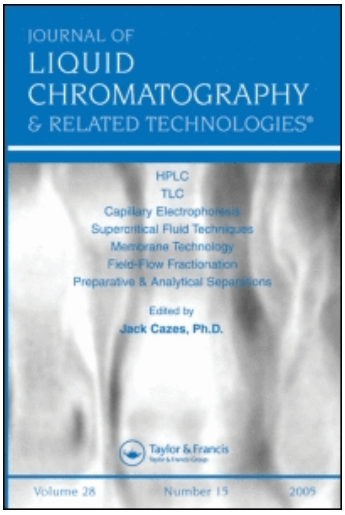

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Determination of B_6 Vitamers in Serum by Simple Isocratic High Performance Liquid Chromatography

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DETERMINATION OF B_6 VITAMERS IN SERUM BY SIMPLE ISOCRATIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the determination of individual B_6 vitamers was developed, and its application to dog, rat, monkey and human serum is described. Serum samples were deproteinated with 8% aqueous perchloric acid and the extracts were purified using an anion-exchange column. Analytical separation of the B_6 vitamers and the internal standard (4'-deoxypyridoxine) was achieved by isocratic elution with a nitrile phase column in less than 30 minutes. The described methodology is simple and fast, and yields high percentage recoveries (> 90) of the vitamers with low inter- and intra-day variations.

INTRODUCTION

Vitamin B_6 is a collective term used for the metabolically related 2-methyl-3-hydroxy-5-hydroxymethyl pyridines. It exists in three interconvertible forms: pyridoxine, pyridoxal, and pyridoxamine, and each of these three has its corresponding 5'-phosphate. It is well known that vitamin B_6 is required for the growth and development of normal and neoplastic tissue. In general, pyridoxal-5'-phosphate is the co-enzyme of many classes of enzymes (1). The principle urinary metabolite of all forms of vitamin B_6 is 4-pyridoxic acid, which has no biological activity, but is often monitored to determine vitamin B_6 status (2). The chemical structures of the vitamers and the metabolite are given in figure 1.

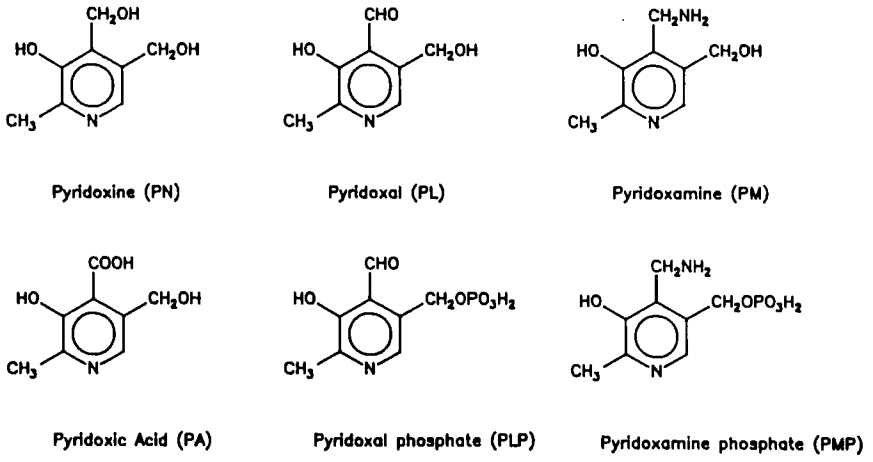

FIGURE 1. Chemical structures of the B_6 vitamers and metabolite.

Traditionally, vitamin B_6 is determined microbiologically or enzymatically. However, these assays are subject to the criticism that they fail to distinguish between the various forms of B_6 which can be of hindrance in metabolic studies. There are literature reports of the separation of these vitamers by TLC (3), and gas chromatography (4-6), but due to the poor resolution and/or instability of these compounds, development of a reliable and efficient method has not been achieved. More recently, high performance liquid chromatography has been utilized extensively for the separation and quantitation of these compounds (7-16) with or without their respective 5'-phosphates. Different approaches tried have included ion-exchange and reversed phase chromatography, with or without ion-pairing reagent, and always using a gradient mode of elution. Chromatographic separation in the isocratic mode is preferred over the gradient mode of elution because of its simplicity, and reproducibility. Tsuge et al (15) reported a method, capable of separating the B_6 vitamers in biological fluids using the isocratic mode, but this yielded higher than expected concentrations of the pyridoxal-5'-phosphate, the most important form of the B_6 vitamer. In this report, we describe a method which is capable of separating the B_6 vitamers and their metabolite pyridoxic acid in a single run in less than 30 minutes by isocratic HPLC, and its application to the serum samples of different species including humans.

