SEPARATION AND QUANTITATION OF B-6 VITAMERS IN RAT PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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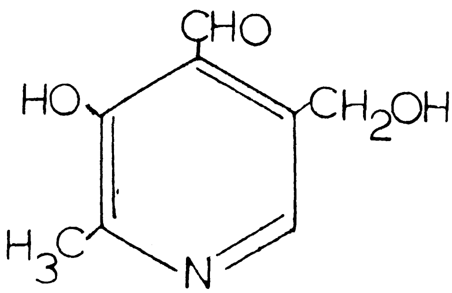
INTRODUCTION

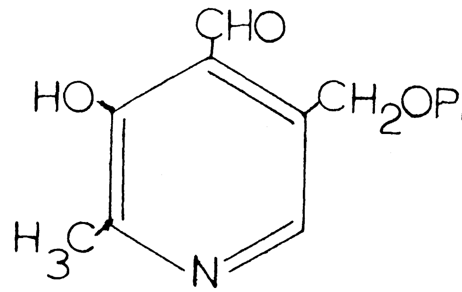
The first half of the 20th century was marked by an intensive exploration concerning the structure and role of vitamins. In 1934 Paul Gyorgy identified "rat pellegra preventative factor" as vitamin B-6, thus differentiating this factor from riboflavin. During this decade, vitamin B-6 was isolated in pure crystalline form by several independent laboratory groups (1). Research continued and before the close of the 1930's the chemical structure of B-6 was characterized and the compound had been synthesized.

Vitamin B-6 exists in 3 nonphosphorylated forms: pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM). The chemical names of these vitamers are: PN, 3-hydroxy-4 -5-bis (hydroxymethyl) - 2-2methylpyridine; PL, 3-hydroxy-4- formyl-5-hydroxymethyl-2-methylpyridine; and PM, 3-hydroxy-4-aminomethyl-5-hydroxymethyl-2-methylpyridine (2). Functional substitution occurs at the number 4 position. PN, PL, and PM exist in a 5'-phosphate form designated as pyridoxine-5'-phosphate (PNP), pyridoxal-5'-phosphate (PLP), and pyridoxamine-5'-phosphate (PMP). (Structural formulas, Figure 1).

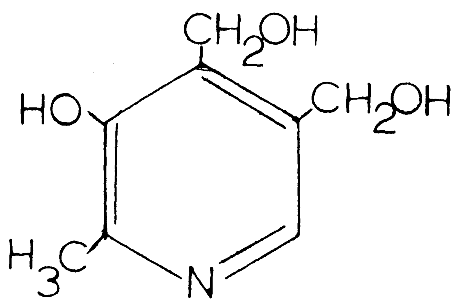
Vitamin B-6 in complex biological matrices presents diverse challenges to the investigator qualitating and quantitating this group of compounds. Presently the methodology most often employed for assessing vitamer content of serum or plasma is the microbiological assay using Saccharomyces uvarum (3); in addition, status is determined measuring PLP levels via decarboxylation of L-tyrosine (4-6), and the coenzyme stimulation of alanine adinotransferase (EC 2.6.1.2,



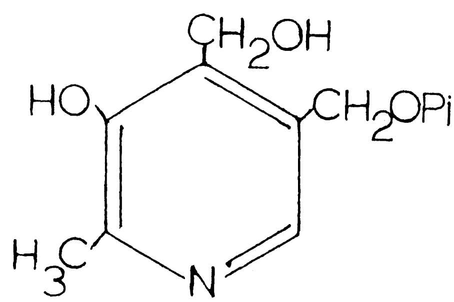
Pyridoxal (PL)



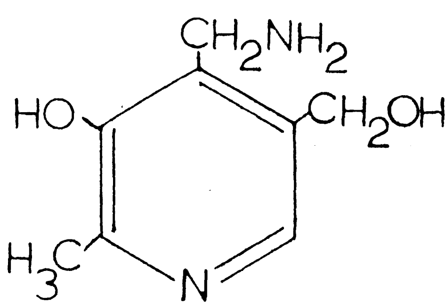
Pyridoxal 5'-phosphate (PLP)



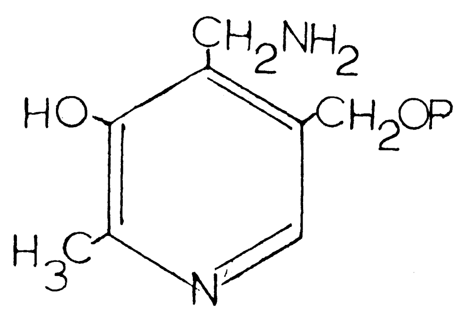
Pyridoxine (PN)



Pyridoxine 5'-phosphate (PNP)



Pyridoxamine (PM)



Pyridoxamine 5'-phosphate (PMP)

Figure 1

Structural formulas of the B-6 vitamers

L-Alanine: 2-oxoglutarate aminotransferase) in erythrocytes (7, 8).

High performance liquid chromatography (HPLC) is very useful in separating and quantitating compounds that are similar in structure (9). HPLC is capable of accurate quantitation and speed in analysis as well; thus, HPLC is an analytical tool suitable for vitamin B-6 analysis.

In view of the analytical capabilities of HPLC, the goals formulated for this research were:

1) the development of an HPLC separation and quantitation of the B-6 vitamers;
2) the development of a method of sample preparation compatible with HPLC analyses;
3) the development of an HPLC methodology of adequate sensitivity to detect and quantitate the B-6 vitamers in rat plasma; and
4) to quantitate the B-6 vitamer content in the plasma of rats in varying states of vitamin B-6 nutriture.

REVIEW OF LITERATURE

The analysis of vitamin B-6 in biological samples presents the researcher with several challenges which must be met in order to obtain accurate results. First, vitamin B-6 is extremely light sensitive; at a pH 7.8 a 10 ng sample of PLP is half destroyed when exposed to 30 min of laboratory "daylight" (10). In addition to light sensitivity, the analytical method employed must be able to detect and resolve a group of structurally similar compounts--PN, PL, and PM. Furthermore, vitamin B-6 is present in ng quantities in tissues that are of significance to the researchers concerned with nutritional status (11). Lastly, one of the major forms of vitamin B-6 in plasma is protein-bound principally to albumin (12, 13).

Various methods have been developed for the separation and quantitation of vitamin B-6. Such methodologies can be classified into three categories: microbiological, enzymatic, and chemical. The first classification discussed is microbiological.

Microbiological assays have been the basis of vitamin B-6 determination in food and other biological materials (14). Early work in this field focused on selection of an organism suitable for determining PN, PL, and PM (15). Organism selection is an ongoing concern. Much work was done developing an appropriate sample extraction as outlined by Storvick and Peters (16). Presently, _Sacchromyces uvarum_ (ATCC 9080) is the organism of choice for B-6 analysis (17).

Although favored by some as the most reliable method of B-6 analysis, the microbiological assay has several disadvantages.

Foremost lengthy procedure time, as well as variability in growth response, interfering impurities, which in turn would contribute to a degree in error in the spectrophotometric determination of turbidity (18), and lastly, differences in reported values among laboratories (19).

The second category discussed are enzymatic assays. These procedures employ B-6 dependent enzymes, most notably L-tyrosine carboxylase, EC 4.1.1.25, and L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2.

In plasma, PLP levels are determined radiometrically using labeled L-tyrosine. The reaction monitored is: L-tyrosine $^{14}C \longrightarrow$ tyramine + $^{14}CO_2$ involving PLP as the cofactor and L-tyrosine carboxylase (5). In the presence of excess apoenzyme and substrate, the amount of product, $^{14}CO_2$, is proportional to the level of PLP (11).

In erythrocytes, the coenzyme stimulation of alanine aminotransferase is useful in determining the stage of vitamin B-6 depletion or repletion (20). The reaction of interest is: L-alanine + α keto-glutarate $\longrightarrow$ pyruvate + L-glutamate involving PLP and the PLP dependent enzyme L-alanine:2-oxoglutarate aminotransferase.

Erythrocytes contain a higher aminotransferase activity than does plasma; in addition, a wide range of activity has been observed in plasma aminotransferase values (21). As a result, monitoring of this enzymatic reaction is confined to red cells and not monitored in plasma.

Enzymatic analysis, by their nature, are highly specific and in the case of the L-tyrosine carboxylase and the L-alanine:2-oxoglutarate aminotransferase reactions, the cofactor PLP is the only vitameric form of vitamin B-6 evaluated. Conversely, two positive aspects of enzymatic analysis are: the ability to quantitate PLP to 10^{-11} mole range and the convenience of using biological extracts without extensive purification or isolation prior to analysis (22).

The third category of discussion is chemical analysis of vitamin B-6. Grouped under this classification is the rapidly growing field of chromatographic analysis. Advantages of chemical assays, particularly chromatographic methods are the potential of directly quantitating the different vitameric forms of B-6 (18). Unlike enzymatic analysis, chromatographic methods require extensive sample preparation prior to analysis. In this respect chromatographic sample preparation objectives are similar to those of B-6 microbiological assays.

Most of the work with vitamin B-6 has been confined to either ion exchange chromatography or reverse phase chromatography. The B-6 vitamers are ionogenic, thus, ion exchange chromatography has been extensively investigated as a means of qualitating and quantitating B-6 vitamers. Ion exchange chromatography is accomplished using packing material that possesses charge bearing functional groups. The mechanism of separation is as follows: sample ions that interact weakly with the charged functional group of the packing, the ion exchanger, in the presence of competing ions in the mobile phase are weakly retained and consequently eluted first. A stronger interaction between sample ions and the ion exchanger, results in stronger retained sample ions, thus compounds of this nature elute later (23).

Ion exchange analysis of B-6 has centered on the use of basically 2 packing materials: Aminex A-5 (Bio-Rad Laboratories, Richmond, CA) and Zipax SCX (E. T. Dupont de Nemours and Co., Instrument Products Division, Willington, DE). Aminex A-5 packed columns are composed of polystyrene divinylbenzene which is 8% crosslinked and has SO_3 as the ion exchanger functional group (23). Research has progressed from separation of vitamers in simple matrices to separation of vitamers in complex matrices such as systems and biological materials. Yasumoto et al. (24) separated PN, PL, and PM on a column packed with Aminex A-5 thermostated at 65^oC. The solvent system was composed of 0.4M NaH_2PO_4 buffer at 3 pHs:pH 4.30, 5.15, and 5.80. Peak shape and retention time were very sensitive to buffer changes, therefore, exact timing of buffer transitions was essential. Linearity, for the vitamers was determined to be between 5 mg/ml to 60 mg/ml. Separation time was quite lengthy--approximately 2 hours. Williams and Cole (25) separated PN, PL, and PM on Aminex A-5 resin employing 0.7M ammonium formate pH 5.60 and UV detection. Isocratic and gradient elution profiles were investigated; changing buffer concentration in a gradient elution resulted in superior separation of PN from interfering compounds, as well as increased retention time and concurrent peak broadening. The researchers were able to analyze PN in vitamin capsules. Chicken muscle, dried beans, and whole wheat samples were attempted, but peak overlapping and interference resulted in an unsatisfactory separation.

Preliminary work in separation of vitamin B-6 in pharmaceutical preparations has been done by several researchers using Zipax SCX packed columns. Zipax SCX contains a sulfonated fluorocarbon, a strong cation exchanger, which is mechanically coated on to a pellicular support (23). Williams et al. (26) using a system composed of Zipax SCX column, UV detector, and 0.1M KH_2PO_4 pH 4.4 solvent was able to separate PN, PL, and PM within a 15 min time period. Changing the composition of the mobile phase by replacing the ionic modifier with NaH_2PO_4 and retaining the same pH separation of PMP, PL, and PN were achieved in a 25 min period.

Callmer and Davis (27) separated several water-soluble vitamins, including B-6 in the PN form, using a similar pellicular coated strong cation exchanger. Column efficiency was examined with respect to ionic strength, pH, and temperature. Results indicated that retention time was influenced by pH and ion exchange effects.

Cation exchange has been used to determine PN, PL, and PM content of fruits and vegetables--specifically apple, carrot, chard, mango, pear, spinach, and squash (28). The enzyme, taka-diastase (EC 3.4.23.6) was used to dephosphorylate and the enzyme papainase (EC 3.4.22.2) was used to deproteinate the sample. Dowex AG 50W in the K^+ form was used to remove interfering components from the sample. The Zipax SCX column was kept at 40^oC and the solvent used was 0.1K KH_2PO_4 pH 4.35.

An anion exchange chromatographic system has been developed by Vanderslice et al. (18, 19). The system developed consisted of: an Aminex A-25 resin column; a solvent system composed of buffers at

2 pHs:0.4M NaCl/0.01M glycine pH 10.0 adjusted with NaOH and 0.4M NaCl/ 0.01M glyine pH 2.5 adjusted with HCl. Solvent delivery was regulated by a 6 way switching valve; and detection was done by a fluorescence spectrophotometer. PL, PN, PM, PLP, PNP, and PMP were separated and detected using a complex system of 2 columns and 2 fluorescence wavelength combinations. PMP, PM, PNP, and PN were separated on the first column and detected using 310nm excitation and 380nm emission wavelengths. The second column was put online at this point and PLP was resolved; concurrently the fluorometric wavelength was changed to 280nm excitation and 487nm emission to enhance PLP detection. The detector was switched to original wavelengths (310nm, 380nm) for 3-hydroxypyridoxine, the internal standard, detection. Elution of PL was accomplished by switching solvent flow to the first column exclusively. Separation of the 6 vitamers and the internal standard was completed in approximately 60 min. This chromatographic system was used to analyze ready-to-eat breakfast cereals (29) and human plasma (30, 31). Only 2 subjects were used for human plasma separation. In contrast to previously reported values, PN was found to be the major form of the vitamin present.

In conjunction with the chromatographic system developed a compatible sample preparation method was developed (32). Previous extraction methods, such as acid hydrolysis and autoclaving, cleave the phosphates from PNP, PLP, and PMP. Acids which do not cleave phosphates, such as metaphosphoric and perchloric (33) were found to be incompatible with the chromatographic system developed by Vanderslice. Sulfosalicylic acid (SSA) in combination with NaF, to inhibit tissue phosphatase activity, were used to extract the 6 vitamers. NaF blocked dephosphorylation by tissue phosphatases and the subsequent addition of SSA resulted in deproteinization of the sample. SSA was removed from the sample before analysis due to its highly fluorescent nature. SSA removal was performed using Dowex AG 2-X8 anion exchange resin; SSA binds tightly to this resin.

Reverse phase chromatography is the term given to chromatographic conditions in which a relatively nonpolar stationary phase is used in conjunction with a polar mobile phase. In most cases the stationary phase contains a chemically bonded C_8 or C_{18} hydrocarbon and the mobile phase is aqueous based combined with a miscible organic (34).

As with ion exchange, preliminary work in reverse phase separation of B-6 involved separation of water-soluble vitamins in a multivitamin sample (35). PN was separated using a μBondapak C_{18} and μBondapak NH_2 column. Composition of the solvent system was varied with respect to: ratio of water:methanol, type of salt-phosphate, citrate, tartrate, and chloride, concentration of salt, and ion pairing reagent.

