Journal of Chromatography, 424 (1988) 315–323
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3985

SIMPLE ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SEPARATION OF THE SIX VITAMERS OF VITAMIN B_6

CHRIS J. ARGOUDELIS

University of Illinois, Department of Food Science, 580 Bevier Hall, 905 S. Goodwin, Urbana, IL 61801 (U.S.A.)

(First received July 22nd, 1987; revised manuscript received September 30th, 1987)

SUMMARY

A simple, sensitive and fast isocratic high-performance liquid chromatographic method has been developed for the separation of all six biologically active forms (vitamers) of vitamin B_6. The separation is accomplished using a strong cation-exchange column and a mobile phase of 0.1 *M* ammonium dihydrogenphosphate adjusted to pH 4.0. All six vitamers are separated within 20 min at a flow-rate of 1 ml/min. The concentration of the vitamers is determined with a fluorescence detector (excitation 290 nm; emission 389 nm). The within-run precision of the method expressed as the coefficient of variation is below 5% at the 25 pmol level. Pyridoxal 5′-phosphate can be determined using either pre- or post-column derivatization with sodium bisulfite. Application of the method to cell-free yeast culture media is presented.

INTRODUCTION

Numerous high-performance liquid chromatography (HPLC) methods are available for the determination of vitamin B_6 [1]. However, only a few methods have been reported in the literature for the separation of all six biologically active forms (vitamers) of vitamin B_6 [2–5] [pyridoxal (PL), pyridoxine (PN) and pyridoxamine (PM) and their corresponding 5′-phosphate esters].

The fact that one of the vitamers, PM, is very basic while the phosphorylated forms are very acidic makes it difficult to separate all six forms on one column and within a reasonable amount of time without resorting to either step-gradient [2] using two columns [3] or ternary gradient [4,5] elution techniques. The advantages of an isocratic elution are well known. Fluorescence detectors are used to enhance the specificity and sensitivity of vitamin B_6 determination in biological systems.

0378-4347/88/$03.50

In this paper a simple, sensitive isocratic HPLC method is reported that separates all six vitamers within 20 min.

EXPERIMENTAL

Reagents

PL, PN, PM, 4′-deoxypyridoxine (4-dPN), pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) were obtained from Sigma (St. Louis, MO, U.S.A.). Pyridoxine 5′-phosphate (PNP) was prepared from PLP [6]. Isopyridoxal (isoPL) was made by hydrolyzing isopropylidene isopyridoxal [7] with 0.1 *M* hydrochloric acid and evaporating to dryness. All other chemicals were of reagent grade.

High-performance liquid chromatography

The liquid chromatograph consisted of an M-45 pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector with 20-μl loop and a Model 980 fluorescence detector (Kratos Analytical, Ramsey, NJ, U.S.A.) equipped with a 5-μl flow-cell. A 290-nm excitation wavelength and a 389 nm long pass cut-off emission filter were used. The detector was connected to a Model A-25 strip-chart recorder (Varian Aerograph, Walnut Creek, CA, U.S.A.) and to a Model 3390A reporting integrator (Hewlet-Packard, Avondale, PA, U.S.A.). A guard column (70×2.1 mm) was followed by a 250×4.6 mm I.D. Partisil-10SCX analytical column (Whatman, Clifton, NJ, U.S.A.). A silica gel saturation column preceded the injector. The mobile phase, 0.1 *M* ammonium dihydrogenphosphate ($NH_4H_2PO_4$) in glass-distilled water, was adjusted to pH 4.0 with a few drops of 85% phosphoric acid and passed through a 0.45 μm filter (Millipore, Bedford, MA, U.S.A.). The column was operated at ambient temperature, and the flow-rate was 1 ml/min (pressure ca. 28 bar).

Reference solutions

Solutions of the five vitamers (1.25–5.00 μM) and the internal standard 4-dPN (6.25–25 μM) in glass-distilled water were kept in the refrigerator and used within one week. Solutions of PLP (1.25–5.00 μM) in 0.1 *M* $NH_4H_2PO_4$ pH 4.0 and 15 μM 4-dPN containing 1 mg/ml sodium bisulfite were freshly made and used within 2 h.

Standard curves

Five replicate injections of 20 μl from the standard solutions were used to construct linear regression lines (peak area versus pmol). The injected quantities of the compounds were in the linear range of the dececor, and the correlation coefficients of the straight line graphs of all six vitamers injected were better than 0.997. The quantities of the six vitamers injected were in the range 25–100 pmol. Similar correlation coefficients were obtained when the internal standard plot method was used (peak-area ratios of each of the six vitamers over 4-dPN versus the corresponding ratios of their respective weights). The fluorescence intensity (area under the peaks) of five of the vitamers did not change appreciably from

injection to injection during the day or within three days. The fluorescence intensity of the addition compound of PLP with sodium bisulfite changes appreciably after 2 h. However, the ratio of the peak areas PLP/4-dPN or PLP/PNP remains constant for a few days.