EXPERIMENTAL

Materials

Pyridoxine (PN), pyridoxamine (PM), pyridoxamine phosphate (PMP), pyridoxal (PL), pyridoxal phosphate (PLP), pyridoxic acid (PA), 4-deoxypyridoxine (internal standard, I.S.), and n-octanesulphonic acid were purchased from Sigma (St. Louis, MO). Anion-exchange AG1-X2 was obtained from Bio-Rad (Richmond, CA).

All other chemicals and solvents such as citric acid and perchloric acid were of analytical grade. All aqueous solutions were prepared using double distilled water.

Chromatographic Conditions

The HPLC system consisted of a Spectra Physics (San Jose , CA) SP 8700XR pump, SP8780 autosampler with 20 μl of sample loop along with SP 4270 recorder-integrator. For detection, a Perkin Elmer 650-10S fluorescence detector (Norwalk, CA) was used. The fluorometer was set at an excitation wavelength of 300 nm and emission wavelength of 375 nm. Both slit openings were set at 20 nm.

Chromatographic separations were achieved on a 5 μm nitrile column (25 x 0.46 cm) obtained from Regis (Morton Grove, IL). A 2 cm size Pelli guard column (LC-CN) from Supelco Canada was used. The mobile phase was prepared by dissolving 3.15 g (0.015M) of citric acid and 0.293 g (0.00125M) of n-octanesulphonic acid in 1l of water. The pH of the mobile phase was 2.5. To this, 2 ml/l of acetonitrile was added to retard mold growth. The flow rate of the mobile phase was set at 1 ml/min, which yielded a back pressure of 2500 psi at ambient temperature.

Standard Solution

Aqueous stock solutions of 1.0 mg/ml of all the compounds were prepared separately. These solutions were further diluted individually with water and mixed together to prepare a working standard solution, having the following concentrations: 2.40 μg/ml of PMP, 4.80 μg/ml of PA and PL, 9.60 μg/ml PN and PM, and 24.0 μg/ml of PLP, and 100 μl of this solution was used for spiking purposes.

Internal Standard Solution

An aqueous solution of 1.0 mg/ml of 4-deoxypyridoxine was prepared and further diluted with water to give a working solution of 48.0 μg/ml.

Sample preparation

Preparation of Ion-exchange Column: Approximately 100 ml of AG1-X2(Cl^-) resin was placed in a 600 ml size glass beaker and washed once with water. To this 500 ml of 1 N NaOH solution was added while gently stirring to replace the Cl^- with OH^-, and then the resin was thoroughly rinsed with 6x500 ml of water, until the pH of the washing corresponded to that of the water used. Two nylon cloth discs were placed in a 3 ml disposable syringe, and the column was packed with the prepared AG1-X2(OH^-) resin to a 2 ml wet bed volume. At this stage, these columns can be stored for later use. Prior to use, columns were once again rinsed with double distilled water.

Extraction Procedure: To 500 μl of the serum sample, in a 15 ml centrifuge tube fitted with a PTFE-lined screw cap, were added 100 μl of I.S. solution, 400 μl of water to maintain a constant volume of 1.0 ml for extraction, and 1.0 ml of a solution of perchloric acid in water (8%). This mixture was vortexed for one minute, then centrifuged at 2.5 K rpm at 4 °C for 5 minutes. The supernatant was transferred to another tube and the sediment was suspended in 1.0 ml of water. After agitation and centrifugation the supernatant was combined with the previous one. These two combined supernatants were applied to the ion-exchange column followed by 6.0 ml of 0.1 M citric acid in water. The first 2.0 ml of the eluent were discarded, while the next 6 ml fraction containing all the vitamers was used for the HPLC analysis.