Gregory and Kirk (36) investigated the vitamin B-6 content of dehydrated food systems using a μBondapak C_{18} column, fluorescence detector, and a mobile phase of 0.033M postassium phosphate buffer pH 2.2. The food samples were prepared for analysis using a 2 step procedure; first, potato acid phosphatase--orthophosphoric monoester phosphohydralase (EC3.1.3.2) was added to hydrolyze the phosphate esters and a 20% TCA solution was added to stop this reaction. Secondly, further protein precipitation was accomplished through the use of a 15 min $50^{O}C$ water bath. Such sample preparation allowed for the chromatographic separation and quantitation of PN, PL, and PM. The same chromatographic conditions were used in determining the B-6 content of various fortified breakfast cereals (37). Sample preparation was similar to the afore mentioned procedure; the dephosphorylation step was eliminated due to the negligible presence of PLP. Detection limits were established at 0.1-0.5 mg/g for PN, PL, and PM. The predominate vitamer isolated was PN. The HPLC results were found to correlate well with data obtained from the microbiological Saccharomyces uvarum method.

Expanding the use of phosphate buffer pH 2.2 mobile phase/C_{18} column, Lim, Young, and Driskell (38) isocratically separated PL, PN, PM, and deoxypyridoxine, the internal standard, in an 11 min period. Bread, milk, and peas were analyzed for PN, PL, and PM content using the same chromatographic conditions (39).

The B-6 content has been determined in various biological matrices through the use of reverse phase chromatography. In order to accommodate the differences between biological matrices and food systems, Gregory (40) modified the sample preparation and chromatography used previously fortified breakfast cereals (37). PLP content was determined in rat liver, and muscle, as well as, rabbit liver and brain. The samples were homogenized in a phosphate buffer, 0.1M KH_2PO_4 pH 7.0, and 3N perchloric acid (PCA) was added to precipitate the proteins. PCA was removed from the sample by the addition of 3N KOH which resulted in the formation of insoluble $KClO_4$. Semicarbazone was added to form a PLP-semicarbazone derivative, thus enhancing retention and fluoresence yield for the derivative compared to the underivatized PLP. Detection limits were established to be about 0.2 µg PLP/ml, however, inspite of derivatizing, human and rabbit plasma PLP could not be accurately measured.

Further modification (41) of the semicarbazone derivatization method was used to analyze calf liver, ground beef, rat brain, and whole milk. The modification consisted of the use of sodium glyoxylate to convert PM and PMP to PL and PLP respectively prior to the addition of the semicarbazone. Sample homogenization and protein precipitation techniques remained the same. Quantitation of the vitamers was based on peak heights of semicarbazone derived PL and PLP in the presence and absence of the sodium glyoxylate. Thus, the addition of the glyoxylate allowed for PM and PMP to be indirectly quantitated as well as PL and PLP which were previously quantitated with the sole addition of semicarbazone. The use of a C_{18} column with a 5 mm particle size in this set of analyses served to increase column efficiency and facilitate quantitation. The detection limits were reported as 0.2 µg/g tissue and 0.05 µg/ml of the milk sample. These detection limits preclude analysis of human plasma.

Most researchers have used a simple reverse phase system with nonpolar stationary phase and polar solvent, although, a few researchers preferred an ion pairing system. Ion pair chromatography was developed to deal with separating compounds that are either very polar, multiple ionized or strongly basic. Advantages of ion pair chromatography when compared to ion exchange chromatography are: ion pair columns tend to be more efficient than ion exchange columns, reverse phase columns used for ion pair chromotography tend to be more reproducible from lot to lot than ion exchange columns, and ion pair columns tend to be more stable than ion exchange columns (42).

At this time, there are 2 schools of thought on the possible mechanism for ion pairing chromatography, otherwise referred to as pair ion chromatography. In the first model, positive charge of the cationic molecule (the charged sample molecule) pairs with the negatively charge organic counter ion of the mobile phase ion pair to form a neutral ion pair complex which partitions into the nonpolar stationary phase. The basis of separation for this model is the hydrophobic interaction between the nonpolar stationary phase and the neutral ion pair. The second model for ion pair chromatography assumes that the alkyl chain of mobile phase ion pair partitions into the alkyl chain of the nonpolar stationary phase, thus exposing the charged ion of the ion pair to the sample. In essence, the nonpolar stationary phase will act like an ion exchange column. Regardless of conflict over mechanism of separation, ion pair chromatography is dependent upon: counterion size, type, and concentration; temperature; mobile phase pH; and type of concentration of the organic modifier (43, 44).

Stewart et al. (45) used reverse phase ion pair chromatography to separate PN and isoniazid in a pharmaceutical preparation. The column was a nonpolar C_{18} column and the mobile phase was methanol: water, 60:40, at pH 2.5, containing 0.01M dicotyl sodium sulfosuccinate as the ion pair. Various ratios of methanol:water were investigated, as well as the effect pH exerts on resolution.

Kirchmeier and Upton (46) separated PN from a multivitamin blend of niacin, niacinamide, pyridoxine, thiamin, and riboflavin using reverse phase ion pair chromatography. The column was μBondapak C_{18} column and the mobile phase was 25:75, methanol:water with the aqueous portion composed of 0.005M sodium hexanesulfonate. The complete elution of all 5 water-soluble vitamin was completed in 35 min.

HPLC methods for analysis of vitamin B-6 have the advantages of being rapid, precise, and are able to directly measure the vitamers. Presently, most methods for analysis of B-6 in plasma use ion exchange chromatography. This research attempted to expand the use of chromatography for the analysis of B-6 in rat plasma by utilizing the advantages of ion pair chromatography for a rapid analysis of the B-6 vitamers.

MATERIALS AND METHODS

Method development - HPLC of standards

The chemicals and vendors utilized in the HPLC analyses are listed in Appendix 1.

A. Analytical System

The HPLC instrumentation used for the experimental procedures consisted of a Waters Associates (Waters Associates, Inc., Milford, MA) liquid chromatograph. The HPLC components are listed below.

1) Model 730 Data Module
2) Model 720 Systems Controller
3) Model M-45 Solvent Delivery Pumps - quantity 2
4) Model U6K Universal Chromatograph Injector
5) Guard column packed with Bondpack C_{18}/Corasil 37-50 μm
6) μBondapak C_{18} analytical column (3.9 mm x 30 cm) 10 μm particle size
7) Model 440 Absorbance Detector, 280 nm filter
8) Model 420 E/420 AC Fluorescence Detector fitted with a G4T5 mercury lamp, 300 nm band-pass with phosphor excitation filter, and 375 nm long-pass emission filter.

B. Standards

Stock standard solutions of PN, PL, PM, PLP, PNP, PMP, and DPN were made on the basis of free base weight at a concentration of 1,000,000 ng/ml. Serial dilutions were performed to obtain working standard solutions. Instructions for making standards are given in Appendix 2.

C. Internal standard

The method of quantitation chosen for this research was the internal standard quantitation. An internal standard is used to compensate for analytical error such as loss of component during pretreatment or extraction procedures, imprecise injection volume, or changes in chromatographic conditions (47). When using a syringe type injector such as the U6K injector, internal standard quantitation is the primary mode of quantitation.

The compound considered for use as an internal standard must elute close to the compounds of interest, in other words, have a similar K' value, and the compound must also be completely resolved from all other peaks. In addition, the internal standard must be structurally similar to the compounds of interest and behave in the same manner as the compounds of interest in the sample during sample extraction; however, the internal standard must not be present in the sample. Lastly, the internal standard must be stable and not react with the mobile phase, column packing, or sample components (47). For these analyses, deoxypyridoxine (DPN) was chosen to be the internal standard since DPN met the criteria for being an appropriate internal standard. The structure for DPN is shown in Figure 2.

D. Reverse phase chromatography - phosphate buffer

A reverse phase C_{18} column and 0.033M KH_2PO_4:CH_3CN solvent have been used to separate and quantitate the B-6 vitamers (37, 38). The instrumentation used by the current researcher to investigate the KH_2PO_4:CH_3CN solvent system is included in the Analytical

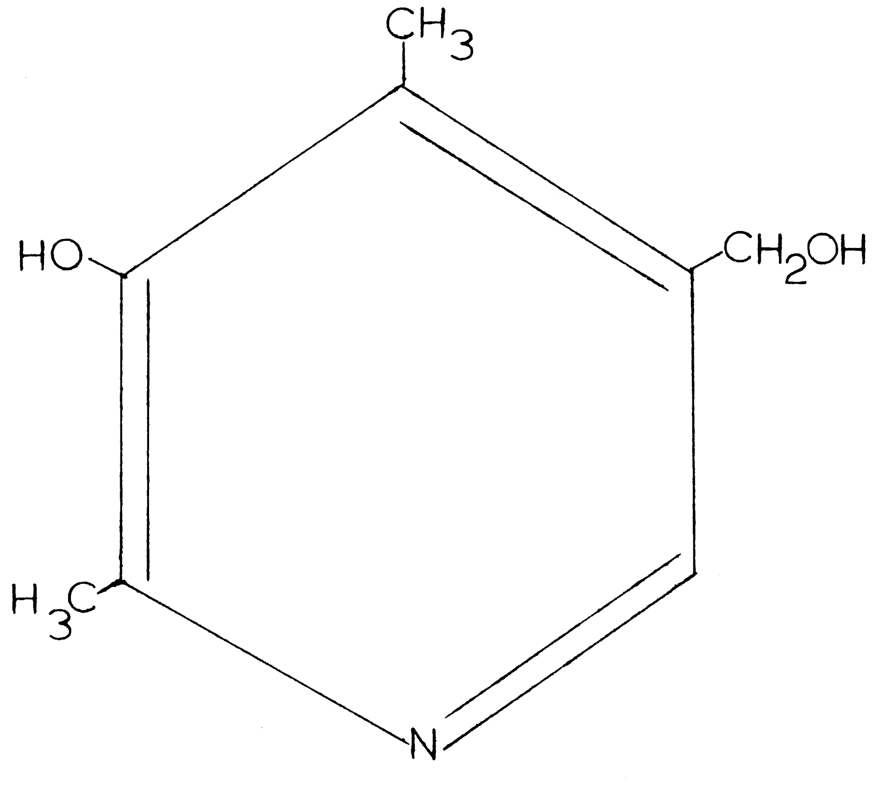


Figure 2

Structure of deoxypyridoxine (DPN)

system section. For this preliminary work, the Model 440 Absorbance Detector equipped with a 280 nm filter assembly was used. The preparation instructions for the 0.033M KH_2PO_4 buffer is given in Appendix 3.

E. Reverse phase ion pair chromatography

For the separation of ionic compounds, reverse phase ion pair chromatography may be used as an alternative to ion exchange chromatography. Thus far, PN has been separated in a multivitamin blend (46) using 0.005M sodium hexanesulfonate as the ion pair. In order to optimize separation of B-6 vitamer standards, counterion size, and concentration were tested.

F. Detection - fluorescence

When quantitating compounds in a complex matrix, fluorescence detection is advantageous due to the selectivity of this type of detector (48). The fluorescent nature of B-6 vitamers has been determined in their cationic, neutral, and anionic forms (49). The mobile phase of this system was at pH 3.3, thus rendering the vitamers to their cationic form. In these forms, the vitamers exhibit similar excitation wavelengths, thus suggesting use of a band-pass type filter. The excitation filter selected as optimum was the 300 nm phosphor coated band-pass filter. Emission wavelengths were determined to lie in the range of 378-402 nm, thus indicating the use of a long band-pass filter. The optimum emission filter selected was a 375 nm long band-pass filter.

G. Gradient

Isocratic and gradient programs were investigated to determine which program resulted in optimum resolution of the vitamers. Gradient elution is used to optimize the K' values of individual peaks during a separation. Several gradients were examined in which time, solvent composition, and curve number were varied. The curve number represents the degree to which the curve is curvilinear with curve number 1 having the greatest degree of concavity; curve number 6 being linear; and curve number 11 having the greatest degree of convexity (50).

H. Injection volume

To obtain maximum sensitivity the volume injected should be as large as possible and still maintain a sharp band. Injection volumes of 250 and 500 μl were investigated to determine the largest possible injection volume without the occurrence of band broadening.

J. Minimum detectable quantity

The minimum detectable quantities (MDQs) were determined for PL, PN, and PM using 250 μl injection volume. MDQ is defined as being the quantity equalivalent to a detector response twice the noise level (48).

Method development - sample extraction

When considering extraction methods for B-6 in plasma, several factors must be dealt with. B-6 is present in very low quantities in plasma, therefore, the method of sample preparation must have few dilutions. In addition, the extraction procedure must be able to cleave PLP from albumin, to which it is tightly bound (12). Also, the sample preparation method must be compatible with the HPLC chromatographic conditions and instrumentation.

Several methods of sample extraction appeared to warrant investigation into the suitability for use with rat plasma. The methods examined were: sulfosalicylic acid extraction, use of Sep-Paks (Waters Associates, Inc., Milford, MA), autoclaving, isoelectric point precipitation, salting out, and use of potato acid phosphatase.

A. Sulfosalicylic acid

Vanderslice et al. (32) proposed a method of sample preparation using sulfosalicylic acid (SSA) to deproteinate food samples and some biological samples (30, 31). The major problem with this method was the removal of the SSA from the sample prior to analysis when using a C_{18} column. To remove the SSA ion exchange resins were tested.

B. Sep-Pak C_{18}

Sep-Pak C_{18} cartridges used in conjunction with solvents of varying polarities have been used in sample extraction methods. In plasma, mebendazole has been extracted (51) and nucleosides have been extracted from serum (52). Variables tested for extraction of B-6 vitamers in plasma were: cartridge pretreatment, composition of eluting solvent, strength of eluting solvent, and volume of eluting solvent.

C. Autoclaving

The next set of sample extraction methods investigated were the sample extraction methods used in conjunction with microbiological assay (16). Basically the procedure involves two steps: deproteination, and dephosphorylation. The variables tested were: concentration of acid for deproteination, concentration of acid for dephosphorylation, and length of autoclaving time.

D. Isoelectric point

Proteins, especially globular type, are least soluble at their isoelectric pH. At this pH the protein molecules have no electrostatic repulsion, therefore the molecules coalesce and precipitate (53). The major protein in plasma is albumin, a globular protein (54). Hence, isoelectric point precipitation was attempted to precipitate the protein. The variables tested were time, temperature, and pH.

E. Salting out

The inorganic salt ammonium sulfate $(NH_4)_2SO_4$ was used to precipitate the proteins present in the plasma (55). The $(NH_4)_2SO_4$ binds H_2O, thus making the H_2O unavailable for the protein and results in protein precipitation. The variables tested were various concentrations of $(NH_4)_2SO_4$ added to the plasma.

F. Potato acid phosphatase

The method of Gregory et al. (36) was the basis for potato acid phosphatase, PAP, procedure for sample extraction. The procedure utilizes PAP to dephosphorylate and TCA to deproteinate the sample. The variables tested were concentration of PAP and concentration of TCA with the goal of extracting the sample with the least dilution volume and complete extraction of the B-6 vitamers.

Sample analyses - validation and quantitation techniques

A. Peak identification

In the sample, peak identification of vitamers was accomplished by comparing retention times of standards with retention times of the corresponding vitamer peaks in the sample; comparison of the ratio of adjusted vitameric retention time to adjusted retention time of the internal standard was also done; and external spiking was performed.

