recoveries. The coefficients of variation for B_6 vitamers and 4-PA concentrations of plasma samples that were extracted and analyzed on different days were around 5%. The plasma samples were also treated with potato acid phosphatase (orthophosphoric-monoester phosphohydrolase; EC 3.1.3.2) to convert the phosphorylated forms to the non-phosphorylated forms of the vitamin; 94–99% of the phosphorylated forms were recovered in this conversion as non-phosphorylated B_6 vitamers. B_6 vitamer and 4-PA concentrations of plasma from freshly drawn blood and plasma which had been frozen for as long as twenty months were compared using samples from three females. The concentrations of all B_6 vitamers and 4-PA in the frozen samples were within 5% of the values found in freshly drawn samples from these subjects. Thus, freezing does not appear to affect the separation and quantitation of the B_6 vitamers and 4-PA in human plasma.

B_6 Vitamer and 4-PA analyses of plasma from subjects

The subjects included adult males who were white, black or of other races (Ecuador, Sri Lanka, China, Philippines, Taiwan, South Korea and Bangladesh). The 22 subjects, 20–37 years of age, were in good health, non-smokers and within normal weight ranges. The subjects were on controlled diets for eight weeks at which time blood was collected; plasma was frozen at $-20°C$ for future analyses. The mean daily B_6 content of these diets as analyzed using *Saccharomyces uvarum* was 0.75–0.98 mg; the mean protein content as analyzed by the Kjeldahl method were 80.8–84.5 g. Plasma PLP concentrations of the subjects were measured radioenzymatically. Subjects, diets and the radioenzymatic assay have been described previously in detail [13]. The B_6 vitamer and 4-PA levels present in plasma from these subjects were quantitated using the developed HPLC methodology.

Statistical analysis

All data were evaluated by analysis of variance and Duncan's multiple range test [14]. The general linear model procedure was utilized to evaluate race–dietary group interaction. Means and standard errors of the mean (S.E.M.) were calculated. Pearson r correlation coefficients were determined between data obtained by the various assay parameters.

RESULTS AND DISCUSSION

Twelve whites, three blacks and seven men of other races volunteered earlier as subjects. The ages, heights and weights of the subjects were statistically similar [13]. None of the variables were affected by race.

Plasma PLP measurements

Plasma PLP levels of the subjects as determined using radiometric, LTD [13], as well as HPLC, UV and fluorometric, methods are listed in Table III. The plasma PLP values of the subjects were in the range of HPLC-derived values reported by other researchers [2,4–6,10]. Values for plasma PLP concentrations obtained by LTD and HPLC, both UV and fluorometric, analyses were highly correlated ($r=0.80$, $P<0.001$; $r=0.87$, $P<0.0001$, respectively); high correlation ($r=0.86$, $P<0.0001$) was also observed between values obtained using the two HPLC detectors. Thus, similar data were obtained utilizing all three of these techniques.

The plasma PLP concentrations of all 22 of the subjects who consumed diets containing 0.75–0.98 mg (mean) of vitamin B_6 daily for eight weeks were well above levels suggested as being indicative of vitamin B_6 inadequacy [15–17]. Plasma PLP levels of approximately 75 nmol/l have been reported for humans consuming regular diets [18]. Plasma PLP values (mean ± S.D.) for 35 healthy

TABLE III

B_6 VITAMER AND 4-PA CONCENTRATIONS OF HUMAN PLASMA

Congener	Assay*	Number of subjects with detectable levels	Concentration** (mean ± S.E.M.) (nmol/l)
PLP	LTD	22	90.3 ± 3.5
	H-FL	22	88.0 ± 3.9
	H-UV	22	89.2 ± 3.8
PNP	H-FL	12	6.4 ± 1.6
	H-UV	9	6.1 ± 1.6
PMP	H-FL	14	12.2 ± 5.7
PL	H-FL	22	38.4 ± 5.0
PN	H-FL	22	41.4 ± 7.5
PM	H-FL	22	17.6 ± 2.0
4-PA	H-FL	16	39.3 ± 11.4

*LTD = radioisotopic tyrosine decarboxylase; H-FL = HPLC using fluorometric detection; H-UV = HPLC using UV detection.

**Non-detectable levels calculated as zeros.

men who did not take supplements containing vitamin B_6 were 51.9 ± 19.3 nmol/l [15]. Steady-state plasma PLP levels reportedly are reached within three to four weeks following alteration of the dietary vitamin B_6 intake [19]. Hence, the subjects in the current study had been on the diets long enough to have steady-state plasma PLP levels; these levels, indicative of adequate vitamin B_6 status, were attained when the men had consumed diets analyzed to contain 0.75–0.98 mg (mean) of the vitamin daily, an amount less than the 1980 Recommended Dietary Allowance (R.D.A.) [20] of 2.2 mg for men.

Plasma PNP, PMP, PL, PN, PM and 4-PA measurements

Plasma B_6 vitamer and 4-PA levels of the subjects are given in Table III. A high correlation ($r = 0.91$, $P < 0.0001$) was observed between the subjects' plasma PNP concentrations as measured utilizing the two HPLC techniques. Twelve of the subjects in the current study had detectable plasma levels of PNP utilizing the HPLC–fluorometric method and nine of these twelve had detectable levels utilizing the HPLC–UV technique. To our knowledge, others have not reported detecting PNP concentrations in plasma from any of their subjects.

Plasma PMP values of the subjects in the current study were similar to those reported by Chauhan and Dakshinamurti [4] but were also in the range of values reported by others [5,6]. Mean plasma PL values were higher than reported values [2,6,7,10] but values were in the range of those of Coburn and Mahuren [6]. Plasma PN values were in the range of values reported by Coburn and Mahuren [6] and Lumeng et al. [2] although mean values were higher than their reported mean concentrations. Plasma PM values were in the range of those reported by Lumeng et al. [2]. Plasma 4-PA values of the subjects were similar to reported values [2,6,7]; two of the research groups [4,5] did not determine 4-PA concentrations. Overall, the B_6 vitamer and 4-PA values of subjects in this study were rather similar to those reported by others. However, it should be emphasized that the subjects of all the researchers including ourselves consumed varying quantities of the vitamin and each research group has a somewhat different HPLC method/system.

Significant correlation ($r = 0.62$. $P < 0.005$) was observed between plasma PN and 4-PA levels of the subjects as well as between plasma PN and PMP concentrations ($r = 0.78$, $P < 0.0001$). The B_6 vitamers but not 4-PA are easily interconvertible; however, 4-PA cannot be converted back to any of the B_6 vitamers [21].