Calibration

Linear calibration curves were prepared by least square regression analysis of peak height ratio of analyte to I.S. against standard concentration of the vitamers. The amounts of B_6 vitamers in 500 μl of serum for calibration curves ranged from 300 to 2400 ng for PLP, 60 to 480 ng for PA and PL, 120 to 960 ng for PN and PM, and 30 to 240 ng for PMP. These concentrations levels were obtained by mixing 100 μl of the spiking standard solution with 0.5 ml serum sample for the highest concentrations. For lower concentrations, the working standard solution was serially diluted to the required concentration prior to spiking with 100 μl of the diluted solution.

RESULTS AND DISCUSSION

Figure 2 shows a typical chromatographic separation of the B_6 vitamers. All of the vitamers are very well separated, and their separation is accomplished in less than 30 minutes. Although the separation of all these vitamers can be achieved without using n-octanesulphonic acid, its use is beneficial for two reasons: firstly, it

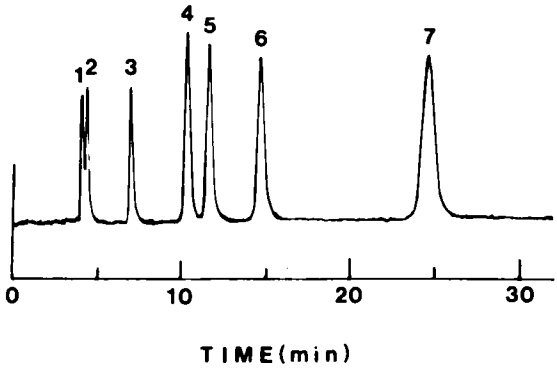

FIGURE 2. Chromatographic traces of B_6 vitamers and metabolite.

Peaks 1= 8 ng PLP; 2= 1.6 ng PA; 3= 0.8 ng PMP; 4= 1.6 ng PL; 5= 3.2 ng PN; 6= 16 ng 4-deoxypyridoxine (I.S.); 7= 3.2 ng PM. See Fig 1 for the chemical names of the abbreviations used.

helps in reducing elution times of the vitamers (especially that of PMP); and secondly it improves the quality of peak shape. This behaviour could possibly be explained by ion-pair formation.

The HPLC separation methods for B_6 vitamers described by Coburn and Mahureen (11) and Shephard et al (16) use three different solvents to elute the vitamers, and need 50 minutes for each run. On the other hand, the method describe by Vanderslice and Maire (7) uses two different chromatographic columns connected by a 6-way valve system and requires an analysis time of more than 60 minutes. The method described by Gregory and Feldstein (13) uses a two pump system with three solvents. Compared to the complexities of solvent programming and column switching, the present methodology provides a simpler alternative using one solvent and one column for the chromatographic separation of B_6 vitamers and metabolite PA. The entire run is completed in less than 30 minutes.

The present extraction procedure, which is compatible with the HPLC conditions used, is based upon the deproteination of the serum samples with perchloric acid, one ml of 8% perchloric acid solution in water being found to be sufficient for the precipitation of proteins. A second wash step was then included to improve the recovery of the B_6 vitamers. The combined supernatants of the extract were passed through the ion-exchange column. These columns were easily prepared

in the laboratory and were preferred to the columns available from Bio-Rad, as in the latter case the elution volume was much higher and the conversion of the Cl^- to OH^- form took much longer. All the vitamers and the metabolite were eluted quantitatively from the ion-exchange column in a 6.0 ml volume of 0.1 M citric acid solution.

The extraction procedure employed in this study is essentially similar to that described by Gregory and Feldstein (13), in which a peristaltic pump is used with a fluorometer to monitor the elution of vitamers from the clean-up column. However, PA was not included in that study, as it was difficult to elute from the clean-up column. In the present method, a much smaller, laboratory-prepared disposable column was used and all of the vitamers along with the metabolite PA were eluted in 6.0 ml of citric acid, allowing both the vitamers and the metabolite to be measured in the same run.

Typical chromatograms of dog serum, blank and serum with B_6 vitamers added are shown in figure 3, there was no interference from serum. A peak at PL retention time is assumed to be due to endogenous PL. While most of the method developmental work was done using dog serum samples, the described methodology was found to be equally valid for monkey, rat and human serum samples. Representative chromatograms of spiked and blank serum samples are given in figure 4. Pyridoxine phosphate was not evaluated in this study because of its minor significance as a naturally occurring form of vitamin B_6 (11,13).