In addition to retention times, relative retention time and external spiking, extrachromatographic spectrofluorometry was utilized. The peak of interest was collected in a 2 ml amber bottler; the eluent for the peak was collected at the outlet of the fluorometric detector and collection was done in the time span which the apex of the peak of interest appeared. In cases when limits of detection for the spectrofluorometer were approached, several sample injections were made and the eluents were collected and concentrated to facilitate spectrofluorometry. Emission scanning was done from 330 nm to 500 nm at an excitation wavelength of 300 nm. Excitation scanning was done from 220 nm to 380 nm with an emission wavelength performed using a Perkin-Elmer 650-105 (Norwalk, CN) fluorescence spectrofluorometer.

B. Quantitation

Internal standard quantitation was the method used to calculate the concentration of each vitamer in the sample. A calibration curve was obtained by injection a calibration solution. The linear response of the vitamers permits the use of a single point calibration (47).

Calculations were performed using the following equation:

$$\text{Vitamer amount injected} = \frac{\text{Response factor x peak area}}{1000} \times \frac{\text{Area of the internal standard in the calibrating solution}}{\text{Area of the internal standard in the sample}}$$

Response factors are used to account for the unequal fluorometric response of each vitamer. Response factors were calculated using the following formula:

$$\text{Response factor} = \frac{\text{Amount of standard}}{\text{Area response}} \times 1000$$

C. Recoveries

Recoveries of vitamers were determined by adding the vitamers to plasma samples prior to sample extractions. The areas of the B-6 vitamer peaks in the "unspiked" sample were substracted from the areas of the corresponding peaks in the "spiked" sample. The ratio of the actual areas, obtained from subtracting "unspiked" from "spiked" sample, and the expected areas, obtained from injection of standards corresponding in concentration to the spike, was used to calculate the % recoveries.

Animal experiments

A. Rat experiment 1

Forty-eight weaning male albino rats (Sprague-Dawley) were randomly divided into 4 groups of 12 animals each. The animals were given a diet which contained 15% protein and was nutritionally adequate in all components (56) except for B-6, the experimental variable. Group 1 was fed a diet intending to provide a 15 μg B-6/day; group 2, 30 μg B-6/day; group 3, 45 μg B-6/day; and group 4, 90 μg B-6/day. The vitamin B-6 in the diets was in the form of PN HCl. Composition of the diets are listed in Appendix 4. The diets were fed ad libitum and actual daily dietary intakes means of vitamin B-6 were calculated from group average daily food consumption. The actual intakes were higher than the intended values and are listed in Table 1.

The vitamin B-6 requirement for growth and gestation of rats has been determined to be 6.0 mg/kg diet (56). On a daily intake basis, the B-6 requirement was found to be 45 μg for the rat (7, 57-59). Thus, several levels of PN were utilized in order to produce corresponding states of nutriture.

During the 15 week experimental period, the rats were housed in individual suspended galvanized cages and supplied with distilled water and diet ad libitum. Weekly body weight and food consumption values were recorded. The animals were sacrificed by electrocution and the blood was obtained via cardiac puncture using 7 ml vacutainers with EDTA anticoagulant and a 21 gauge needle. The blood was placed immediately in crushed ice and covered to protect the samples from light. The blood samples were centrifuged for 15 min at 5^{o}C at 5000 x g and the plasma portion was removed and stored at -20^{o}C until extraction.

B. Rat experiment 2

Thirty weanling male albino rats (Sprague-Dawley) were randomly assigned to 5 groups of 6 rats each. The diets, as detailed previous for rat experiment 1, were prepared with levels of PN HCl which would provide daily intakes of 15, 30, 45, 90, and 150 μg. Average group daily food consumption was used to calculate the actual daily dietary intakes of vitamin B-6. Table 1 lists the intended and actual vitamer intakes of these rats. The rats were kept on the

Table 1

Vitamin B-6 intakes - comparison of intended and actual intakes

Rat experiment 1		Rat experiment 2		Rat experiment 3	
Intended[1]	Actual[2]	Intended	Actual	Intended	Actual
		μg/day			
15	23	15	24	2	3
30	41	30	53	90	124
45	60	45	75		
90	127	90	152		
		150	248		

[1]Calculated from projected average daily consumption.

[2]Calculated from actual average group daily food consumption.

diets for 12 weeks and then were sacrificed by eletrocution. Samples were collected and treated in the same manner as in rat experiment 1.

C. Rat Experiment 3

Twenty-five weanling male albino rats (Sprague-Dawley) were randomly assigned to 2 groups. Group 1, the deficient group, contained 10 rats and was fed a diet (described in experiment 1) that had no additional B-6 added to the diet, although the casein component contained 0.65 μg PN/g casein; therefore the diet was not completely free of B-6. Group 2, the control group, contained 15 rats that were fed a diet (described in rat experiment 1) that intended to provide 90 μg B-6/day. The deficient group was supplemented with control diet at week 5, 10, and 18; supplementation was done when the deficient group of animals failed to gain weight and began to lose weight. Actual intakes of vitamin B-6 were calculated from average group daily food consumption (Table 1).

The animals were kept on the diet for a 27-31 week period and were sacrificed by electrocution or by use of CO_2. The use of CO_2 was necessitated by the malfunctioning of the electrocution unit. Samples were treated using the same procedure as in rat experiments 1 and 2 with the exception that the samples were extracted and analyzed on the same day as the animals were sacrificed.

Statistical analyses

Means ($\overline{X}$) and standard deviations (SD) were calculated for all appropriate data. Calculations of Pearson's correlation coefficients (r) were done for HPLC vitamer values for all 3 rat experiments. Analysis of variance (ANOVA) was used to analyze data of rat experiments 1 and 3. Duncan's multiple range test were used in the evaluation of rat experiment 1 data (60).

RESULTS AND DISCUSSION

HPLC method development

A. Reverse phase chromatography - phosphate buffer

Separation of the nonphosphorylated vitamers, PN, PL, and PM, as well as the internal standard DPN was attempted using modifications of the method of Lim et al. (38). Several v:v ratios of KH_2PO_4:CH_3CN were tested including 90:10, 95:5, 97:3, and 99:1. The best peak shapes and resolutions of standards were obtained utilizing a 95:5 mixture. A typical HPLC chromatogram of standards is given in Figure 3. Chromatographic conditions were as follows: flow rate - 1.0 ml/min, solvent composition - isocratic 95% KH_2PO_4:5% CH_3CN, injection volume - 25 μl, standard concentration - 1000 ng/ml, UV detection - 280 nm, and attenutation - 1.0 AUFS. The order of elution was PM, PL, and PN, and DPN; elution was completed in under 15 min.

Considering that PLP and PL are the major forms of B-6 in plasma (61), the previously described conditions were utilized in attempts to separate the phosphorylated (PLP, PNP, and PMP), as well as the nonphosphorylated vitamers (PL, PN, and PM). These attempts resulted in unresolved peaks and problems with K' values.

Drawbacks to this solvent system were: inability to separate the 6 vitamers; stress on the C_{18} column due to silica dissolving readily at pH 2.0 and below (34); and band broadening of the internal standard which would affect quantitation. Therefore, investigation of another solvent system was undertaken in order to rectify these problems. An overview of ion pair chromatography indicated possible utility for separation of the B-6 vitamers and was the next system investigated.

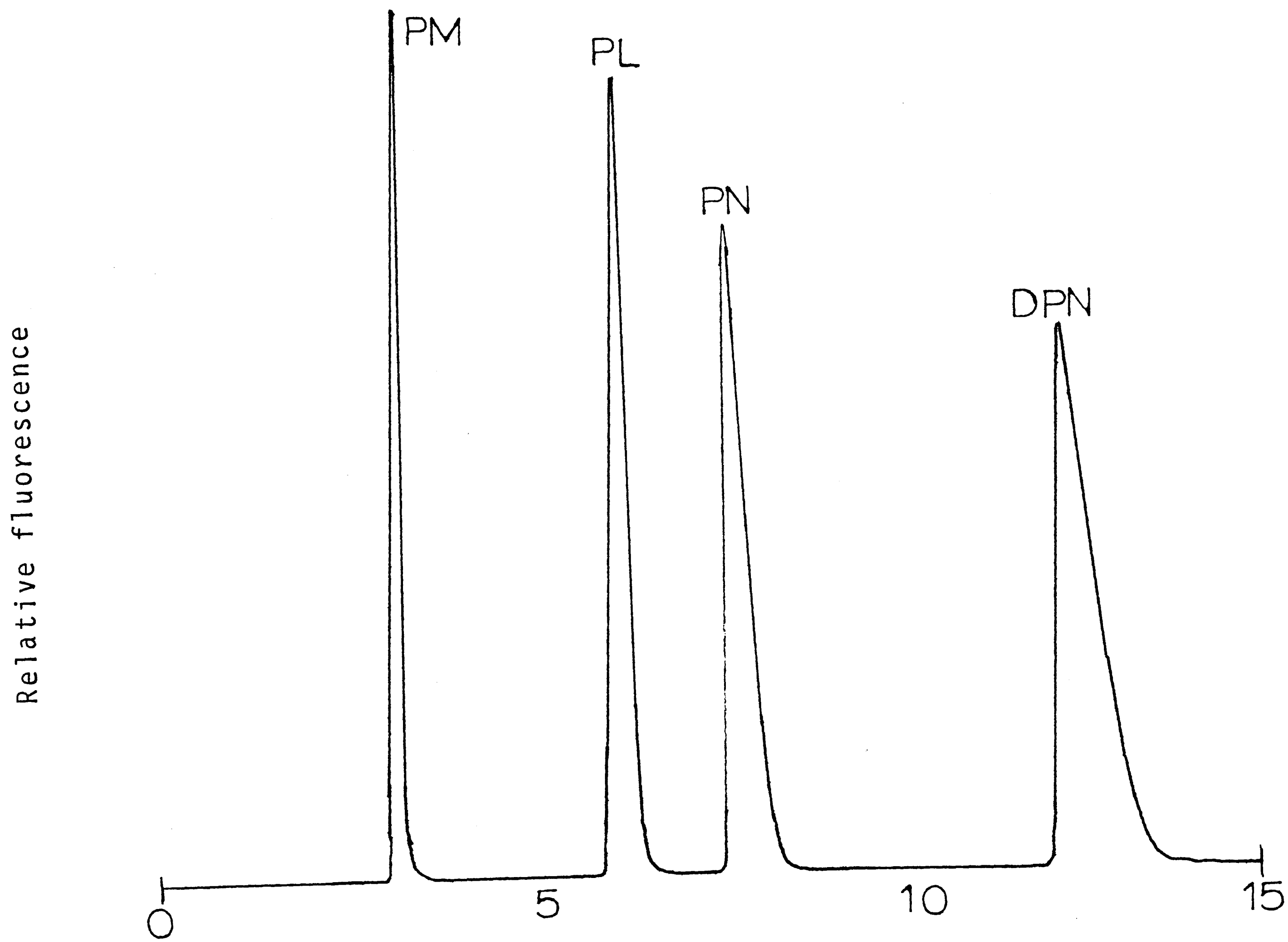


Figure 3
Typical chromatogram of standards using reverse phase chromatography

B. Reverse phase ion pair chromatography

The ability of the pyridine ring of the B-6 vitamers to protonate at a low pH indicates the potential utility of a reverse phase ion pair system for chromatographic resolution of the B-6 vitamers. In view of the positively charged ring N, a sulfonic acid ion pair was selected. Various alkyl chain lengths were tests including: octane sulfonic acid, heptane sulfonic acid, and hexane sulfonic acid; PIC B-8, PIC B-7, and PIC B-6 (Waters Associates, Inc., Milford, MA) respectively. Use of PIC B-8 resulted in a separation time of over 1 h for PL, PN, PM, and DPN; use of PIC B-7 resulted in a separation time of just under 20 min for the same vitamers; and use of PIC B-6 resulted in a separation time of less than 10 min, unfortunately the resolution was not baseline for the PIC B-6. Therefore PIC B-7 was selected based on the resolution and speed of separation.

Initially PL, PN, PM, and DPN were separated using an isocratic solvent profile and fluorescent detection (300 nm emission and 375 nm excitation). Isocratic profiles tested were: 10:90, 15:85, 20:80, 25:75, and 30:70 MEOH:PIC B-7, v:v. The best separation was achieved using the 30:70 mobile phase.

Attempts were made to resolve phosphorylated and nonphosphorylated vitamers. PMP remained unresolved under the isocratic conditions. Hence, several gradient programs, both linear and curvilinear, were tried without success. In an attempt to resolve PMP, PIC B-7 was added to the organic phase; the organic phase was changed to 85% MeOH:15% H_2O (v:v) since PIC is insoluble in 100% MeOH. Resolution of PMP was not improved. Increasing the concentration of the ion pair to 0.01 M $CH_3(CH_2)_6HSO_3$ did not improve PMP resolution either. Thus, PLP, PNP, PL, PN, PM, and DPN were the vitamers resolved under these conditions. Figure 4 shows a typical chromatogram for the separation of phosphorylated and nonphosphorylated B-6 vitamers using PIC B-7.

C. Gradient

The gradient program yielding optimal resolution for the B-6 vitamers is listed in Table 2. An illustration of this gradient is given in Figure 5. A typical chromatogram of standards is shown in Figure 6. The conditions were: flow rate - 1.5 ml/min, fluorescence detection - 300 nm excitation and 375 nm emission, and attenuation - 32.

D. Injection volume

Injection volumes of 250 and 500 μl for standards were investigated. The 500 μl volume resulted in band broadening and poor peak shape, whereas 250 μl injection did not exhibit these characteristics. Therefore, 250 μl was selected as the injection volume for standards. A 250 μl glass barrel Hamilton syringe was used equipped with a blunt needle.