B_6 Vitamer distribution

The plasma B_6 vitamer distribution of subjects in this study as quantitated by the HPLC–fluorometric method is depicted in Table IV; S.E.M. were quite large for all except PLP. Over half of the B_6 vitamers in the subjects' plasma was PLP. Other [1,2,6,10] but not all [4,5] researchers have reported PLP to be the predominant vitamer in plasma from seemingly healthy adults some of whom took supplements containing the vitamin. The men in the current study had practically equal plasma distributions of PL and PN; Coburn and Mahuren [6] also reported finding nearly equal distributions of PL and PN in plasma from their subjects. PNP was the B_6 vitamer with the lowest percentage distribution in plasma from men in the current study.

TABLE IV

B_6 VITAMER DISTRIBUTION IN HUMAN PLASMA

Vitamer	Distribution (mean ± S.E.M.) (%)
PLP	54.1 ± 2.1
PNP	3.4 ± 0.8
PMP	5.0 ± 1.5
PL	15.2 ± 1.8
PN	15.1 ± 1.7
PM	7.9 ± 1.0

This HPLC methodology provides a rapid, reproducible and sensitive procedure for determining plasma B_6 vitamer and 4-PA concentrations in humans. The methodology has potential for use in the assessment of vitamin B_6 status in humans.

ACKNOWLEDGEMENT

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APPENDIX D

FEMALES
12-14 YRS OLD
TO PARTICIPATE IN
NUTRITION RESEARCH
(VITAMIN B-6)

CONTACT:
BARBARA CHRISLEY, B.S., M.T.
JUDY A. DRISKELL, PH.D., R.D.
HUMAN NUTRITION AND FOODS
VIRGINIA TECH
BLACKSBURG, VA 24061
674-4021
OR
961-5939

You may have a vitamin deficiency! Stress, growth, maturation and poor nutrition contribute to vitamin inadequacy. Participate in a nutrition project at VPI&SU. Find out your vitamin B-6 status, determination for anemia, nutritional consultation and supplementation, if necessary. FREE T-SHIRT DESIGNED FOR PROJECT.

CONTACT: Barbara M. Chrisley, B.S., M.T.
Home: 674-4021
Dr. Judy A. Driskell
Office: 961-5939

APPENDIX F

COLLEGE OF HUMAN RESOURCES

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Blacksburg, Virginia 24061

DEPARTMENT OF HUMAN NUTRITION AND FOODS

Dear Parents (or Guardian):

Nutritional knowledge is acquired each day through research and is passed on to the public. Good nutrition practices promote physical and mental health. I solicit your help to further this cause.

This research is designed to determine and evaluate the Vitamin B-6 status of adolescent females. Twelve to fourteen year old girls 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.

Since Vitamin B-6 has been determined to be essential, the National Research Council has established a Recommended Dietary Allowance by interpolations from adult studies. However, virtually no controlled studies have been conducted on this age group of females. This research needs to be done to determine the accuracy of these recommended levels; also to help establish "normal" values and requirements for these females.

Participation in this study is on a voluntary basis. Subjects needed are twelve to fourteen year old white females in good health and with ideal body weights within 20%.

Involvement in this study requires collecting:

1. a consent form,
2. one 20 ml intravenous, fasting blood sample which will be done by a Registered Medical Technologist; also a urine sample (50 ml) is requested,
3. three days of dietary records,
   a. a 24-hour recall will be carried out by interview taking approximately one half-hour of your time,
   b. two-day food records which your daughter will be asked to complete,
4. subject background information questionnaire, and
   a. bring supplement and medication bottles to researcher to review,
5. parents/guardians questionnaire,

6. In the event that an inadequate B-6 status is established, I agree to take 10 mg (or 25 mg) of pyridoxine hydrochloride (vitamin B-6) and provide additional blood, urine, and dietary information.

Although not required for my study, we will provide hemoglobin determinations using the same blood sample. Any cases of anemia will be reported to you.

Information secured will be kept confidential and used for statistical purposes only. All findings wil be reported to you.

Therefore, each female will benefit by acquiring her B-6 status, free supplementation if necessary, and determination for the incidence of anemia. In addition, she will receive a T-shirt designed especially for this nutrition study. In the event she should have an inadequate B-6 status, then she will be monetarily compensated for further participation in the study.

Thank you for your support!

Sincerely,

Barbara Mc. Chrisley, B.S., M.T.
Ph.D. Candidate
Office Phone - 961-5939
Home Phone -

Judy A. Driskell, Ph.D.
Professor
Office Phone - 961-5939

JAD/BMC/sks

APPENDIX G

SUBJECT CODE NUMBER_________

CONSENT FOR PARTICIPATION

in

Vitamin B-6 Nutrition Study of
12-14 year old Females

Virginia Polytechnic Institute and State University

My parent(s)/guardian(s) and I have received a verbal explanation of the study and have had an opportunity to ask questions regarding the procedure. We understand the following:

Purpose of research project

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. Therefore, the purpose of the study is to determine the vitamin B-6 status and perhaps requirements of healthy, white twelve to fourteen year old females (wt ± 20% IBW) by the use of selected methods utilizing blood samples. These values will help to establish "normal" values and requirements for this age group of females.

Protocol for participation in the study

The undersign consent to participate in the vitamin B-6 study.

Information to be secured will include dietary records and socioeconomic information pertinent to the study.

A fasting blood sample, approximately 20 milliliters, will be taken by venipuncture by a Registered Medical Technologist; also a urine sample (approximately 50 ml) will be collected as close to blood drawing time as possible.

I agree to be supplemented with 10 mg pyridoxine hydrochloride (vitamin B-6) and provide additional blood, urine, and dietary information in the event that I am found to have inadequate vitamin B-6 status. I will agree to adhere to this protocol for 1-3 months or until adequate status is obtained.

All information obtained in the study will be held strictly confidential and will be used for statistical purposes only.

Any inquiries I may have concerning the procedures utilized in this study will be answered at any time.

No compensation will be offered if injury is incurred as a result of participating in this study. The probability of injury is very low. I will be expected to advise the researcher of any medical problems that might arise in the course of the study and I am free at any time to withdraw consent and discontinue participation in the project.

I understand the above and agree to participate in the study.