Calibration lines were drawn using dog serum samples, and were linear over the ranges mentioned in the text with minimal intercepts and high correlation coefficients (Table 1).

Percentage recoveries of vitamers were also determined (Table 2). Serum samples were spiked with 100 μl of the standard solutions and passed through the whole extraction procedure. Percentage recoveries were determined by comparing the ratio of the peak-height of I.S. to the respective extracted vitamer to that of the ratio of the standards after injecting in water. In general, the recoveries were well above 90% with minimal variations. Inter- and intra-day variations of the whole procedure, including extraction and chromatographic separation, were also assessed and found to be minimal (Table 3).

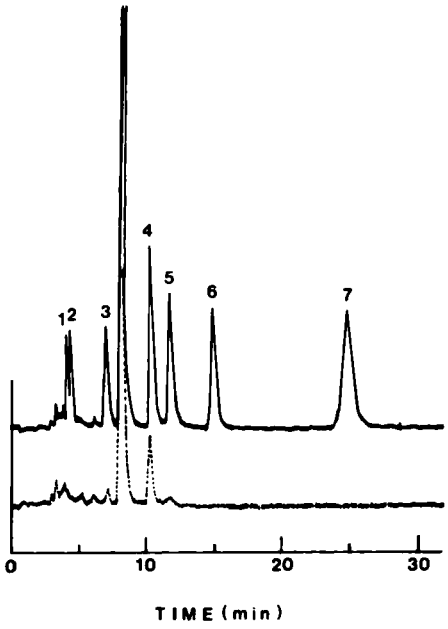

FIGURE 3. Chromatographic traces of B_6 vitamers and metabolites. (-----) blank: (____) spiked dog serum samples. For peaks order and amounts see figure 2.

TABLE 1. Slopes and Intercepts of the Calibration Lines for the B_6 Vitamers and Metabolite.

Vitamer	Slope	Intercept	Correlation Coefficient (r)
PLP	0.0018 ± 0.0006	0.0098 ± 0.0134	0.991
PA	0.0097 ± 0.0002	0.0220 ± 0.0111	0.995
PMP	0.0186 ± 0.0006	0.0758 ± 0.0123	0.993
PL	0.0124 ± 0.0006	-0.0289 ± 0.0286	0.990
PN	0.0063 ± 0.0002	0.1010 ± 0.0182	0.991
PM	0.0061 ± 0.0002	0.0071 ± 0.0185	0.990

TABLE 2. Percentage Recoveries of B_6 Vitamers and Metabolite from Dog Serum.

Compound	Amount added (ng)	% Recovery (± %CV)
PLP	2400	91.43 ± 7.65
PA	480	95.62 ± 5.24
PMP	240	98.29 ± 5.93
PL	480	88.63 ± 1.77
PN	960	102.56 ± 5.67
PM	960	96.85 ± 6.69

TABLE 3. Inter- and Intra-day Variation in Percentage Recoveries of B_6 Vitamers from Dog Serum.

Compound	Amount added (ng)	CV (%) Intra-day	CV (%) Inter-day
PLP	2400	4.34	6.64
PA	480	6.42	4.99
PMP	240	3.61	1.55
PL	480	5.84	5.03
PN	960	4.97	4.58
PM	960	2.76	2.41

Since this methodology was developed for the determination of B_6 vitamers after megadose therapy, high sensitivity was not of great concern. However, for improved sensitivity of the method, extracts after ion-exchange purification step were freeze dried, and redissolved in HPLC mobile phase to give a 10-fold concentration of the standards and applied onto the HPLC column without significant deterioration of the peak shape.

In summary, the described method is not only capable of separating the B_6 vitamers but also the metabolite pyridoxic acid by an isocratic HPLC system coupled with the extraction procedure, which is capable of extracting all the vitamers from a given serum sample. The method is simple, fast, reproducible and inexpensive.

FIGURE 4. Chromatographic traces of B_6 vitamers and metabolites. (------) blank: (_____) spiked serum samples: (a) Rat, (b) monkey and (c) human. Peaks order and amounts are the same as described in figure 2.

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