E. Minimum detectable quantity

The MDQs determined for PL and PN were 5 ng and for PM was 4 ng. Attempts were made to reduce background noise so that MDQs could be decreased. Argon was bubbled through the PIC B-7 solvent to displace

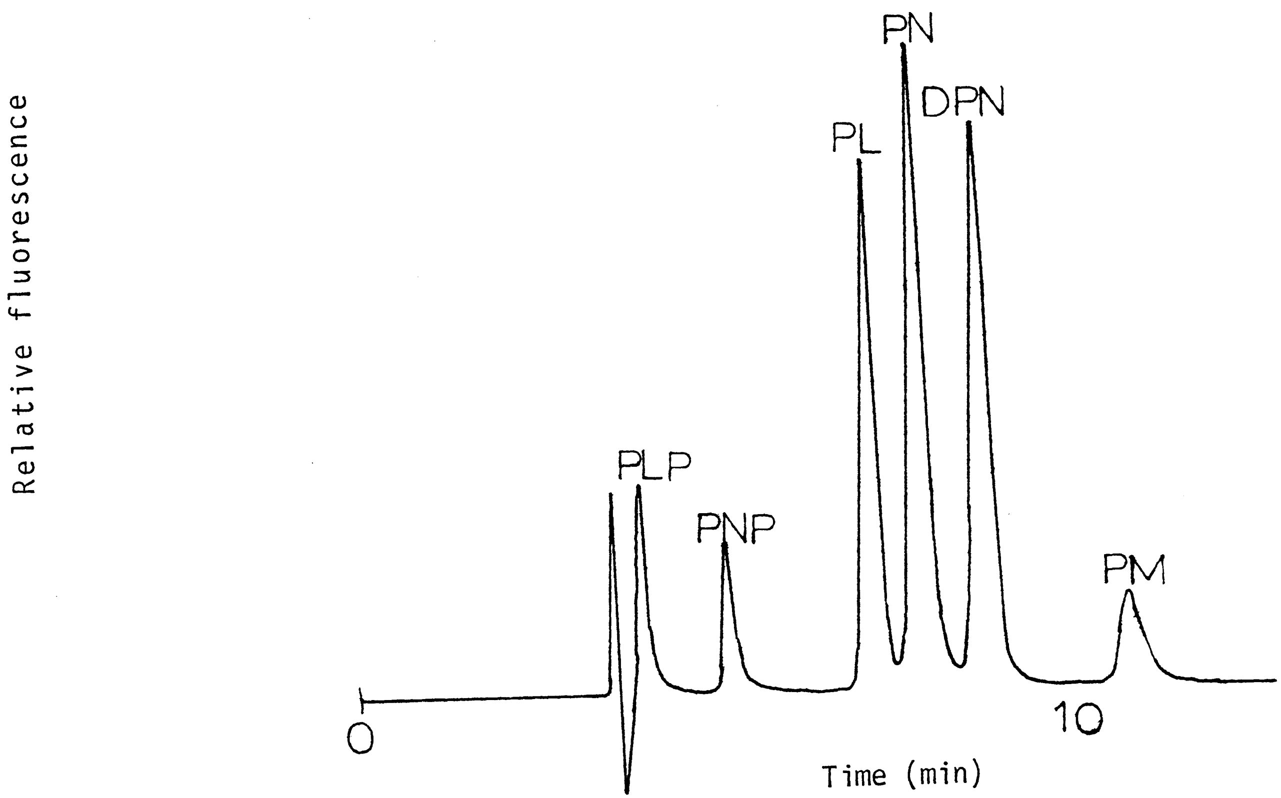


Figure 4
Typical chromatogram for the separation of phosphorylated and nonphosphorylated vitamers (standards)

Table 2

HPLC gradient program for resolution of

PL, PN, PM, and DPN

Time	Flow rate	Mobile phase composition A[1]	B[2]	Curve
min	ml/min	%		type
0	1.5	0	100	--
8	1.5	25	75	3[3]
12	1.5	40	60	9[4]
16	1.5	40	60	6[5]
20	1.5	0	100	6

[1]85% MeOH:15% H_2O, v:v.

[2]PIC B-7.

[3]Concave.

[4]Convex.

[5]Linear.

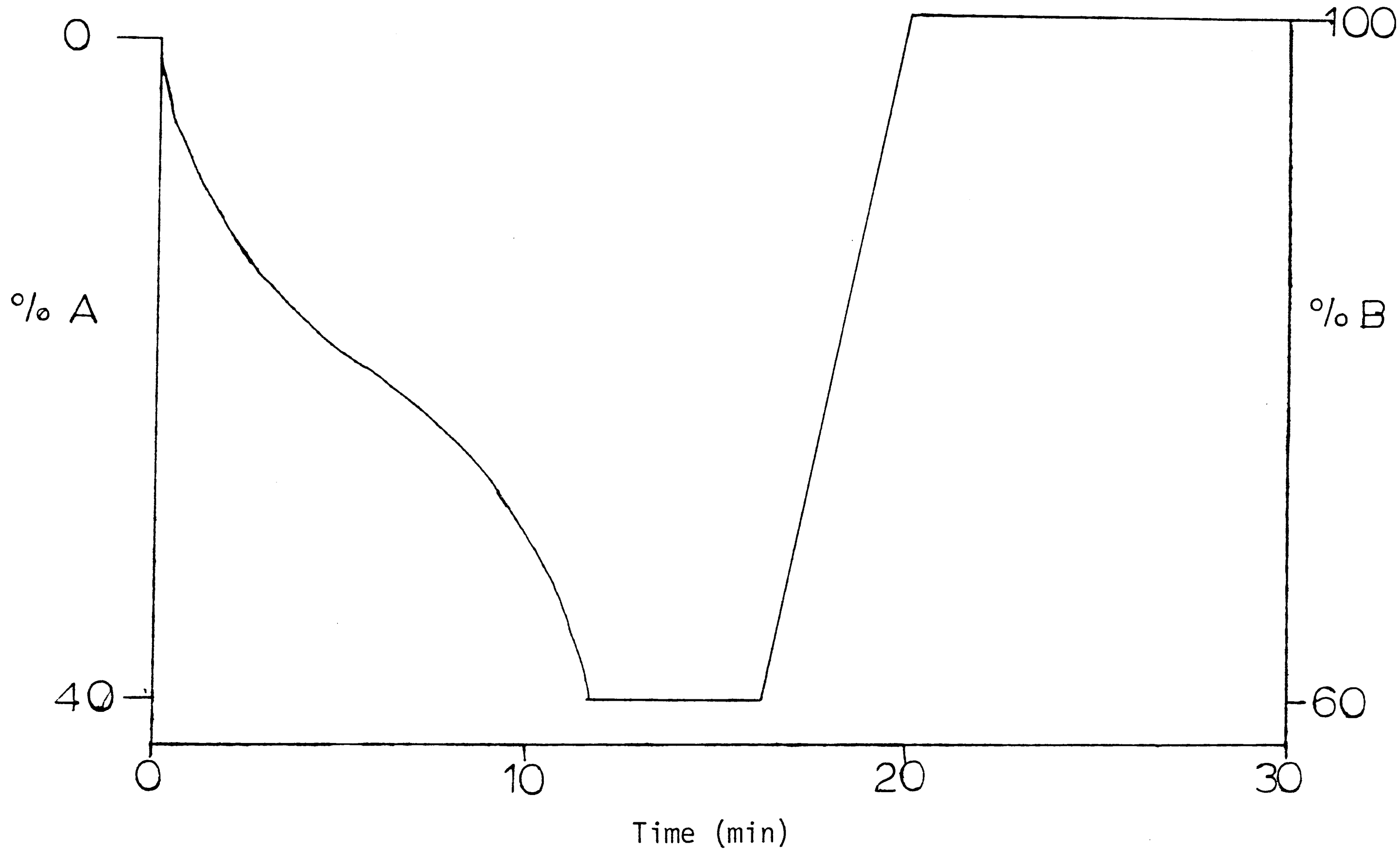


Figure 5

Illustration of gradient

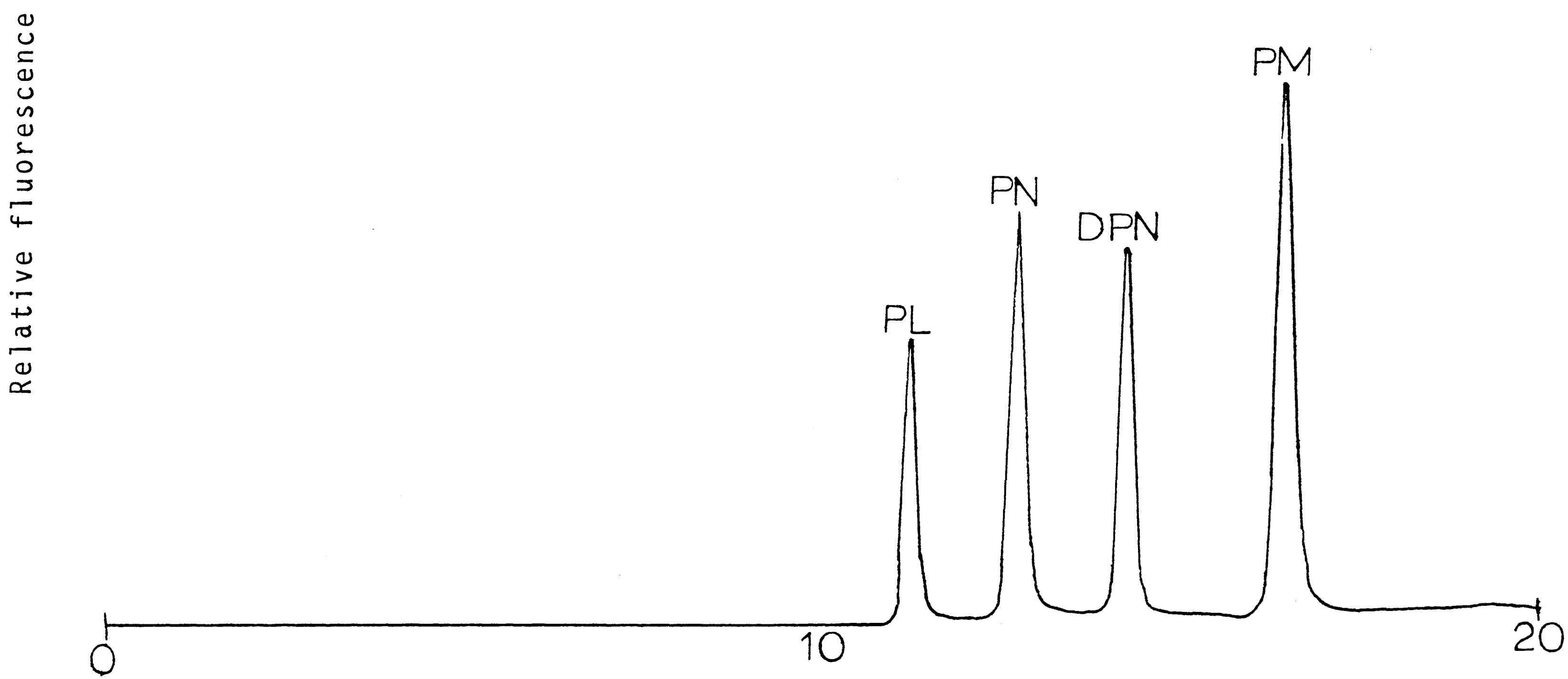


Figure 6

Typical chromatogram of B-6 vitamers (standards) only O_2 dissolved in the solvent; O_2 in solvents tends to increase background noise. The Ar had a minimal positive difference in the noise level.

Vitamers were tested for linearity between the range of 5-500 ng/ml and found to be linear. External calibration curves for standards were determined using 5 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml, 250 ng/ml, and 500 ng/ml. Table 3 shows the coefficient of correlation for the external standard calibration curves.

Sample extraction method development

A. Sulfosalicylic acid

The method of Vanderslice et al. (32) was the basis for the development of the modified SSA extraction method presented in Appendix 5. Several changes were attempted in order to adapt this extraction method for use with plasma. The major problem with this method was that the SSA must be removed from the sample prior to injection on to a C_{18} column. Several combinations of ion exchange resins, both anionic and cationic in series, were tried in conjunction with various pH washes. In attempting to remove SSA, the resin and wash must be such that the SSA is retained and the vitamers eluted. One problem with plasma is that the amount of solvent required to wash the column diluted the sample too greatly. The AG 2-X8 resin (Bio Rad Laboratories, Richmond, CA) equilibrated in 0.1 N HCl was selected as the resin which could remove the SSA. Unfortunately, recoveries of standards were less than 60% and the standards and vitamers in the sample matrix did not behave in an

Table 3

Coefficients of correlation for external standard calibration curves

Vitamer	Coefficient of correlation
PLP	0.9769
PNP	0.9976
PMP	0.9973
PL	0.9977
PN	0.9971
PM	0.9988
DPN	0.9978

[1]Coefficient of correlation computed via linear regression analysis.

identical manner. At such a low pH the PM peak was found to spontaneously split, therefore accurate quantitation was impossible.

B. Sep-Pak

Another sample extraction method investigated was the use of C_{18} Sep-Pak cartridges. Various pretreatments of the cartridges were tried including no treatment, $CH_3OH:H_2O$ washes of differing volumes and concentrations, and $CH_3CN:H_2O$ washes of differing volumes and concentrations. A 50:50 $CH_3OH:H_2O$ (v:v) wash appeared to be the best pretreatment. Several solvent systems were examined for use to remove the vitamers from the Sep-Pak. Solvent composition, strength, and volume were the variables tested and no system tested was able to yield satisfactory recoveries of standards. In addition, the bond of PLP to albumin must be cleaved prior to the sample application on the C_{18} cartridge. To deproteinate, SSA was tried in conjunction with the Sep-Pak and proved to be unsuccessful due to the fact that the SSA was not fully retained on the Sep-Pak or fully eluted. In order to completely remove the SSA, the use of ion exchange resins would be necessary. The use of ion exchange resins and SSA would result in dilution and pH problems, thus defeating the purpose of using a Sep-Pak.

C. Autoclaving

Modifications of the 2 step deproteination and dephosphorylation of B-6 vitamers for microbiological assays (16) were tried. Two levels of perchloric acid (PCA) were tested - 8% and 16% PCA. For dephosphorylation, several concentrations of HCl were examined - 0.2N, 0.1N, and 0.055N HCl. In addition both 2h and 5h periods of autoclaving were tested. The sample was diluted 1:1 (v:v) at the PCA stage (to deproteinate) and diluted 1:1 (v:v) once again at the HCl stage (to dephosphorylate), thus, regardless of the treatment the dilution factor was too great for the amount of B-6 vitamers present in plasma.

D. Isoelectric point

The use of isoelectric point for deproteination was investigated. The pH of the plasma was adjusted to pH 5.0 using HOAc and NaOH. This pH was selected in order to precipitate albumin; the isolectric of albumin is pH 4.7 to 5.2. The variables of temperature and time were tested. Two temperatures were used - 37^oC and 100^oC; the time variables were 10, 20, 30, 40, 50, and 60 min for both temperatures. These trials did not yield an extract "clean" enough for an HPLC analysis.

E. Salting out

Ammonium sulfate trials consisted of adding: 90% saturated $(NH_4)_2SO_4$, 0.6 g solid $(NH_4)_2SO_4$, or 0.9 g solid $(NH_4)_2SO_4$ to 3 ml plasma. These were stirred for 30 min and centrifuged 1h at 0^oC at 12,000x g. The supernant was treated with CH_2Cl_2 and centrifuged for 20 min at 4^oC at 7000x g. The 90% saturated $(NH_4)_2SO_4$ treated resulted in too great a dilution and the addition of the solid was not easily controlled. Extracts that were run on the HPLC were not sufficiently clear of interfering compounds and column pressure problems were apparent, thus indicating incomplete protein removal.

F. Potato acid phosphorylase

With the failure of the previous extraction methods, modifications of the Gregory et al. (36) potato acid phosphorylase method was investigated. The variables tested are concentration of PAP and concentration of TCA. Three levels of PAP were tested - 2, 5, and 10 ng/ml, as well as 2 volumes, 0.5 and 1.0 ml, of the differing PAP concentrations for sample dephosphorylation. Both 20% and 100% TCA solutions were tested for deproteination; the 100% TCA treatment was selected as optimum since a lesser volume was required thereby decreasing the dilution factor. The extraction procedure utilizing 0.5 ml of the 10 ng PAP/ml, 0.02 M KOAc buffer and the 100% TCA was selected as the optimum sample extraction method. This procedure resulted in a "clean" sample and recoveries greater than 85%.

Of all the extraction procedures tested, the modified potato acid phosphorylase method provided extracted samples that were reproducible, "clean" and had acceptable recoveries. Therefore this method was selected as the extraction method for vitamin B-6 in rat plasma.

Sample analysis - method of choice

Ultimately, successful chromatographic operating conditions for the separation and quantitation of B-6 vitamers in rat plasma were determined. Instructions for the preparation of reagents for the HPLC mobile phase are contained in Appendix 6. Detailed below is the modified method for rat plasma extraction using PAP and the chromatographic conditions used to separate PL, PN, PM, and DPN in rat plasma.