________________	________________________________
DATE	VOLUNTEER

________________	________________________________
DATE	PARENT/GUARDIAN

Principal Investigators: Dr. Judy A. Driskell
Office Phone - 961-5939
Barbara Mc. Chrisley, B.S., M.T.
Office Phone - 961-5939
Home Phone -

Chairman, Institutional Review Board for Research Involving Human Subjects: Dr. Charles Waring (Office Phone - 961-5284).

APPENDIX H

SUBJECT CODE NUMBER_________

PRE-EXPERIMENTAL QUESTIONNAIRE

NAME____

ADDRESS_______________________________________ DATE____________

PHONE NUMBER (HOME/OFFICE)_______________________________

PHYSICIAN'S NAME (OR CLINIC)______________________________

HEIGHT (INCHES)____________ WEIGHT (POUNDS)____________ AGE _______/________ (YEARS / MONTHS)

1. Do you or have you had any illness or disease? Yes_____ No_____
   If yes, please describe_______________________________
   ____
2. Do you take vitamin or mineral supplements? Yes_____ No_____
   If yes, please specify what brands and for how long (example: 3 times a week since January, 1987)_______________________________
   ____
   a. seldom _____ c. most of the time _____
   b. frequently _____ d. always _____
3. Do you take any other kinds of medication? Yes_____ No_____
   If so, give brand name_______________________________
   ____
4. Are you on a special diet? Yes_____ No_____
5. Do you choose not to eat any specific food(s) because of religious or other beliefs? Yes_____ No_____
   If so, what foods? _______________________________
6. Do you have a good appetite? Yes_____ No_____
7. Are your present eating habits similar to those that you have practiced throughout your life? Yes_____ most of the time_____ No_____
8. Have you lost or gained weight in the last year? Yes_____ No_____
   lost_____ gained_____ how many pounds? _________
9. Is what you ate the day of the recall the way you usually eat?
   Yes_____ No_____
   If no, give recall for another day.
10. Do you take birth control pills? Yes_____ No_____
    If so, give brand name_______________________________
11. Length of time that you have taken birth control pills (example: for 1 year - 8-82/8/83)_______________________________
12. Give day of menstrual cycle on which blood sample was taken______________
    The first day of menstrual bleeding (period) is day 1.
13. Are you a student? Yes_____ No_____
14. County in which you attend school_______________________________
15. Do you eat meals at school? Yes_____ No_____
    a. cafeteria _____ once a day_____ twice a day_____
    b. brown bag _____ once a day_____ twice a day_____
    c. snack machine _____ if so, what food items?_______________
    ______________________________ once a day_____ twice a day_____
16. How often do you eat in commercial establishments such as cafeterias and restaurants?
    a. seldom _____ c. most of the time _____
    b. frequently _____ d. always _____

APPENDIX I

SUBJECT CODE NUMBER________

PARENTS/GUARDIANS QUESTIONNAIRE

1. Years of education completed for father (or male guardian):

   _____6 years
   _____High School
   _____College
   _____Masters Degree
   _____Ph.D.

2. Years of education completed for mother (or female guardian):

   _____6 years
   _____High School
   _____College
   _____Masters Degree
   _____Ph.D.

3. What approximately is your family income per year?

   _____under $5,000
   _____$8,000-$10,000
   _____$10,000-$15,000
   _____$15,000-$25,000
   _____$25,000-$35,000
   _____$35,000-$50,000
   _____over $50,000

4. The income level given in #3 supports how many people? ________

5. How much do you spend for food each week?

   _____$10-$15
   _____$15-$25
   _____$25-$35
   _____$35-$50
   _____over $50

6. For how many people is this amount spent? ________

APPENDIX J

Two girls from county take part in Tech nutrition study

and , members of the Pulaski Porpoise Swim Team, participated, in a Nutrition Study from the department of Human Nutrition and Food at Virginia Polytechnic Institute and State University.

Nutritional knowledge is acquired each day through research and is passed on to the public. Good nutrition practices promote physical and mental health.

This research is designed to determine and evaluate the Vitamin B-6 status of adolescent females. Twelve to fourteen year old girls 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.

Since Vitamin B-6 has been determined to be essential, the National Research Council has established a Recommended Dietary Allowance by interpolations from adult studies. However, virtually no controlled studies have been conducted on this age group of females.

This research needs to be done to determine the accuracy of these recommended levels; also to help establish "normal" values and requirements for these females.

Therefore, each female will benefit by acquiring he B-6 status, free supplementation if necessary, and determination for the incidence of anemia. In addition, she will receive a T-shirt designed especially for this nutrition study. In the event she should have an inadequate B-6 status, then she will be monetarily compensated for further participation in the study.

Participation in this study is on a voluntary basis. Subjects needed are twelve to fourteen year old white females in good health and with ideal body weights within 20%.

Girls that are interested in participation in the study contact:

Barbara Mc. Chrisley, B.S., M.T., Ph.D. Candidate, office phone - 961-5939; home phone - or Judy A. Driskell, Ph.D, Professor, office phone - 961-5939.

APPENDIX K

SUBJECT CODE NUMBER__________

VITAMIN B-6 NUTRITION STUDY
24-HOUR RECALL QUESTIONNAIRE
DATE OF RECORD________________
DAY OF WEEK TAKEN: M T W TH F S SUN (CIRCLE)

CODE NO.	G/ FOOD	FOOD AND BEVERAGE CONSUMED WHAT DID YOU EAT?		COOKING METHOD	AMOUNT
		EXAMPLE:	EGGS	FRIED	2 MED.
			OIL		1 TBS.
		BREAKFAST			
		SNACK			
		LUNCH			
		SNACK			
		DINNER			
		SNACK			
		ANY OTHER TIME			

APPENDIX L

SUBJECT CODE NUMBER__________

VITAMIN B-6 NUTRITION STUDY
TWO-DAY DIETARY FOOD RECORD FORM
DATE OF RECORD__________________
DAY OF WEEK TAKEN: M T W TH F S SUN (CIRCLE)

CODE NO.	G/ FOOD	FOOD AND BEVERAGE CONSUMED WHAT DID YOU EAT?	COOKING METHOD	AMOUNT
		EXAMPLE: EGGS	FRIED	2 MED.
		OIL		1 TBS.
		BREAKFAST		
		SNACK		
		LUNCH		
		SNACK		
		DINNER		
		SNACK		
		ANY OTHER TIME		