A. Sample extraction - potato acid phosphatase extraction

One hundred µl of a 20,000 ng/ml aqueous solution of DPN was added to 2.5 ml of plasma. The tube was covered with parafilm and vortexed for 5 sec. To dephosphorylate, 0.5 ml of a 0.1 g/ml of 2U/ml potato acid phosphatase in 0.2M KOAc, pH 4.5, was added to the plasma and vortexed for 15 sec. The covered sample tubes were incubated for 1h in a shaking $37^{o}C$ waterbath. To deproteinate, 0.2 ml 100% TCA was added to the sample and the tubes were vortexed for 15 sec and incubated at $50^{o}C$ for 15 min in a waterbath. The tubes were cooled to room temperature and 3 ml of cold CH_2Cl_2 were added to each tube. The tubes were covered with plastic wrap and shaken vigorously for 1 min. The samples were then centrifuged for 20 min at $5^{o}C$ at 7000 x g. The supernant was pipetted off and the volume recorded. The pH was adjusted to pH 5-6 using 10% HOAc and 33% NaOH and the amount used to adjust pH was recorded. The samples were filtered through a 0.2 μm Gelman Acrodisc filter and stored in amber vials. A schematic of the sample extraction is given in Figure 7. Instructions for preparation of reagents for PAP extraction method are in Appendix 7.

B. HPLC separation of B-6 vitamers in rat plasma

The B-6 vitamers in rat plasma were separated using a 250 μl injection volume and the gradient listed in Table 2. Samples were injected in duplicate. A typical chromatogram of the B-6 vitamers in rat plasma is shown in Figure 8. No difference was observed between frozen extracts and extracts that had been thawed and refrozen; this was tested on extracts that had been refrozen for 1 day, 1 week, and 1 month. The effect of storage longer than 1 month on

2.5 ml plasma spiked with 100 μl DPN, vortex

↓

add 0.5 ml PAP, vortex, incubate 1h at 37^oC

↓

add 0.2 ml 100% TCA, vortex, incubate 15 min at 50^oC

↓

cool, add 3 ml cold CH_2Cl_2, shake 1 min
centrifuge 20 min, 5^oC, 7,000 x g

↓

remove supernant and adjust to pH 5-6

↓

filter through 0.2 μm filter

Figure 7. Schematic of sample extraction method for rat plasma

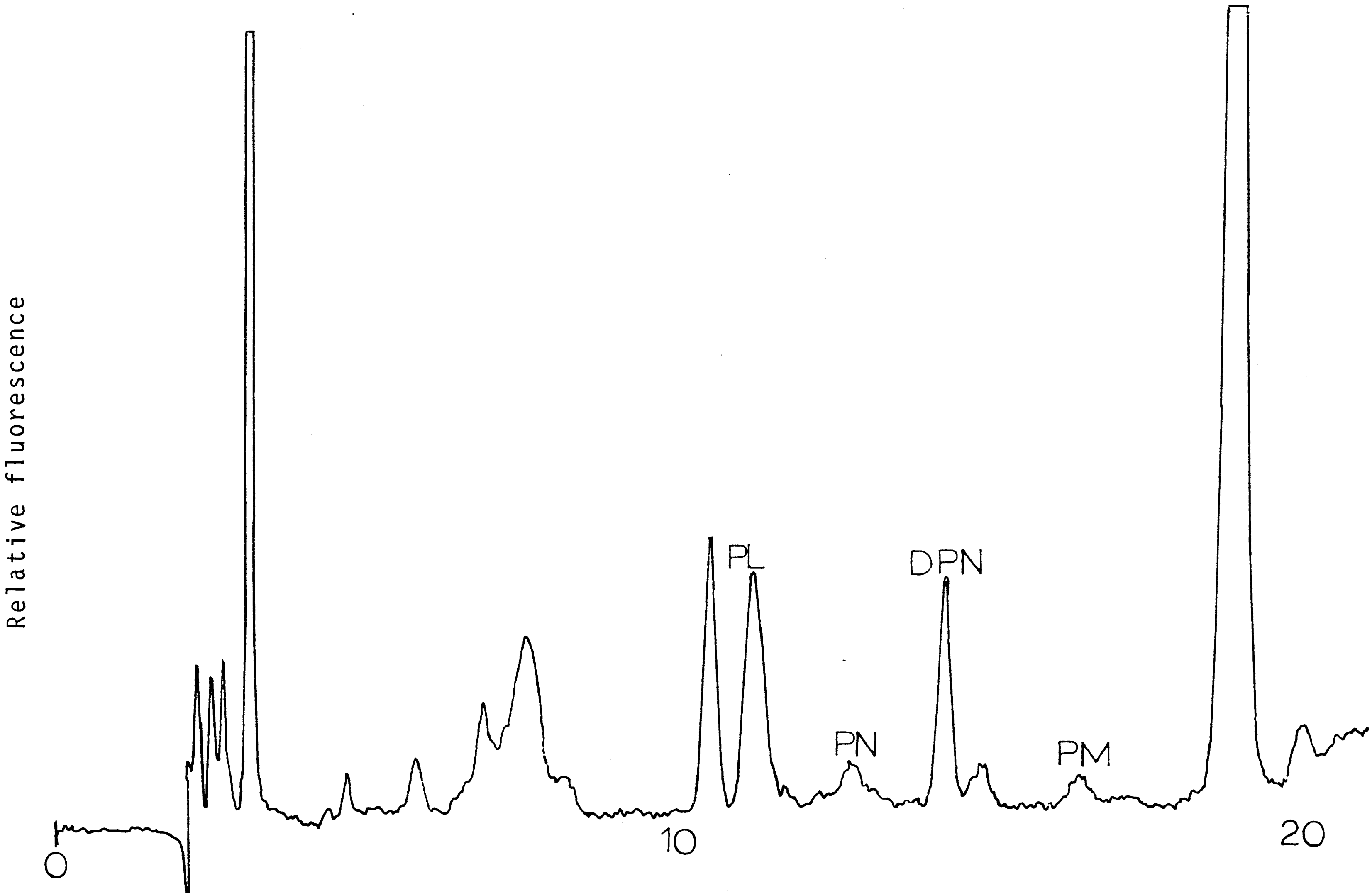


Figure 8
Typical chromatogram of B-6 vitamers in rat plasma

refrozen extracts is not known. Vanderslice et al. (63) found no difference in vitamer concentration in extracts of human milk when the sample was immediately frozen and stored overnight prior to extraction; however, when the extracts were stored overnight there was a loss in vitamer concentration. In the preliminary work performed in this laboratory with the SSA extraction method of Vanderslice et al. (32), the pH of the sample after extraction was pH 1 or less. Further investigation with standards and samples at pH 1 revealed instability of the vitamers which was manifested in HPLC analyses by splitting peaks. The resulting instability may be responsible for the loss of vitamer observed by Vanderslice in stored extracts.

C. Spectrofluorometric scan

Spectrofluorometric scans were peformed in accordance with the procedures outlined in the Peak identification section of the Material and Methods. Scans of standards and sample peaks were compared and Figures 9-12 shows these scans. The spectrofluorometric scans were among the methods used to confirm peak identities in the samples.

D. Recoveries

Recoveries were determined by spiking the sample prior to extraction. Table 4 shows the recoveries obtained for rat plasma using the modified potato acid phosphorylase extraction method.

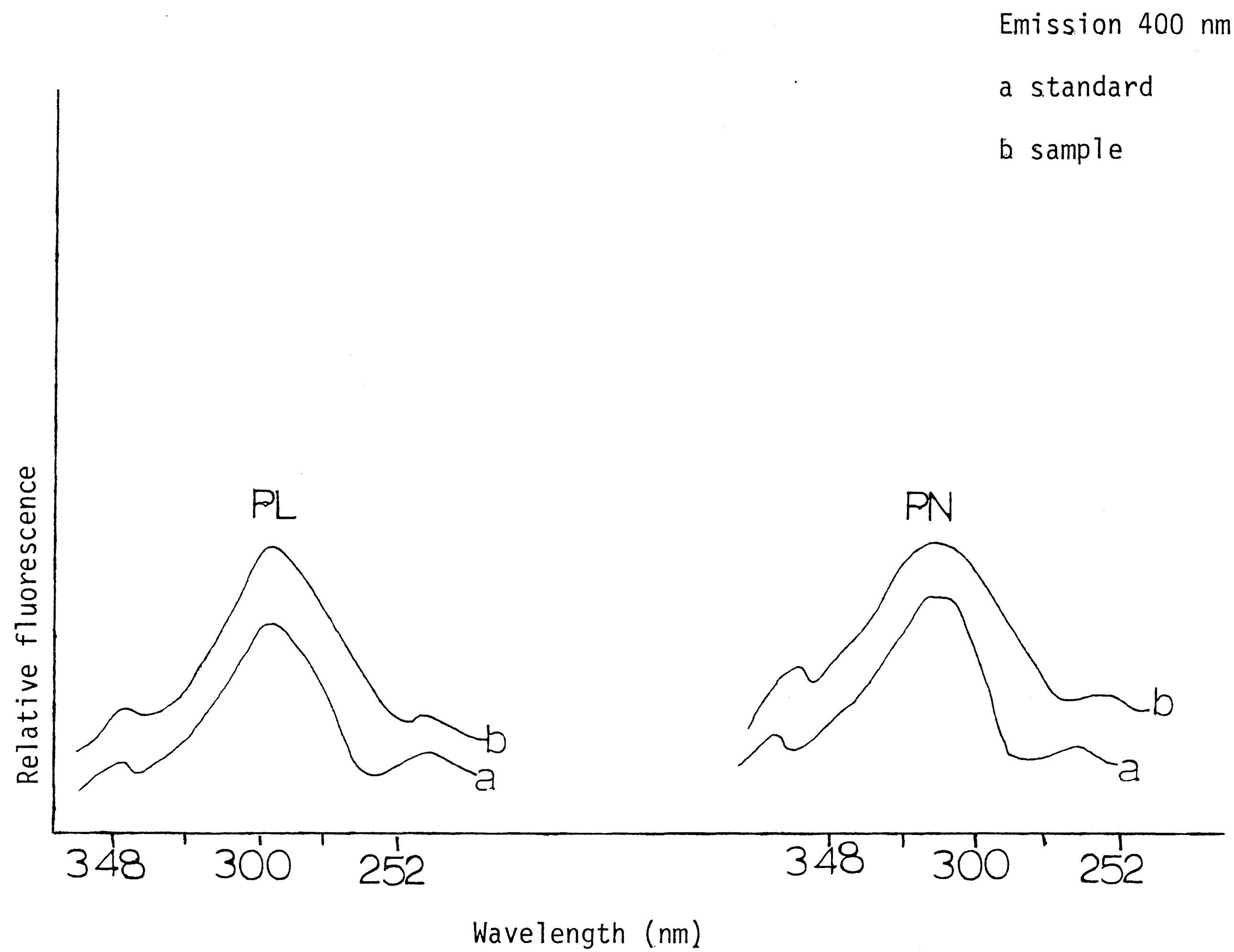


Figure 9

Fluorescence excitation scans of PL and PN - comparison of standard and sample

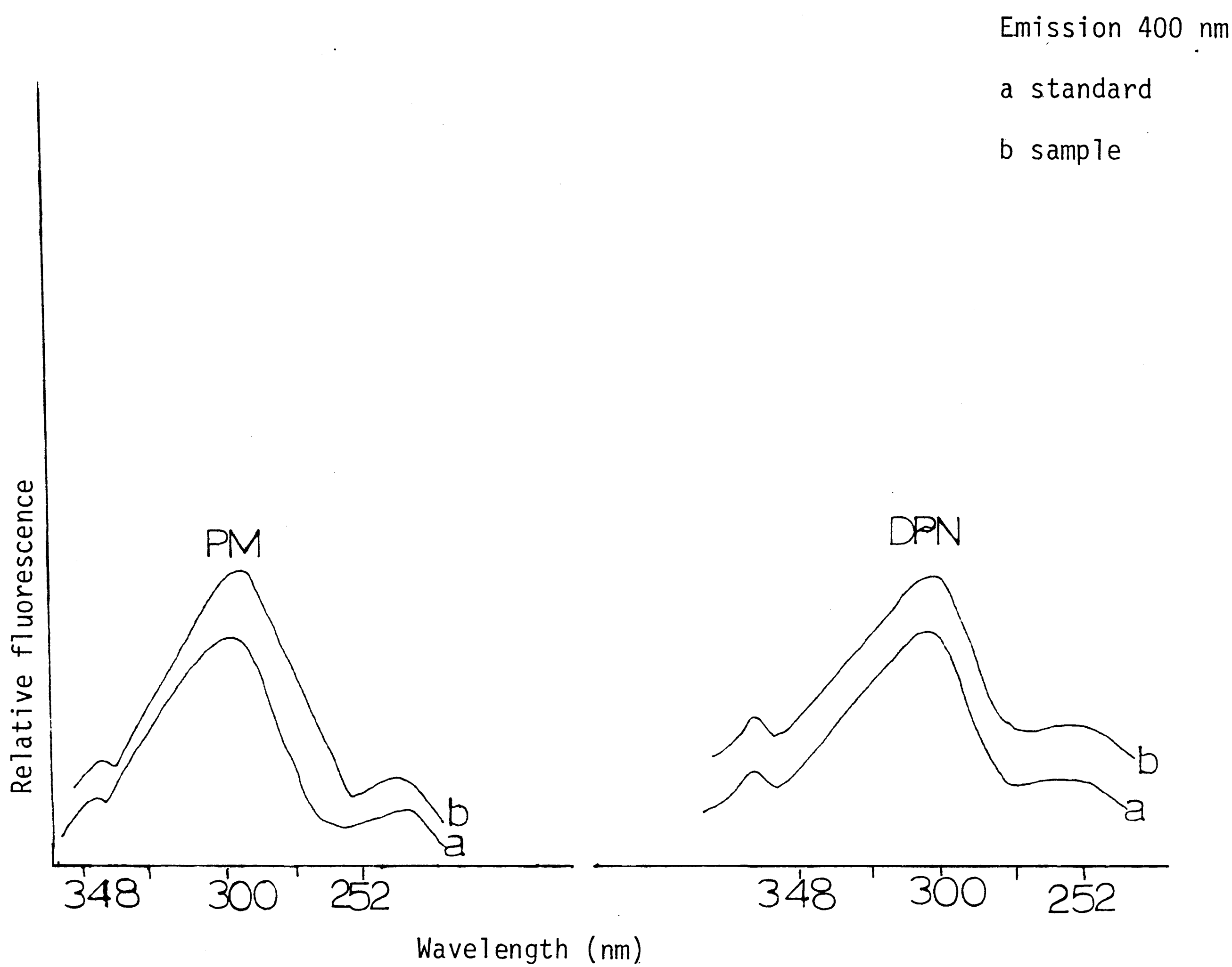


Figure 10

Fluorescence excitation scans of PM and DPN - comparison of standard and sample

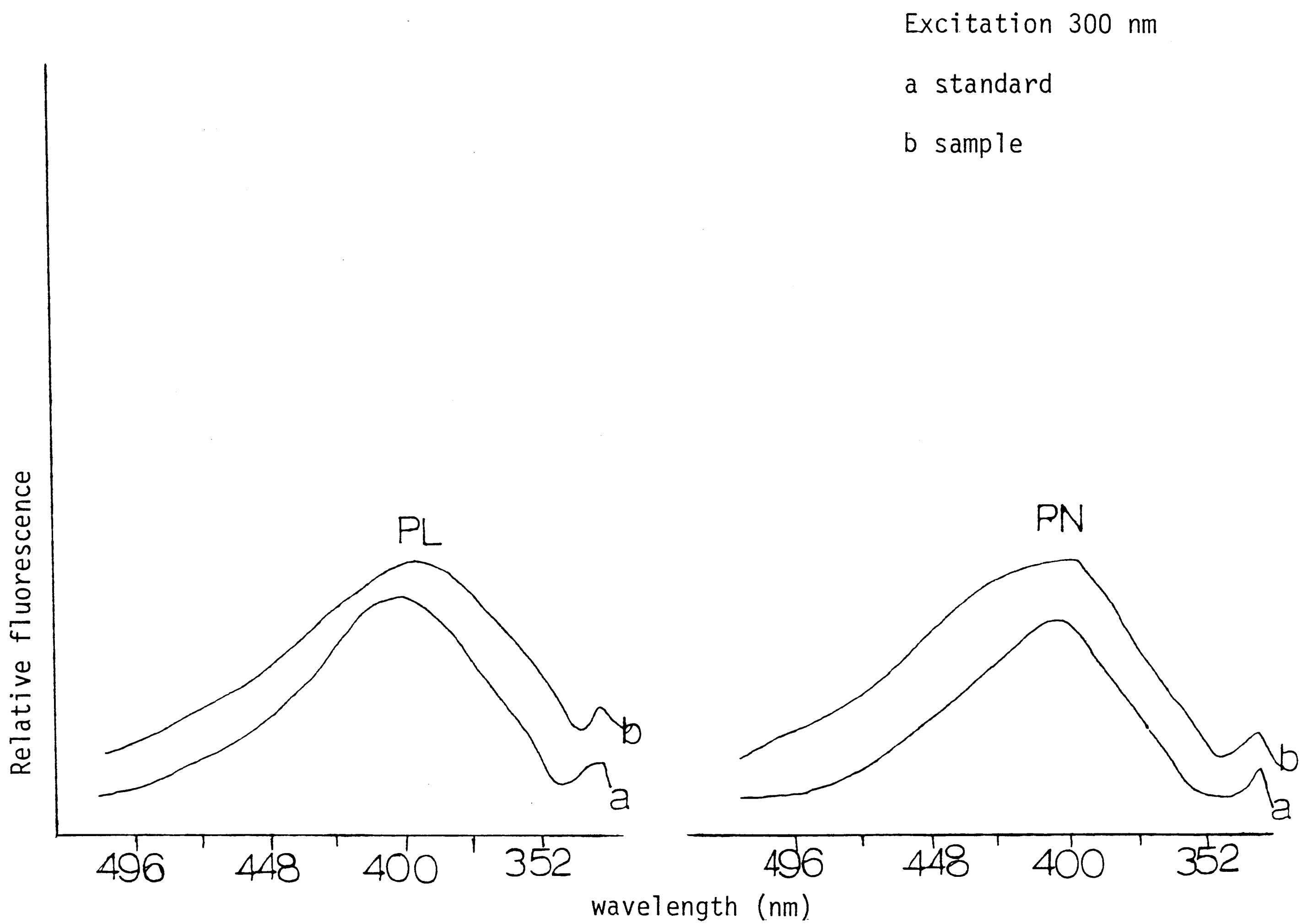


Figure 11

Fluorescence emission scans of PL and PN - comparison of standard sample

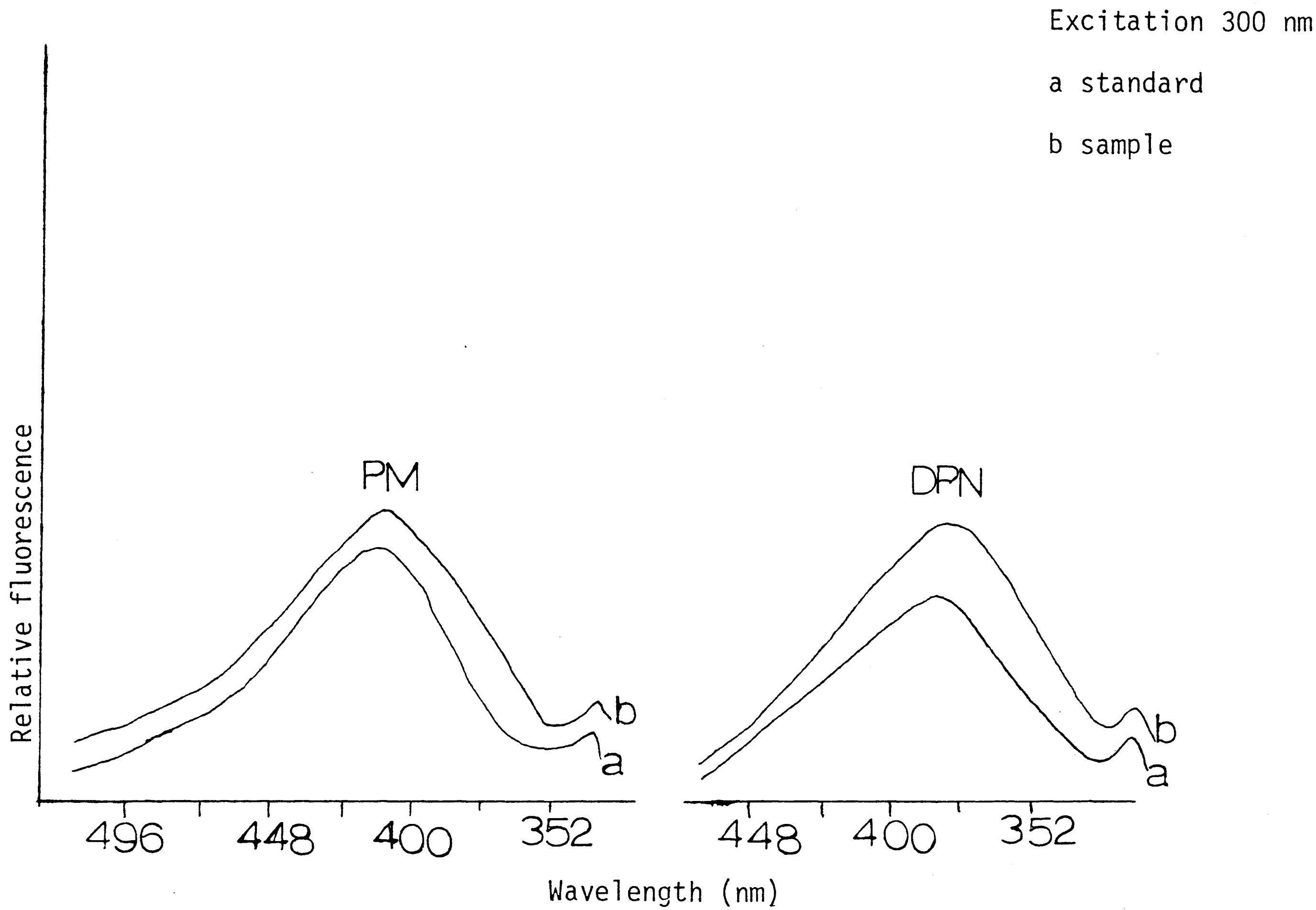


Figure 12

Fluorescence emission scans of PM and DPN - comparison of standard and sample

Table 4

Rat plasma B-6 vitamer recoveries[1]

Vitamer	Recoveries
	%
PL	98
PN	95
PM	95
PLP (dephosphorylated to PL)	85
PNP (dephosphorylated to PN)	91
PMP (dephosphorylated to PM)	97

[1]Samples were externally spiked prior to extraction done in triplicate.

Animal Experiments

A. Vitamin B-6 intakes

The rats in experiment 1 were given diets that were intended to provide 15, 30, 45, and 90 µg of vitamin B-6/day. The diets were fed ad libitum and the animals consumed a greater portion of the diet daily than had been anticipated. The actual intakes of vitamin B-6 were calculated from daily food consumption data. Based on the 45 µg/day level as a basal requirement, the actual intakes were calculated as a % basal requirement (Table 5). On this basis, rats in group 1 consumed about half of the requirement and this would represent animals having inadequate status; rats in this group were proven to have deficient status by other researchers in our laboratory group. Groups 2 and 3 consumed just below (group 2) and slightly above (group 3) the basal requirement. Group 4 animals consumed in excess of 2 1/2 times the basal requirement. The animals in experiment 1 were kept on the diets for 15 weeks.

On the whole, the animals in experiment 2 had a higher mean daily food consumptions than the rats in group 1. Hence, rats in groups 2-5 were in excess of the basal requirement (Table 6). The actual mean intake of group 1 was slightly over half the basal requirement and represents inadequate status; also proven to have deficient status by other researchers in our laboratory group. These animals were kept on the diet for 12 weeks.

In rat experiment 3, the mean level of vitamin B-6 intake for the deficient group was only 7% of the basal requirement (Table 7),

Table 5

Actual intakes of vitamin B-6 and % basal requirement for rat experiment 1[1]

Group	Intake	Basal requirement[2]
	μg/day	%
1	23±2	51±4
2	44±4	91±9
3	60±6	133±13
4	127±12	282±27

[1]Values are $\overline{X} \pm$ SD.

[2]Values are % $\overline{X} \pm$ SD of 45 μg/day.

Table 6

Actual intakes of vitamin B-6 and % basal requirement for rat experiment 2[1]

Group	Intake	Basal requirement[2]
	µg/day	%
1	24±3	53±7
2	53±8	117±18
3	76±10	169±22
4	152±23	338±51
5	248±32	551±71

[1]Values are $\overline{X} \pm$ SD.

[2]Values are % $\overline{X} \pm$ SD of 45 µg/day.

Table 7

Actual intakes of vitamin B-6 and % basal requirement for rat experiment 3[1]

Group	Intake	Basal requirement[2]
	μg/day	%
Deficient	3±0.2	7±0.4
Control	124±8.0	276±18.0

[1]Values are $\overline{X} \pm$ SD.

[2]Values are % $\overline{X} \pm$ SD of 45 μg/day.

thus manifesting a severely deficient state. The control animals had vitamin B-6 intakes similar to the intakes of group 4 rats in experiment 1. The animals were kept on the diets for 27-31 weeks.

An overview of the actual mean vitamin B-6 intakes of rats in all 3 experiments compared with the basal requirements revealed that the % basal requirements ranged from a low of 7% for the deficient group in experiment 3 to a high of 551% for the rats in group 5 of experiment 2. Therefore, the 3 experiments provided rats in the range of vitamin B-6 nutriture from severely deficient to quite adequate nutriture.

B. Body Weight

For the rats in experiment 1 there was no significant difference in the initial body weights (Table 8). The initial body weight for each animal was determined at week 4 of life; the animals were received at week 3 and adapted for 1 week. The initial body weight was determined at the end of this period. At the end of the 15 week experiment there were still no significant differences in body weights of these animals (Table 9).

The initial body weights for the group of rats in experiment 2 (Table 8) were similar. Initial body weights were determined in the same manner as for experiment 1. The final body weights, presented in Table 9, were determined at the termination of the 12 week study. Animals in group 5 were found to have significantly lower ($p < 0.01$) body weights than rats in groups 3 and 4, but no significant difference in body weight was observed between groups 1, 2 and 5. In

Table 8

Initial body weights for rat experiments 1, 2, and 3[1,2,3]

Group	Rat experiment 1	2	3
		g	
Deficient			95 ± 5
1	88 ± 8	72 ± 8	
2	88 ± 5	75 ± 8	
3	85 ± 7	71 ± 2	
4	91 ± 8	74 ± 4	97[4] ± 7
5		67 ± 7	

[1]Values are $\overline{X} \pm$ SD.

[2]Values are not significantly different.

[3]Body weights taken at 4 wk of life.

[4]Corresponds to control group rat experiment 3.

Table 9

Final body weights for rat experiments 1, 2, and 3[1,2,3]

Group	Rat experiment 1	2	3
		g	
Deficient			324[a] ±20
1	422 ±16	350[a,b] ±18	
2	431 ±30	367[a,b] ±13	
3	431 ±33	385[a] ±13	
4	448 ±22	370[a] ±30	502[b,4] ±37
5		334[b] ±10	

[1]Values are $\overline{X} \pm$ SD.

[2]Values not followed by the same letter in experiments 2 and 3 are significantly difference, $p < 0.01$.

[3]Body weights taken at wk 15, experiment 1; wk 12, experiment 2; and wk 27-31, experiment 3.

[4]Corresponds to control group in experiment 3.

addition, rats in groups 1 and 2 were not significantly different in final body weights than groups 3 and 4. Previous experiments (63, 64) have shown lower body weights in animals fed levels of vitamin B-6 exceeding the requirement.

The initial body weights for the rats in experiment 3 (Table 8) were determined as in experiment 1. There was no significant difference observed in initial body weights for the animals in experiment 3. The final body weights (Table 9), determined prior to sacrifice at 27-31 wk, exhibited a significant difference between deficient and control groups at $p < 0.01$. The animals were sacrificed and the plasma was pooled, extracted and analyzed in the same day. Thus, the time needed to obtain, extract, and analyze the fresh, unfrozen plasma limited the number of animals that could be killed at one time. Consequently, some deficient and some control animals were taken for each sacrifice.

B-6 vitamer content of rat plasma

The phosphorylated B-6 vitamers (PLP, PNP, and PMP) were converted to the nonphosphorylated forms by the PAP sample extraction method. Therefore, the individual nonphosphorylated B-6 vitamer values are the sum of both the phosphorylated and the nonphosphorylated B-6 vitamers. Tracer studies done by Shane (65) utilizing (^{14}C) PN in the blood indicated that the nonphosphorylated B-6 vitamers accounted for only a minor portion of the vitamin B-6 found in blood. In addition, Hämfelt (66) and Shane (67) observed no presence of PL Kinase (EC 2.7.1.35 ATP:pyridoxal 5′-phosphotransferase) in plasma; PL Kinase is responsible for the phosphorylation of each of the nonphosphorylated forms to their phosphorylated congeners.

A. Rat experiment 1

Pooling of the plasma from the rats was necessary in order to obtain a sufficient volume of extract for HPLC and microbiological analyses. This volume of plasma was determined to be 2.5 ml; the 2.5 ml of plasma yielded about 1.5 ml of extract which was sufficient for 1.0 ml extract for microbiological analysis and 0.5 ml extract for HPLC analysis. There were several animals from which less than this volume of plasma was obtained; as a result, plasma was pooled in equal volumes from sets of 2 or 3 animals to make each pooled sample. The pooled plasma B-6 vitamer concentrations for the animals in experiment 1 are given in Appendix 8.