APPENDIX M

SUBJECT CODE NUMBER__________

VITAMIN B-6 NUTRITION STUDY
TWO-DAY DIETARY FOOD RECORD FORM
DATE OF RECORD__________________
DAY OF WEEK TAKEN: M T W TH F S SUN (CIRCLE)

CODE NO.	G/ FOOD	FOOD AND BEVERAGE CONSUMED WHAT DID YOU EAT?		COOKING METHOD	AMOUNT
		EXAMPLE:	EGGS	FRIED	2 MED.
			OIL		1 TBS.
		BREAKFAST			
		SNACK			
		LUNCH			
		SNACK			
		DINNER			
		SNACK			
		ANY OTHER TIME			

APPENDIX N

PROCEDURE FOR DETERMINATION OF PLASMA PYRIDOXAL PHOSPHATE (PLP) LEVELS (1)

Equipment & Supplies

a. automatic pipets and tips (10,20,50,100 µl)

b. 25 ml reaction flasks - 2 for each blank and standard (8 total) plus 3 for each sample. Label all flasks appropriately with tape. Color coding is helpful. (ex: blue-blanks, red-standards, a color for each sample) number or letter each flask as to position in the metal rack.

c. metal rack

d. dish pan with crushed ice and salt

e. 2-5 ml beakers
2-40-50 ml beakers

f. refrigerated centrifuges

g. shaker water bath 37° and shaker ice bath

h. filter paper - Whatman No. 42

i. rubber stoppers

j. center wells

k. 250 µl and 1 ml leur lock syringes with 1½ in, 21 gauge needles

l. gloves and lab coat

m. scissors

n. scintillation vials with caps and a rack for them

o. sorval centrifuge tubes and corex clinical centrifuge tubes - appropriately labeled

p. polypropylene tubes with blue caps

q. vortex mixer

r. kimwipes

Reagents

1. L-tyrosine-1-^{14}C working standard

2. PLP working standard (20 ng/ml)

3. 0.1 M sodium acetate buffer solution

4. Trichloroacetic acid (TCA) 50% solution
5. TCA 10% solution
6. Turner's scintillation medium
7. Hyamine hydroxide
8. Amine/Freon solution
9. Purified tyrosine apodecarboxylase solution
10. Decasol

General

1. All procedures must be carried out in the dark.
2. All samples and reagents must be kept cold (in the refrigerator or in ice brine) unless otherwise noted.
3. Gloves and lab coats must be worn at anytime the radioactive material is being used.

No material such as pipets, scissors, beakers, syringes, etc. should ever be removed from the radioactive area without thorough cleaning with decasol.

After each run a swipe should be made from an item in the radioactive area such as the tray or counter top and counted on the scintillation counter to verify that clean up procedures have been adequate.

Procedure

I. Prepreparation

1. Turn on refrigerated centrifuge in 302
    a. Depress main switch button
    b. Set temperature control to 4-5°C - pull out knob and move red pointer then push in knob and move blue pointer until desired temperature setting is between the two pointers. Approximately 20 min is needed for the centrifuge to reach the proper temperature.
2. If samples are frozen insert the tubes of plasma into a beaker of ice water and cover. Thawing takes about 30-45 min.
3. Fill shaker water bath with water and turn on. Check thermometer and be sure the water temperature is stable at 37°C. Adjust temperature control only if needed. If bath is filled with cold water, it may take 45 min to 1 hr to reach the proper temperature. (Using warm water will decrease the time needed to reach 37°C).

II. Deproteination of plasma samples

Prior to assay, plasma samples are deproteinized to stop the activity of the tyrosine apodecarboxylase in the plasma. The TCA is the agent used to accomplish the above. As TCA would inhibit the activity of the tyrosine apodecarboxylase added in a subsequent step, it is extracted from the sample with amine/freon solution using the method of Khym (2).

1. Pipet 0.9 ml plasma into a sorval centrifuge tube - label tube appropriately
2. Add 100 µl of 50% TCA to each sample (TCA may be at room temperature)
3. Vortex each sample
4. Centrifuge 20 min at 17,300 x G and 5°C
5. After centrifugation, pour supernatant into a corex/clinical centrifuge tube. (transfer label from sorval tube to corex tube)
6. Under the hood, add 1 ml freon/amine solution to each tube (use 1000 µl automatic pipet or 1 ml volumetric pipet) and vortex each sample.
7. Centrifuge samples for 20 min at 5°C at 2000 x G. (use clinical centrifuge in the walk-in refrigerator). Two clear organic phases should be evident after this step.
8. Using a 50-100 µl automatic pipet or pasteur pipet, remove at least 150 µl of the top phase without disturbing the bottom phase. The top phase contains the PLP and will be used in the analysis procedure. Place the sample in a small polypropylene tube (blue cap).
9. Keep tubes of sample on ice and covered until analysis.

III. Analysis of plasma PLP

The analysis for plasma PLP is accomplished using the method of Chabner and Livingston (1) as modified by Reinken (3). The basis of this enzymatic assay is measurement of $^{14}CO_2$ emitted when L-tyrosine-1-^{14}-C is decarboxylated by the PLP dependent enzyme tyrosine decarboxylase. The activity of the enzyme tyrosine apodecarboxylase varies directly with PLP concentration. All procedures must be performed in the dark as the vitamin is light sensitive.

1. Cut 1 strip of filter paper (5.1x0.6 cm) for each flask. Fold strips uniformly and place one into each center well. Insert the stem of the center well into a rubber stopper. These stoppers will be inserted into the flasks during the procedure.
2. Set up rack with flasks (see diagram) and place in pan of ice-salt mixture. Place 1 tube of enzyme in the pan of ice.
3. Pour needed amount of the following cold solutions into properly labeled beakers: (1)-PLP standard, (2)-0.1 M sodium acetate buffer, (3)-hyamine hydroxide and place the beakers in the ice salt mixture.

Example of flask organization in rack - write flask name and position code on appropriate color of tape

Martin 1 Green	Martin 2 Green	Martin 3 Green		Smith C Pink	Jones C Yellow
Blank B Blue	10 B Red	20 B Red	50 B Red	Smith B Pink	Jones B Yellow
Blank A Blue	10 A Red	20 A Red	50 A Red	Smith A Pink	Jones A Yellow

Name / Position / Tape Color

4. Add the following to the front of each flask:

Blank	-
10 µl standard	10 µl PLP standard
20 µl standard	20 µl PLP standard
50 µl standard	50 µl PLP standard
sample	20 µl of sample

5. Add 10 µl of enzyme to the back of each flask

6. Add 1.5 ml of 0.1 M sodium acetate buffer to each flask and swirl flasks to mix.

7. Add 200 µl hyamine hydroxide to the filter paper in each center well and insert the rubber stopper into the neck of the flask and pull down to seal. Hyamine hydroxide is used to capture the $^{14}CO_2$.