The plasma B-6 vitamer content for rats in experiment 1 is given in Table 10. There were no significant differences between groups for PN and PM values. However, the PL values for group 1 were significantly lower ($p < 0.01$) than values for groups 2-4. There was no significant difference in the PL concentration for groups 2 and 3. Interestingly, the PL values for groups 3 and 4 were significantly higher ($p < 0.01$) than the values for group 1. The same trend among groups seen for PL values was also observed for total B-6 vitamer concentration. At $p < 0.01$ the total B-6 vitamer values for group 1 were significantly lower than the values for the other groups. No significant difference was observed for the values of rats in groups 2 and 3. The animals in group 4 was

Table 10

Plasma B-6 vitamer content determined for rats in experiment 1[1,2]

Group	n[3]	Dietary PN	PL	PN	PM	Total
		μg/day		ng/ml		
1	5	23	61.5[a] ±12.3	12.6 ±3.8	19.3 ±16.5	93.4[a] ±26.0
2	5	41	205.8[b] ±19.8	19.2 ±4.7	20.8 ±10.3	245.8[b] ±20.5
3	5	60	237.2[b,c] ±30.0	14.2 ±8.1	14.7 ±14.8	266.1[b,c] ±39.8
4	4	127	283.7[c] ±33.9	17.5 ±7.1	16.7 ±9.2	317.9[c] ±28.7

[1]Values are $\overline{X} \pm$ SD.

[2]Values not followed by the same letter are significantly different, $p < 0.01$.

[3]n = the number of pooled samples; each pooling represents plasma from 2 or 3 animals.

was shown to have significantly higher ($p < 0.01$) total B-6 vitamer values than groups 1 and 2 and significantly higher ($p < 0.05$) than the values for group 3.

The % distributions of plasma B-6 vitamers for rats in experiment 1 are given in Table 11. The % distributions of PL for group 1 were significantly lower, $p < 0.01$, than the values for groups 2-4. The PN values for group 1 were found to be significantly higher ($p < 0.01$) than the PN values for groups 3 and 4. There was no significant difference between groups for the PM values. The % distributions of B-6 vitamers for individual animals are listed in Appendix 9.

The plasma B-6 vitamer content and % distributions values indicated that PL was the primary form of vitamin B-6 in rat plasma. At this time, no published data are available for B-6 vitamer content of rat plasma. However, several researchers have published results for human plasma B-6 vitamer content. Vanderslice et al. (31) reported PN as being the major B-6 vitamer present in human plasma. In order to confirm this finding, which did not agree with unpublished reports on similar samples, a second chromatographic system was set up. A new anion exchange method (68) was developed and different buffers and resins were used than in their previous method. Their newly developed system was unable to confirm PN as the major form of vitamin B-6 in human plasma.

Reynolds (69) reported the range of B-6 vitamer concentrations in human plasma determined by 14 laboratory groups. The

Table 11

% distributions of plasma B-6 vitamers for rats in experiment 1[1,2]

Group	PL	PN	PM
		%	
1	67.2[a] $\pm$7.4	14.8[a] $\pm$6.7	18.0[a] $\pm$12.7
2	83.7[b] $\pm$2.6	7.9[a,b] $\pm$2.5	8.4[a] $\pm$4.1
3	89.4[b] $\pm$3.7	5.5[b] $\pm$3.3	5.1[a] $\pm$4.0
4	89.1[b] $\pm$4.2	5.5[b] $\pm$2.1	5.4[a] $\pm$3.2

[1]Values are $\overline{X} \pm$ SD.

[2]Values not followed by the same letter are significantly different, $p < 0.01$.

subjects prior to sampling were dosed with 11.8 μmole of PL, PN, and PM; or 2.41 mg PL, 2.43 mg PN, and 2.85 mg PM. The recommended dietary allowance for adult males is 2.2 mg daily of vitamin B-6 and for adult females 2.0 mg daily (70). PNP was not detected by any of the investigators; the range for PN was 1.6-7.4 ng/ml; for PL 13.0-50.0 ng/ml; for PM was 0.2-1.0 ng/ml; for PLP was 11.8-420.2 ng/ml, and for PMP was 3.4-17.4 ng/ml. In discussions at a meeting of vitamin B-6 researchers in April 1983 (71), the ranges for human plasma B-6 vitamers were reported to be similar to those given above with the exception of PL which was reported to range from 13.1-19.5 ng/ml and PMP 3.4-11.4 ng/ml. The predominate forms of B-6 vitamers in human plasma following injection of PL, PN, and PM were PLP, PL, and PMP in decending order. The results obtained from the plasma of the rats in experiment 1 concurs with PL and/or PLP being the predominate form of vitamin B-6 in plasma as reported by Reynolds (69).

Gregory, in unpublished work (72), has determined PL and PLP in rat plasma via a post column semicarbazone deviation method. The rat plasma was reported to have 11.6 ng/ml PLP fluorometrically measured as the PLP semicarbazone derivative. No information was given for the PL fraction. The value for the PLP derivative is somewhat lower than the values obtained for PL in rat plasma in experiment 1 by the current researcher.

Pearson r correlations were calculated for the B-6 vitamer values obtained in experiment 1 (Table 12). A highly significant

Table 12

Pearson r correlation coefficients for B-6 vitamers for rats in experiment 1[1,2]

Variable	Pearson r	p Value
PL:total	0.99	<0.0001

[1]n = 19 pooled samples.

[2]All other correlation coefficients not significant.

correlation was found for PL and total B-6 vitamer values. All other correlations for the B-6 vitamers were not significant.

B. Rat experiment 2

The plasma B-6 vitamer concentrations for the samples are given in Table 13. Pooling of plasma was required as a result of the small volume of plasma obtained from some animals. The plasma was pooled using an equal aliquot from each of the 6 animals in the group. Hence, the data presented in Table 13 were a mean of duplicate sample injections for the pooled plasma for each group.

The pooled plasma B-6 vitamer concentrations followed a pattern similar to the B-6 vitamer concentrations determined for rat experiment 1. The level of PL for the groups in experiment 2 increased with increasing vitamin B-6 intake as shown in Table 13. Lumeng et al. (73) observed increasing levels of PLP in plasma with increased intakes of vitamin B-6; the highest vitamin B-6 level tested by Lumeng and coworkers was 100 µg vitamin B-6/day. The presence of albumin in plasma and its binding capacity of 800 µg/ml was cited as a possible reason for the continuing rise in PLP when contrasted to saturation of liver and brain at lower levels of vitamin B-6 intake. The binding of PLP to albumin in plasma may be responsible for the continuing rise in PL concentration demonstrated by the data of rat experiment 2. However, the pooling of the plasma samples and the resulting $n = 1$ for each group limits the interpretation of this data.

Table 13

Plasma B-6 vitamer content for rats in experiment 2[1]

Group	Dietary PN	PL	PN	PM	Total
	μg/day		ng/ml		
1	24	54.6	23.0	49.4	127.0
2	53	238.0	23.8	26.5	288.3
3	76	295.8	21.7	26.7	344.2
4	152	398.5	22.2	13.3	434.0
5	248	411.8	18.5	5.7	436.0

[1]n = 1 pooled sample for each group.

The % distributions of B-6 vitamers in rat plasma are listed in Table 14. The values for rats in experiment 2 showed the same trends as the % distributions of B-6 vitamers for experiment 1. PL was shown to be the predominate form of vitamin B-6; in addition, there was a general trend for PL to constitute an increasing % as the intake of vitamin B-6 increased.

Pearson r correlations were calculated for the B-6 vitamer values obtained from the rat plasma in experiment 2 (Table 15). As with rat experiment 1 data, a highly significant correlation was found between PL and total B-6 vitamer values. In addition, significant negative correlations were found for PL and PM ($p < 0.003$), and for PM and total B-6 vitamer values ($p < 0.005$). These correlations were determined with $n = 5$ (1 value per group).

C. Rat experiment 3

Pooling of the plasma samples for experiment 3 was done in order to provide enough sample for extraction to be done on fresh samples and frozen samples that were to be frozen for about 1 year. The vitamin B-6 concentrations of the frozen samples will be determined by another researcher in our laboratory group. The plasma volume obtained was especially critical for the smaller deficient animals. The pooled samples consisted of equal volumes of plasma from 2 animals. All extracts were analyzed prior to freezing except for C-01, C-12 extract which was run after being frozen. Values obtained for this sample were consistent with the values from other animals in this group. The plasma B-6 vitamer concentration for individual pooled samples is given in Appendix 10.

Table 14

% distributions for B-6 vitamers in rat plasma from experiment 2

Group	PL	PN	PM
		%	
1	43.0	18.1	38.9
2	82.6	8.2	9.2
3	85.9	6.3	7.8
4	91.8	5.1	3.1
5	94.5	4.2	1.3

Table 15

Pearson r correlation coefficients of B-6 vitamers for rats in experiment 2[1]

Variable	Pearson r	p value
PL:PM	-0.98	<0.003
PL:total	0.99	<0.0001
PM:total	-0.97	<0.005

[1]n = 5 pooled samples; each pooled sample was derived from the plasma of 6 animals.

For statistical analyses, the values for D-10 were dropped because the PL value was found to be 3 times higher than the PL values for the other animals in the deficient group. The electrocution unit malfunctioned and the animal was exposed to electrical current for a longer period of time than the other animals. Carbon dioxide was ultimately used to sacrifice this animal. Cheney et al. (20) demonstrated that the injection of cortisone, an adrenal hormone, increases alanine aminotransferase (ALAT) values. The resulting stress from the malfunctioning electrocution unit may have induced synthesis of adrenal hormones such as cortisone, epinephrine, and norepinephrine. PLP is needed for the synthesis of norepinephrine at the stage where DOPA (3, 4-dihydroxyphenylalanine) is converted to DOPAMINE (3, 4-dihydroxyphenylamine) (74). The use of CO_2 to sacrifice the remaining rats did not seem to affect the vitamin B-6 values.

The pooled plasma B-6 vitamer concentrations for rats in experiment 3 are presented in Table 16. Significantly higher ($p < 0.0001$) PL values were found for plasma from the control group than for the deficient group of animals. The PN values were significantly higher ($p < 0.03$) for the control animals than the deficient animals. The values for PM did not exhibit a significant difference. The total B-6 vitamer values were found to be significantly different, $p < 0.0001$, for the deficient than for the control groups.

Plasma B-6 vitamer % distributions for rats in experiment 3 are presented in Table 17. The PL % increased with the increased

Table 16

Plasma B-6 vitamer concentrations for rats in experiment 3[1,2]

Group	n[3]	Dietary PN	PL	PN	PM	Total
		µg/day		ng/ml		
Deficient	5	3	10.5[a] $\pm$1.8	10.1[a] $\pm$2.1	68.0[a] $\pm$8.7	88.6[a] $\pm$8.7
Control	9	124	375.3[b] $\pm$51.3	25.8[b] $\pm$17.0	63.0[a] $\pm$2.72	468.2[b] $\pm$63.1

[1]Values are $\overline{X} \pm$ SD.

[2]Values not followed by the same letter are significantly different, $p < 0.0001$ for PL and total, $p < 0.03$ for PN.

[3]n = number of pooled samples; each pool consisted of plasma from 2 animals.

Table 17

% distributions for plasma B-6 vitamers for rats in experiment 3[1,2]

Group	PL	PN	PM
		%	
Deficient	12.0[a] ±2.0	11.4[a] ±2.3	76.6[a] ±3.6
Control	83.0[b] ±5.7	4.7[b] ±3.0	5.1[b] ±8.6

[1]Values are $\overline{X} \pm$ SD.

[2]Values not followed by the same letter are significantly different; $p < 0.002$ for PN values, $p < 0.0001$ for PL and PM values.

vitamin B-6 intake of the control group. Interestingly, the primary B-6 vitamer for the deficient group, which had a mean vitamin B-6 intake of 7% basal requirement, was shown to be PM. The % of PL was significantly higher, $p < 0.0001$, for the control rats than the deficient rats. The % distributions for PN and PM were significantly lower, $p < 0.002$ and $p < 0.0001$ respectively, for the control animals than for the deficient animals. Individual % distribution data for B-6 vitamers in plasma for animals in experiment 3 are listed in Appendix 11.

As with the animals in experiments 1 and 2, PL was found to be the predominate B-6 vitamer in rats receiving vitamin B-6 at or above the basal requirement. This is in agreement with the findings of Reynolds (69) for human plasma. However, the predominance of PM in the plasma for the rats in the deficient group was unexpected. The predominance of PM may be a way in which rats on severely restricted intakes of vitamin B-6 conserve the body's pool of this vitamin. Since PM can be oxidized to PL via PMP oxidase (EC 1.4.3.5 pyridoxamine phosphate: oxygen oxidoreductase) and PL not PM is oxidized to the metabolite pyridoxic acid (61), the predominance of PM may be a conservation mechanism.

Pearson r correlations were calculated for the B-6 vitamer values obtained for rat experiment 3 (Table 18). There was a highly significant correlation between PL and total B-6 vitamer values. The correlations between PL and PN values, as well as between PN and total B-6 vitamer values, were found to approach significance.

Table 18

Pearson r correlation coefficients of B-6 vitamers for rats in experiment 3[1]

Variable	Pearson r	p Value
PL:total	0.99	<0.0001
PL:PN	0.42	<0.13[2]
PN:total	0.48	<0.08[2]

[1]n = 14 pooled samples.

[2]Value approached significance.

D. Comparison of vitamer data from experiments 1-3.

In rat experiment 1 a significant difference, $p < 0.01$, was observed for plasma PL and total B-6 vitamer concentrations between group 4 and groups 1 and 2. There was a significant coefficient of correlation ($r = 0.99$, $p < 0.0001$) between PL and total B-6 vitamer concentration. An overview of the data on a % distribution basis revealed PL as the major vitameric form in rat plasma with a trend for the PL % to increase with increasing dietary intakes of vitamin B-6.

The pooled plasma B-6 vitamer concentration for experiment 2 followed a pattern similar to the pooled plasma B-6 vitamer concentrations in rat experiment 1. An increase in PL concentration was found with increasing vitamin B-6 intakes; the corresponding increase was also found for the total B-6 vitamer concentrations. PN and PM values did not seem to be influenced by vitamin B-6 intake. The % distributions of plasma B-6 vitamers revealed PL as the primary B-6 vitamer in rat plasma, as was found in experiment 1; also the same general trend of increasing PL % as vitamin B-6 intake increased was observed.

The data from rat experiment 3 indicated that a significant difference, $p < 0.0001$, existed for the PL values and the total B-6 values for plasma from control and deficient animals. A significant correlation was observed for the PL and total B-6 vitamer values, as was observed in experiment 1. The % distributions data for PL values of the control animals were similar to the % distributions of PL for rats receiving adequate vitamin B-6 in experiments 1 and 2. PM was found to be the predominate vitamer in plasma from the deficient animals in experiment 3.

For each of the 3 experiments, a Pearson r correlation coefficient ($r = 0.99$, $p < 0.0001$) was observed for plasma PL and total B-6 vitamer values. The % distributions for the B-6 vitamers in all 3 experiments revealed for the rats having adequate intakes of vitamin B-6 that PL was the predominate vitameric form. However, this was not the case for severely deficient animals as demonstrated by the predominance of PM in plasma from the deficient animal group in experiment 3.

Applications of this HPLC methodology holds promise for the evaluation of B-6 vitamers in human plasma. At this time, vitamin B-6 status is determined by measuring coenzyme stimulation in erythrocytes of alanine aminotransferase or by radiometrically measuring the ^{14}C-labeled CO_2 produced enzymatically by plasma tyrosine decarboxylase. These methods indirectly measure PLP, the cofactor in these reactions. Thus far, HPLC analysis of B-6 has been done on the plasma of subjects receiving supplementation prior to sampling (31, 69); the supplementation facilitated the quantitation of B-6 vitamers in plasma at a point within the detection limits of the HPLC system. Thus, in order to detect B-6 vitamers in the plasma of humans with inadequate status, further reduction in the detection limits must be made. If this were to occur, HPLC analysis of plasma for vitamin B-6 content would be very attractive due to the capacity for automation in sample injection and the ability to qualitate and quantitate the vitameric forms of B-6. In addition, the analysis time by HPLC is rapid with separation of the B-6 vitamers in less than 20 min. The extraction procedure using PAP and TCA is about 2.5 h long and is adaptable for the extraction of up to 20 samples at one time. These factors are favorable for multiple sample analyses.

SUMMARY AND CONCLUSIONS

A chromatographic system was developed for the separation and quantitation of PL, PN, and PM. The column used for the analyses was a reverse phase C_{18} column in conjunction with a binary mobile phase. The mobile phase consisted of a MeOH component and an ion pair component, heptane sulfonic acid buffered with HOAc to a pH of approximately 3.3. The separation of the B-6 vitamers was accomplished in under 20 min using a gradient elution program. The mode of detection was a fixed wavelength which employed a set of excitation and emission filters, 300 nm and 375 nm respectively, selected to provide sensitivity and selectivity for quantitating of the B-6 vitamers in rat plasma.

An extraction procedure was developed which utilized PAP to dephosphorylate PLP, PNP, and PMP; 100% TCA was used in this extraction to deproteinate the sample. The extraction procedure converted PLP to PL, PNP to PN, and PMP to PM; therefore the quantitation of PL, PN, and PM represents a sum of the phosphorylated and nonphosphorylated forms of vitamin B-6.

Pooled plasma samples were analyzed from rats which were fed various amounts of vitamin B-6. Quantitation was achieved for PL, PN, and PM in rats consuming 7% basal requirement of vitamin B-6, the lowest level tested, and also in rats consuming 551% basal requirement of vitamin B-6, the highest level tested. A significant difference ($p < 0.01$) was found for plasma PL values in animals of group 1 and the rats in groups 3 and 4 for experiment 1. The same was true for total B-6 values as well. This trend was also observed for plasma PL and total B-6 vitamers for rats in experiments 2 and 3.

The predominance of PL in rat plasma was found for all animals consuming an adequate amount of vitamin B-6 daily in experiments 1-3. The animals in experiments 1 and 2, which consumed about half the basal requirement for vitamin B-6, had PL as the predominate vitamer, but to a lesser degree than the animals fed the basal requirement and above. For the animals receiving half the basal requirement, an increase was observed for the % PM values. Further shifts in the % distributions of B-6 vitamers were observed in the deficient group of experiment 3 in which PM was found to be the predominate B-6 vitamer.

Significant differences were observed between inadequate and adequate vitamin B-6 intake animal groups for PL and total B-6 vitamer concentrations. In addition, the correlation coefficient ($r = 0.99$) for PL and total B-6 vitamer was highly significant, $p < 0.0001$, for all 3 experiments.

The HPLC methodology and sample extraction has demonstrated utility for measuring B-6 vitamer content in plasma of rats with differing states of vitamin B-6 nutriture. Therefore, vitamin B-6 status measurements in rats could be done by HPLC in the future. At this time, human plasma B-6 vitamer content has not been measured by HPLC for subjects not consuming a supplementation of vitamin B-6 prior to sampling. By increasing the sensitivity for detection or by increasing the fluorescent response of the B-6 vitamers by derivatization, human plasma B-6 vitamers could be quantitated by HPLC. Thus, use of this HPLC methodology for analysis of vitamin B-6 status in humans bears further investigation.

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Appendix 1

Vendors of Chemicals

---

1) Fisher Scientific Co., Fairlawn, NJ
   0.2 μm gelman acrodisc filters 09 730 218
   0.3 μm type A/E glass fiber filter 48 mm 09 730 78
   HPLC grade water W-5
   HPLC grade methanol A-452

2) Bio-Rad Laboratories, Richmond, CA.
   AG 2-X8 200-400 mesh 140-2451

3) ICN Nutritional biochemicals, Cleveland, OH.
   Pyridoxine phosphate 102778

4) Pierce Chemical Co., Rockford, IL.
   5-sulfosalicylic acid dihydrate 27800

5) Sigma Chemical Co., Saint Louis, MO.
   Potato acid phosphatase 2U/mg type IV-S
   acid phosphatase p1146
   Pyridoxal hydrochloride P9130
   Pyridoxine hydrochloride P9755
   Pyridoxamine dihydrochloride P9380
   Pyridoxal 5'-phosphate P9255
   Pyridoxamine 5'-phosphate P9505

6) United States Biochemical Corp., Cleveland OH.
   4-deoxypyridoxine hydrochloride 14215

7) Waters Associates Inc., Milford, MA.
   PIC B-7 85103
   μ Bondapak C_{18} 27324
   Corasil/Porasil C_{18} 27248

Appendix 2

Preparation instructions for standards

---

1) Stock standards 1,000,000 ng/ml
   Weight out into separate 100 ml volumetric flasks:

	g
pyridoxal HCl	0.1218
pyridoxine HCl	0.1216
pyridoxamine di HCl	0.1434
pyridoxal phosphate	0.1478
pyriodoxine phosphate	0.1473
pyridoxamine phosphate	0.1686
deoxypyridoxine	0.1238

   Bring volume with HPLC grade water.

2) Serial dilution - working standard 200 ng/ml
   PLP, PNP, PMP, PL, PN, and PM

   a) Volumetrically measure 2 ml of appropriate stock standard into 100 ml volumetric flask. Bring to volume with HPLC grade water. Makes 20,000 ng/ml solution.

   b) Volumetrically measure 1 ml of appropriate 20,000 ng/ml solution into 100 ml volumetric flask. Bring to volume with HPLC grade water. Makes 200 ng/ml working standard.

3) Serial dilution - working standard DPN 800 ng/ml.

   a) Volumetrically measure 4 ml of stock standard 100 ml volumetric flask. Bring to volume with HPLC grade water. Makes solution 40,000 ng/ml.

   b) Volumetrically measure 2 ml of 40,000 ng/ml solution into 100 ml volumetric. Bring to volume with HPLC grade water. Makes 800 ng/ml solution.

All solutions are stored in amber bottles at $5^{o}C$. Stock standards and working standards may be made up as individual standards or combined standards.

Appendix 3

Preparation instructions for 0.033M KH_2PO_4

---

1) Acidify to pH 2.2 a liter of HPLC grade water with H_3PO_4.
2) To approximately 900 ml of the acidified water add 4.491 g KH_2PO_4.
3) Readjust pH to 2.2 using H_3PO_4.
4) Bring to 1 liter with pH 2.2 water.
5) Filter and degas.

Appendix 4

Composition of rat diets

Component	Quantity/ Kg diet
Vitamin-free casein	150 g
Sucrose	290 g
Alphacel	20 g
Jones-Foster salt mixture	40 g
Crisco shortening	100 g
L-Methionine	0.8 g
L-Cystine	0.4 g
Cornstarch which contains the following vitamins:	
Thiamine hydrochloride	2.5 mg
Riboflavin	4.0 mg
Niacinamide	15.0 mg
d-Pantothenic Acid, Ca salt	20.0 mg
Folic acid, crystalline	0.2 mg
p-Aminobenzoic acid	20.0 mg
Biotin	0.2 mg
Vitamin B_{12}	3.0 mg
Menadione	10.0 mg
Choline chloride	3000.0 mg
Corn oil which contains the following vitamins:	
dl-α-tocopherol acetate	60.0 mg
Vitamin D_2	0.19 mg
Vitamin A palmitate	10.3 mg

Experiment 1

Diet 1 contains 1.2 mg PN HCl/kg diet
Diet 2 contains 2.4 mg PN HCl/kg diet
Diet 3 contains 3.6 mg PN HCl/kg diet
Diet 4 contains 7.2 mg PN HCl/kg diet

Experiment 2

Same as experiment 1 for diets 1-4
Diet 5 contains 9.6 mg PN HCl/kg diet

Experiment 3

Deficient diet contains no additional PNHCl
Control diet contains 7.2 mg PN HCl/kg diet

Appendix 5

Modified sulfosalicylic acid sample extraction method

---

1) Thaw plasma in ice bath.
2) Vortex plasma for 5 sec.
3) Pipette 3 ml plasma into 15 ml sorvall tube, keep on ice.
4) Add 10 μl NaF/DPN solution (0.05M NaF/5000 ng DPN/ml).
5) Vortex 15 sec.
6) Add 0.15 g SSA to make 5% W^t/V solution.
7) Vortex 5 sec.
8) Homogenized for 30 sec.
9) Add 3 ml cold CH_2Cl_2, use half to rinse homogenizer blades.
10) Incubate in ice 10 min.
11) Centrifuge 10 min at 4^oC at 7500 x g.
12) Remove aqueous layer with pipette.
13) Place aqueous portion on 10 cm column of AG2-X8 equilibrated in 0.1 N HCl.
14) Collect under vacuum.
15) Filter using 0.2 μm filter.
16) Collect in brown vial.

Appendix 6

Preparation instructions for reagents of the mobile phase

---

1) 85% Methanol

   a) Measure 850 ml HPLC grade methanol, add 150 ml HPLC grade water.

   b) Filter and sonicate.

   c) Store at room temperature.

2) PIC B-7 solution 0.005M

   a) Measure 1000 ml HPLC grade water into Erlenmyer flask.

   b) Add 1 bottle PIC B-7.

   c) Stir mechanically for at least 5 min.

   d) Filter and sonicate under vacuum.

   e) Store at $5^{o}C$.

Appendix 7

Preparation instructions for reagents of potato acid phosphorylase sample extraction method

---

1) Potassium acetate buffer 0.2M, pH 4.5

   a) Dissolve 1.962 g KOAc in approximately 900 ml ion free water.

   b) Adjust pH to 4.5 using 10% HOAc and 1.0 N KOH.

   c) Bring to volume in a 1 liter volumetric flask with ion free water.

2) Potato acid phosphorylase enzyme solution

   a) In a 10 ml volumetric flask, dissolve 1.0 g PAP in 0.2 M KOAc, pH 4.5 bringing to volume.

   b) Stir mechanically until dissolved.

   c) Make fresh daily.

Appendix 8

Pooled plasma B-6 vitamer concentration for rats in experiment 1*

Rat	PL	PN	PM	Total
		ng/ml		
101 106	72.3	13.6	39.6	125.5
102 103	51.5	14.3	ND	65.8
104 107 109	49.1	13.8	11.9	74.8
105 108 110	76.4	5.9	33.3	115.6
111 112	58.0	15.2	11.7	84.9
201 208	224.0	21.6	12.2	257.8
202 204 205	178.1	25.8	8.7	212.6
203 207	208.2	13.9	25.9	248.0
206 209 211	194.4	15.7	33.9	244.0
210 212	224.2	19.1	23.3	266.6
301 303	270.5	16.9	37.5	324.9
302 306 312	252.8	ND	20.5	273.3
304 305	228.3	20.5	ND	248.8
307 308 309	191.1	15.9	9.1	216.1
310 311	243.2	17.7	6.4	267.3

Appendix 8 (Cont.)

Rat	PL	PN	PM	Total
		ng/ml		
401 402	249.5	19.9	28.4	297.8
403 408 409	330.6	22.4	6.5	359.5
404 406 410	275.6	20.7	18.2	314.5
405 407 411	279.1	7.0	13.8	299.9

*ND = non detectable.

Appendix 9

Individual % distribution of B-6 vitamers in rat plasma

for experiment 1

Rat	PL	PN %	PM
101 106	57.6	10.8	31.6
102 103	78.3	21.7	0
104 107 109	65.6	18.5	15.9
105 108 110	66.0	5.1	28.9
111 112	68.3	17.9	13.8
201 208	86.9	8.4	4.7
202 204 205	83.8	12.1	4.1
203 207	84.0	5.6	10.4
206 209 211	79.7	6.4	13.9
210 212	84.1	7.2	8.7
301 303	83.3	5.2	11.5
302 306 312	92.5	0	7.5
304 305	91.8	8.2	0
307 308 309	88.4	7.4	4.2

Appendix 9 (Cont.)

Rat	PL	PN	PM
		%	
310 311	91.0	6.6	2.4
401 402	83.8	6.7	9.5
403 408 409	92.0	6.2	1.8
404 406 410	87.6	6.6	5.8
405 407 411	93.1	2.3	4.6

Appendix 10

Pooled plasma B-6 vitamer concentration of rats in experiment 3

Rat	PL	PN ng/ml	PM	Total
D-01 D-05	11.7	11.2	59.0	81.9
D-02 D-08	11.9	11.1	61.6	84.6
D-03* D-04	11.8	9.2	81.1	102.1
D-06*	8.8	6.8	66.1	81.7
D-07* D-09	8.3	12.1	72.2	92.6
D-10**	35.5	14.1	62.9	112.5
C-01*** C-12	321.7	55.1	41.6	418.4
C-02 C-08*	450.1	12.2	74.5	536.8
C-03 C-15	381.8	21.4	110.1	513.3
C-04	428.1	18.4	63.4	509.9
C-05 C-06	360.8	55.1	73.7	489.6
C-07	319.0	11.6	25.5	356.1
C-09 C-11*	441.8	23.8	63.6	529.2
C-10	305.0	17.0	51.9	374.9
C-13 C-14*	368.3	17.8	77.3	463.4

*Animals sacrificed with CO_2.

**Animal sacrificed with CO_2 and electrocution.

***Sample extract was frozen prior to analysis.

Appendix 11

Individual % distribution for B-6 vitamers in plasma of rats in experiment 3

Rat	PL	PN	PM
		%	
D-05	14.3	13.7	72.0
D-02 D-08	14.1	13.1	72.8
D-03 D-04	11.6	9.0	79.4
D-06	10.8	8.3	80.9
D-07 D-09	9.0	13.0	78.0
C-01 C-12	76.9	13.2	9.9
C-02 C-08	83.8	2.3	13.9
C-03 C-15	74.4	4.2	21.4
C-04	84.0	3.6	12.4
C-05 C-06	89.6	3.3	7.1
C-07	94.0	3.0	3.0
C-09 C-11	83.5	4.5	12.0
C-10	81.6	4.6	13.8
C-13 C-14	79.5	3.8	16.7

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SEPARATION AND QUANTITATION OF B-6 VITAMERS IN RAT PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

Theresa E. Hefferan

(ABSTRACT)

A high performance liquid chromatographic method was developed for the separation and quantitation of the B-6 vitamers in rat plasma. The separation time was under 20 minutes. The analyses were accomplished using a reverse phase C_{18} column; a binary system of methanol and heptane sulfonic acid, the ion pair; and a fluorescence detector using 300 nm excitation and 375 nm emission filters. The minimum detectable quantity was determined to be 5 ng for PL and PN and 4 ng for PM. Samples were extracted using potato acid phosphatase; phosphorylated forms of the B-6 vitamers were converted to nonphosphorylated. The B-6 vitamers were measured in plasma from rats which had intakes of vitamin B-6 ranging from 7% - 551% of the basal requirement for the vitamin. PL was found to be the predominate B-6 vitamer in plasma from animals consuming <50% B-6 basal requirement and PM for the lowest intake group. PL and total B-6 values were significantly correlated ($r = 0.99$, $p < 0.0001$). Significant differences ($p < 0.01$) in plasma PL and total B-6 concentrations were observed for rats with inadequate and adequate B-6 intakes. The method seemed to be a sensitive indicator of vitamin B-6 status in rats thus indicating potential for use with humans.