8. Place flasks in a 37°C shaking water bath for 15 min to allow for formation of the holoenzyme.

9. Place flasks in a shaking ice bath for 10 min to halt holoenzyme formation.

10. Inject 250 µl of L-tyrosine-1-^{14}C working standard into each flask through the rubber stopper. This provides substrate for the reaction. Always wear rubber gloves while handling any radioactive materials. Dispose of needle and any paper that has come in contact with the radioactive material in the radioactive solid waste. Pour any extra radioactive solution into the radioactive liquid waste bottle. Rinse all beakers, flasks, and syringes in decasol solution and pour decasol into the radioactive liquid waste bottle. (Avoid getting solution in the center wells while injecting.)

11. Swirl flasks to mix.

12. Place flasks in 37°C shaking water bath for 15 min. This incubation period allows the reaction to take place.

13. Return flasks to ice mixture and inject 1 ml of 10% TCA (room temperature) through the rubber stopper. (Again avoid getting the TCA in the center well.) The cold and TCA terminate the reaction.

14. Swirl flasks to mix.

15. Place flasks in a 37°C shaking water bath for 1 hr to ensure complete evolution of the $^{14}CO_2$.

16. Set up a corresponding scintillation vial for each flask in rack. Write identifying code on caps of vials.

17. Put on gloves. Remove each rubber stopper from its flask and cut center well with paper into the corresponding scintillation vial.

18. Pipet 5 ml of scintillation fluor into each vial and cap tightly.

19. Take rack of vials to scintillation counter. Each sample is counted for 2 min. 14C is a beta particle emitter.

20. Wear gloves during all clean up procedures. All beakers, flasks, stopper, and other items which have been in contact with the radioactive material must have any liquid waste emptied into the radioactive liquid waste and then must be washed with decasol. Empty all decasol into the radioactive liquid waste.

21. Stems from center wells should be placed on a Kimwipe and then all discarded into the radioactive solid waste container. The counter top, sink ledge, refrigerator handle and tray should all be cleaned with a paper towel and decasol. The paper towel is then disposed of in the radioactive solid waste.

    All waste areas for vials, liquid, and solid radioactive wastes are kept locked when wastes are not being disposed of.

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APPENDIX O

Coenzyme Stimulation of Alanine Aminotransferase Activity
An Indication of Vitamin B_6 Status

Principle

The method is based on the transamination reaction between alanine and α-ketoglutarate in the presence of tissue; the pyruvate formed in the reaction is convected to diphenyl-pyruvate hydrazone and this compound is extracted in toluene. The toluene solution is treated with alkali, and the colored compound that results is analyzed spectrophotometrically. The enzyme involved in alanine aminotransferase, ALAT, EC 2.6.1.2, formerly known as glutamic-pyruvate transaminase. Extra pyridoxal phosphate is added to the tissue in vitro to measure the coenzyme stimulation.

Preparation of Erythrocytes for Analyses

Blood samples are taken by venipuncture from either fasting or non-fasting subjects. EDTA (100 USP units/ml) is used to prevent coagulation -- or heparinized vacutainer tubes may be utilized. Blood samples are held in crushed ice until centrifugation. Whole blood samples are centrifuged for 10 minutes at 2000 x g and 5 C (use refrigerated rotor or head); the supernatant or plasma is removed by use of Pasteur pipettes -- also remove buffy coat. Erythrocytes are washed by mixing (inversion) with twice their volume of cold physiological saline solution (9 g NaCl/l H_2O) followed by centrifuging a second time and discarding the supernatant. A 10% (vol/vol) solution of erythrocytes is made by adding cold phosphate buffer, pH 7.4. It is crucial that the

blood be kept between 0-5 C at all times during this procedure. Hemolysate aminotransferase activity is stable for up to 1 year when the hemolysate is stored frozen. This is the method reported by Driskell, Wiley, and Kirksey (1971).

Determination of Coenzyme Stimulation of ALAT Activity

The first part of this procedure must be performed in the dark and the centrifuge tubes containing the buffered erythrocytes must be kept in an ice-cold salt brine (crushed ice + salt + H_2O).

1. 6 centrifuge tubes are required per sample in order for analyses to be done in duplicate. 2 tubes are needed for zero activity determinations, 2 for basal activity determinations, and 2 for activity determinations when additional cofactor (pyridoxal phosphate, B_6P) is added.
2. To the bottom of 2 "+B_6P" tubes add 10 μl of pyridoxal phosphate solution.
3. Add 0.5 ml cold buffered erythrocytes to each of 6 tubes. Also - 0.5 ml standard solution per tube -- 2 tubes; 0.5 ml H_2O -- 2 tubes -- procedure blank.
4. Mix (Vortex) the "+B_6P" and erythrocyte mixture, then place in 37 C shaker water bath for 20 minutes. Place back in ice-cold brine after the 20 minutes.
5. To the contents of the 2 "zero" tubes, add 1 drop 100% TCA and mix (Vortex).

6. Add 0.5 ml cold alanine reagent to each of the tubes (including procedure blank and standards).
7. Cover tubes with parafilm and mix (Vortex) tube contents.
8. Place tubes in 37 C shaker water bath for exactly 10 minutes (to the second).
9. Remove tubes from water bath and place into ice water brine.
10. Add 1 drop 100% TCA to contents of each tube. Remove tubes from ice water brine and mix (Vortex) contents of tubes.
11. Keep tube contents at room temperature for at least 20 minutes; contents of tubes may be frozen at this point and the analyses completed at a future time.
12. From this point on, the lights may be on in the room.
13. Add 1 ml dinitrophenylhydrazine reagent to each tube by buret, contents of the tubes are mixed (Vortex) and permitted to stand at room temperature for <u>exactly</u> 5 minutes (to the second).
14. Add 2 ml toluene by buret to each tube, cover tube with saran wrap and shake tubes vigorously.
15. Centrifuge tubes for 10 minutes at 3000 x g.
16. Remove 1 ml of the toluene layer from each centrifuge tube to a spectrophotometer tube, cuvette -- do by timed intervals as before. The toluene should be permitted to extract the pyruvate hydrazone from the contents of each centrifuge tube for an equal time interval.

17. Add 5 ml alcoholic KOH and 1 ml distilled water to each spectrophotometric tube.
18. Cover tubes with saran wrap and mix tube contents by inversion.
19. Read absorbance of contents of tubes at 430 nm on spectrophotometer. Color is stable for at least 4 hours.

The above alanine aminotransferase activity of erythrocytes method is that of Tonhazy et al. (1950) as modified by Heddle et al. (1963). The method for determining the pyridoxal phosphate stimulation response is that of Raica and Sauberlich (1964).

<u>Chemicals Required</u>

Phosphate buffer, pH 7.4 -- add 804 ml $K_2 HPO_4$ (11.612 g/l) and 196 ml KH_2PO_4 (9.08 g/l)

Pyruvate standards -- contains 25-125 μg 0.5 ml

Alanine reagent -- 1.78 g D-L alanine + 2.0 g KH_2PO_4 + 0.6 g α-ketoglutaric acid and bring to 100 ml with distilled water; adjust pH to 7.4 using 10% KOH

100% TCA -- may be purchased as such

Dinitrophenylhydrazine reagent -- 0.1 g 2,4 dinitrophenylhydrazine + 20 ml concentrated HCl and bring to 100 ml with distilled water

Toluene -- purchase spect grade

Alcoholic KOH -- 2.5 g KOH and bring to 100 ml with 95% ethanol

Distilled water

Pyridoxal phosphate solution -- 0.5 g PMP -> 10 ml with distilled water

Required Supplies and Equipment

Centrifuge tubes -- that hold approximately 15 ml

Vortex mixer

0.5 volumetric or 1 ml transfer pipettes

10 micropipette

Shaker water bath

Parafilm

Saran Wrap

1 ml volumetric pipettes

1 or 2 25 ml burets

1 or 2 50 ml burets

Spectrophotometric tubes or cuvettes

Spectrophotometer -- such as Bausch and Lomb Spect 20

Kimwipes

References

Tonhazy, N.E. et al. 1950 A rapid method for the estimation of glutamic aspartic transaminase in tissues and its application to radiation sickness. Arch Biochem. 28, 36.

Heddle, J.G. et al. 1963 Penicillamine and vitamin B_6 interrelationships. Can. J. Biochem. Physiol. 41, 1215.

Driskell, J.A. et al. 1971 Alanine aminotransferase activity in liver and erythrocytes of pregnant and nonpregnant rats fed different levels of pyridoxine. J. Nutr. 101, 85.

Raica, N.J. and H.E. Sauberlich 1964 Blood cell transaminase activity in human vitamin B_6 deficiency. Am. J. Clin. Nutr. 15, 67.

APPENDIX P

Procedure for Determination of Urinary 4-PA

<u>Equipment</u>
Waters Associates HPLC

<u>Reagents</u>
Trichloroacetic acid (TCA), 40% (v/v). Pour 40 mL 100% TCA in deionized H_2O and dilute to 100 mL.

4-PA standards. 4-Pyridoxic acid was obtained from Sigma Chemical Co. St. Louis, MO.

A working stock (20 µg/mL) solution was prepared by dissolving 20 µg 4-PA in 1 liter deionized H_2O.

A 10 µg/mL recovery standard solution was prepared by dilution of 25 mL of the working stock solution with 25 mL deionized H_2O.

Calibration standards are prepared over the range of 0.5 to 6.0 µg/mL.

<u>Procedure</u>
Sample preparation. Pipet 4 mL of urine into a 16x100 mm crew cap test tube. Add 1 ml deionized H_2O, or for recovery samples, add 1 mL of the 10 µg/mL recovery standard solution. Add 1 ml 40% TCA and mix thoroughly. Centrifuge at medium speed (approximately 1000 x g) for 15 min in a clinical centrifuge to sediment precipitated urinary proteins. Remove supernatant for analysis and, along with prepared calibration standards, hold in an ice bath until analyzed.

Calculations

$$\mu g \text{ 4-PA/mL} = \frac{\text{Relative fluorescence)(6/4)(100)}}{\text{(slope of standard curve)(0.05)(\% recovery)}}$$

Reference
Gregory, J. and Kirk, J. Determination of urinary 4-Pyridoxic acid using high performance liquid chromatography. Am. J. Clin. Nutr. 32:879-883, 1979.

Stanbio Creatinine Procedure No. 0400

Quantitative Colorimetric Determination of Creatinine in Serum or Urine

Summary and Principle

Early application of the Jaffe reaction[1] for the clinical determination of creatinine in blood was reported by Folin and Wu in 1912.[2] This classical reaction produces a red tautomer of "alkaline creatinine picrate" following treatment of a protein-free filtrate with picric acid and sodium hydroxide.[1]

Attempts to improve specificity were made by noting the difference in absorbance after discharging the final red color with acid.[3,4] Further efforts toward "true" creatinine values involved use of Lloyd's reagent (an aluminum silicate). This agent absorbs creatinine from non-creatinine color-producing compounds in the protein-free solution, followed by its elution into alkali and colorimetric Jaffe-type assay.[5] Similar work toward greater specificity was reported by Teger-Nilsson who utilized column chromotography to separate creatinine from interfering substances.[7]

The method presented employs an excess of picric acid for deproteinization, as well as for developing the Jaffe chromogen on addition of alkali. This technique avoids preparation of the conventional tungstic acid protein-free filtrate or supernatant.[8]

Creatinine, in a picric acid protein-free solution, reacts with added alkali to form a reddish-brown complex, the actual formula of which is unestablished. The absorbance of this compound at 520 nm is proportional to creatinine concentration.

Reagents

Creatinine Picrate Acid Reagent, Cat. No. 0401 (500 mL)
Saturated aqueous solution of picric acid, 1 4% (w/v)

Creatinine Sodium Hydroxide Reagent, Cat. No. 0402 (125 mL)
Aqueous solution of sodium hydroxide, 3 0% (w/v)

Creatinine Standard (Equivalent to 2 5 mg/dL), Cat. No. 0403 (60 mL)

Creatinine Standard (Equivalent to 5 0 mg/dL), Cat. No. 0404 (60 mL)

These standards are solutions of creatinine zinc hydrochloride in aqueous picric acid (1 4% w/v). Stated values apply only when used as directed in method presented.

Precautions: For In Vitro Diagnostic Use.

Reagent Storage and Stability: All reagents are stable at 20-25°C until the expiration date shown on label.

Instrumentation and Material (Not Provided)

Spectrophotometer capable of absorbance readings at 520 nm
Centrifuge with high speed capacity (≥ 1500g)
Pipets capable of accurately delivering 0 5, 1 0, 3 0, 4 5 and 9 0 mL
Test tubes and/or cuvets. Vortex mixer. Interval timer

Specimen Collection and Preparation

Creatinine in serum and urine is reportedly stable 4-7 days at room temperature[9] and indefinitely when frozen.[10] A 24-hour urine specimen should be collected without preservative and the total volume (mL) measured.
A 2-hour single-voided specimen is acceptable, with no volume measurement necessary.

Procedure:

Preparation of sample dilution
1. Serum: To 4 5 mL Picric Acid Reagent add 0 5 mL serum. Mix well, centrifuge for 5 minutes to obtain clear supernatant.
2. Urine: Add 1 part of well-mixed 2-hour or 24-hour specimen to 9 parts distilled water (1:10).
3. Make a further 1:10 dilution using 1 part dilute urine (Step 2) with 9 parts Picric Acid Reagent. If precipitated protein is visible, centrifuge for 5 minutes to obtain clear supernatant.

Assay

1. Pipet into cuvets the following volumes (mL) and mix well.

	Reagent Blank (RB)	Standard (2 5)	Standard (5 0)	Sample* (U)
Distilled Water	0 3	-	-	-
Picric Acid	2 7	-	-	-
Standard 2 5	-	3 0	-	-
Standard 5 0	-	-	3 0	-
Supernatant	-	-	-	3 0
Sodium Hydroxide	1 0	1 0	1 0	1 0

*Diluted serum (Step 1) or diluted urine (Step 3)

2. Incubate all cuvets at room temperature for 10 minutes.
3. Read S[2.5], S[5.0] and U vs RB at 520 nm within 30 minutes.

Quality Control: Use of commercial control serum, or pooled serum previously assayed and divided in frozen aliquots, is recommended with each series of assays.

Results

Values are calculated as follows:

$$\text{Serum Creatinine (mg/dL)} = \frac{Au}{As} \times Cs$$

where Au and As are the absorbance values of the UNKNOWN and STANDARD, respectively, and Cs the concentration of the STANDARD (mg/dL), the absorbance of which is closest to that of the UNKNOWN.

$$\text{Urine Creatinine (mg/dL)} = \frac{Au}{As} \times Cs \times 10$$

$$\text{Urine Creatinine (gm/dL)} = \frac{\text{Urine Creatinine (mg/dL)}}{1000}$$

$$\text{Urine Creatinine (gm/24h)} = \frac{\text{Urine Creatinine (gm/dL)} \times \text{24h Vol (mL)}}{100}$$

Expected Values[11]

Normal Range: Serum 0 8-1 5 mg/dL
Urine 0 8-2 0 gm/24h

Performance Characteristics[8]

Precision: Replicate assays (once a day for 20 days) on each of 2 serum pools having mean creatinine levels of 0 8 and 5 6 mg/dL showed respective standard deviations of 0 10 and 0 12 mg/dL.

Recovery: A serum pool having a mean creatinine level of 1 0 mg/dL was divided into 4 aliquots and creatinine added to the extent of 1, 3, 5 and 7 mg/dL. Reassay revealed recoveries of 95 5, 97 3, and 99 3%, respectively.
Linearity: 0-10 mg/dL

References

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Notes:

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February 1988
Last Revision Feb 1988
Procedure No 0400

APPENDIX R

Age, height, and weight values of subjects

Subject	Age y	Ht cm	Wt kg
200	12	157.5	39.9
201	12	161.3	47.7
202	12	154.9	50.0
203	12	160.0	63.6
204	12	151.0	39.0
205	12	152.4	59.1
206	12	162.6	42.7
207	12	149.9	38.6
250*	12	162.6	43.6
251*	12	160.0	59.0
252*	12	152.4	46.8
253*	12	157.5	56.4
254*	12	160.0	58.2
255*	12	163.8	48.6
256*	12	154.9	45.6
350	13	165.1	47.3
351	13	154.9	50.0
352	13	165.1	50.0
450	14	167.6	63.6
451	14	165.1	50.0
452	14	170.2	59.0
453	14	162.6	56.8
550	15	154.9	62.7
551	15	162.6	61.4
552	15	168.9	56.8
553	15	165.1	56.8
554	15	172.7	59.0
555	15	160.0	61.4

*12 y girls who had experienced menarche.

APPENDIX S

Per capita income and educational levels of parents of subjects.

Subject	PCI*	EF	EM	EP
200	12500	2	2	2
201	7500	2	1	2
202	5000	2	2	2
203	10625	1	2	2
204	21250	2	2	2
205	6700	1	2	2
206	12500	2	2	2
207	6000	2	2	2
250	6700	2	2	2
251	16700	2	2	2
252	10625	2	2	2
253	5000	1	2	2
254	5000	1	2	2
255	5000	1	1	1
256	12500	2	2	2
350	7500	2	1	2
351	10625	2	2	2
352	5000	2	2	2
450	5000	1	2	2
451	6000	1	1	1
452	6700	2	1	2
453	5000	1	1	1
550	10000	1	1	1
551	5000	1	1	1
552	10625	1	2	2
553	12500	2	2	2
554	6700	2	1	2
555	6700	1	1	1

* Per capita income.
EF, Education of father.
EM, Education of mother.
EP, Combined education of father and mother
1, high school; 2, college.

APPENDIX T

Hemoglobin and hematocrit values of subjects

Subject	Hb* g/dL	Hct[t] ratio
200	14.0	0.41
201	14.6	0.38
202	14.5	0.40
203	12.5	0.43
204	13.5	0.44
205	14.0	0.45
206	12.0	0.36
207	14.5	0.40
250	14.0	0.40
251	13.0	0.37
252	13.5	0.40
253	14.0	0.37
254	14.5	0.40
255	12.0	0.37
256	15.1	0.42
350	15.0	0.40
351	13.0	0.37
352	12.5	0.37
450	14.0	0.38
451	14.0	0.38
452	12.5	0.42
453	13.5	0.38
550	12.0	0.36
551	15.0	0.40
552	13.5	0.39
553	13.5	0.40
554	14.5	0.41
555	13.0	0.38

* Hemoglobin.
[t] Hematocrit.

APPENDIX U

Estimated daily nutrient intakes of subjects *

Subject	Kcal	Protein g	Vitamin B-6 µg	B-6/ protein ratio
200	2535.3	81.1	2787.5	0.034
201	2784.2	79.9	2045.0	0.026
202	2135.0	74.3	1360.0	0.018
203	2731.0	63.2	1189.0	0.019
204	2161.5	91.8	1535.5	0.017
205	1404.0	56.0	865.5	0.015
206	2369.0	102.3	1721.5	0.017
207	2153.0	85.4	1891.5	0.020
250	2595.0	77.8	2920.5	0.038
251	1633.0	78.7	1398.5	0.018
252	1940.0	77.6	3449.5	0.044
253	2322.0	111.5	1540.0	0.014
254	1177.0	56.3	971.0	0.172
255	1382.0	76.1	959.5	0.013
256	3206.0	130.1	1396.0	0.010
350	1480.0	57.8	717.5	0.012
351	1995.0	77.5	1884.0	0.024
352	2398.0	93.3	1303.5	0.140
450	2064.0	81.0	1411.0	0.017
451	2349.0	76.9	1529.5	0.020
452	2328.0	58.2	1276.0	0.021
453	1886.0	75.7	732.0	0.010
550	2008.0	65.8	1187.5	0.018
551	2597.0	86.9	1661.5	0.019
552	2233.0	73.4	874.0	0.012
553	2353.0	95.6	960.0	0.010
554	1575.0	54.7	839.5	0.015
555	1140.0	54.1	1121.5	0.021

* Calculated from 24-h recall followed by 2-d dietary record.

APPENDIX V

Plasma B-6 vitamers and EALAT activities of subjects

Sub-ject	RPLP*	FPLP	UPLP	HPNP	UPNP	4-PA	PMP	PL	PN	PM	EALAT-AC
	ng/mL										%
200	28.9	30.1	31.0	0.0	0.0	10.2	0.0	0.0	3.7	0.0	7.1
201	16.5	16.8	16.4	0.0	0.0	0.0	2.0	0.0	0.0	1.0	11.8
202	14.0	13.4	13.7	0.0	0.0	0.0	0.0	0.8	0.0	0.0	14.3
203	18.8	18.4	18.2	0.0	0.0	0.0	1.0	1.6	0.0	1.0	11.1
204	24.6	25.0	24.5	1.0	0.8	2.9	0.0	0.0	0.0	0.5	5.0
205	15.0	15.1	13.7	1.0	0.0	0.0	0.0	0.0	0.0	1.0	11.1
206	12.3	13.4	13.7	0.0	0.0	2.9	0.0	0.0	0.0	1.0	13.7
207	23.7	25.0	24.0	0.0	0.0	2.9	0.0	0.0	0.0	0.5	5.3
250	26.1	25.0	25.5	0.0	0.0	5.8	0.0	0.0	0.0	0.0	0.0
251	25.0	25.0	27.2	0.0	0.0	1.5	0.0	0.0	0.0	0.0	6.3
252	20.0	20.0	18.2	4.0	3.8	0.0	0.0	0.0	0.0	0.0	11.9
253	21.1	20.1	18.0	1.4	1.0	3.3	0.0	0.0	0.0	0.0	11.2
254	21.2	20.1	21.8	0.0	0.0	1.2	0.0	0.0	0.0	0.0	11.2
255	14.1	13.6	13.6	0.0	0.0	0.0	0.0	0.0	0.0	0.5	15.4
256	10.7	10.0	10.0	1.0	0.0	0.0	0.5	0.0	0.0	0.5	12.5
350	18.5	18.0	18.2	0.0	0.0	0.0	0.0	0.0	0.0	2.0	6.7
351	18.2	20.0	18.1	0.0	0.0	0.0	0.0	0.0	0.0	2.0	8.7
352	12.0	13.4	13.7	0.0	0.0	4.0	0.0	0.0	0.7	0.5	13.7
450	17.0	17.0	16.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.7
451	26.3	25.0	24.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0
452	21.2	20.0	23.0	1.0	1.1	0.0	0.0	0.0	0.0	0.0	2.2
453	23.1	22.5	22.8	1.4	1.0	1.5	0.0	0.0	0.0	2.0	2.0
550	15.2	15.1	16.4	1.0	0.0	0.0	1.0	2.5	0.0	1.3	14.5
551	14.7	13.4	13.9	0.0	0.0	0.0	0.0	0.0	0.0	2.5	13.6
552	19.5	20.0	19.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1
553	20.1	20.1	22.8	1.4	0.0	2.0	0.7	0.0	1.1	0.0	5.5
554	20.7	20.1	20.1	2.2	0.0	1.5	0.0	0.0	0.0	0.5	7.4
555	23.4	23.5	24.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.2

* RPLP, Radiomonitored Pyridoxal Phosphate.
FPLP, Fluorometric detector, PLP.
UPLP, UV detector, PLP.
HPNP, Fluorometric detector, Pyridoxine Phosphate (PNP).
UPNP, UV detector, PNP.
4-PA, 4-Pyridoxic acid.
PMP, Pyridoxamine Phosphate.
PL, Pyridoxal.
PN, Pyridoxine.
PM, Pyridoxamine.
EALAT-AC, Erythrocyte alanine aminotransferase activity coefficient.

APPENDIX W

Urinary 4-PA, 4PA/creatinine ratio, and % dietary intake excreted as 4-PA

Subject	4-PA µmol	4-PA/creatinine ratio µmol/mmol	Dietary intake excreted as 4-PA %
200	5.5	0.80	33.3
201	7.8	1.10	64.5
202	4.8	0.56	60.0
203	7.1	1.25	101.2
204	6.0	0.80	65.9
205	3.0	0.58	58.8
206	6.5	1.10	63.7
207	11.5	1.10	102.0
250	13.6	1.22	79.1
251	5.8	1.32	69.9
252	19.1	1.74	93.6
253	5.5	2.50	60.4
254	1.9	1.90	33.3
255	1.8	0.39	31.6
256	3.8	0.22	45.8
350	3.0	0.24	69.8
351	9.8	0.71	88.3
352	5.2	0.63	67.5
450	2.0	0.17	23.0
451	3.5	0.60	38.9
452	4.4	0.70	57.9
453	1.1	0.13	25.6
550	1.9	0.15	27.1
551	5.8	0.35	59.2
552	2.0	0.27	39.2
553	2.1	0.11	36.8
554	3.2	0.46	64.0
555	2.0	0.20	30.3

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