Recovery studies

Quantities of the six vitamers at two levels each were added into 100-ml volumetric flasks containing culture media that were not inoculated and also 1.5 μmol of the internal standard 4-dPN. Recoveries of the individual vitamers were calculated from the internal standard plot curves.

Yeast culture

Yeast mutant P-131 [8] was grown in culture media [9] omitting pyridoxine hydrochloride but adding nicotinic acid (0.2 mg/l). The yeast was grown in conical flasks incubated at 29°C in a Model G25 gyrotory shaker (New Brunswick Scientific, Edison, NJ, U.S.A.) at 70 rpm. After five days, an aliquot from the yeast culture media was filtered through a 0.45-μm filter and injected into the chromatograph.

Microbiological assay

Total vitamin B_6 activity due to PL, PN and PM secreted in the culture media of mutant P-131 was determined in filtered aliquots using *Saccharomyces uvarum* as the test organism by the procedure of Toepfer and Lehmann [10].

All experiments were carried out under conditions of subdued light.

RESULTS AND DISCUSSION

We have been using Partisil-SCX columns for a few years to separate vitamin B_6 compounds [6,11]. The method that is reported here was originally developed [12] using an "aged" column. Attempts to reproduce that method using a brand new column were unsuccessful. PN and PM were not eluted from the column when the mobile phase was 0.1 *M* $NH_4H_2PO_4$. However, their elution was possible when the ammonium phosphate buffer solution was adjusted to pH 4.0 with a few drops of orthophosphoric acid. In Fig. 1 is depicted a chromatogram obtained when 25 pmol of the five vitamers, 250 pmol of PLP, 25 pmol of isoPL and 125 pmol of 4-dPN were injected into the chromatograph. The small peak that precedes PLP is due to a strongly fluorescent impurity present in commercial PLP. There is good separation of all vitamin B_6 compounds. The separation between PLP and PNP is not excellent. However, it has been reported [3,4,13] that PNP has a minor significance as a naturally occurring vitamer; therefore, its concentration will not interfere with the integration of the PLP peak when post-column derivatization [4,5] is used with this method.

Among the six vitamers, PLP is the only one that has a low fluorescence intensity due to the presence of an intact carbonyl group. In order to increase the natural fluorescence of PLP, pre-column [3] or post-column [4] derivatization has been used. In this method, due to lack of instrumentation for post-column

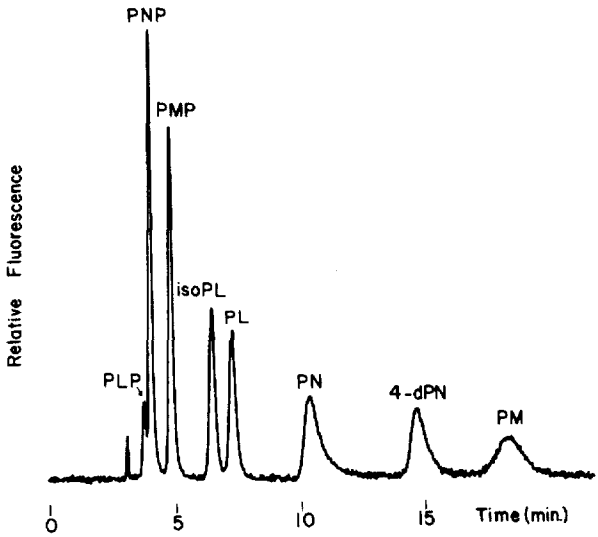

Fig. 1. HPLC tracing of B_6 vitamers and internal standards on Partisil-10SCX (250×4.6 mm) column at 0.05 a.u.f.s. Chart speed was 0.635 cm/min. Amounts injected: 25 pmol of each on the five vitamers, 250 pmol of PLP, 25 pmol of isoPL and 125 pmol of 4-dPN. The mobile phase was 0.1 *M* $NH_4H_2PO_4$, pH 4.0 at a flow-rate of 1 ml/min.

derivatization, pre-column derivatization for only PLP is used. It was observed that under the chromatographic conditions of this method the pre-column reagent, sodium bisulfite, affects (reduces) the fluorescence intensity of all vitamers probably as soon as it is added to the solution of the vitamers. It is, however, detected at the time the vitamer is eluted from the column (area under the peak of the vitamer before and after the addition of solid sodium bisulfite). In Fig. 2 is depicted a chromatogram of 50 pmol each PLP and PNP before (A) and after (B) the addition of solid sodium bisulfite. In Fig. 2A the peak for PLP can hardly be seen; however, in Fig. 2B a large peak due to the adduct of PLP and sodium bisulfite is present. The fact that the PLP–$NaHSO_3$ adduct is eluted with the void volume is a disadvantage of the method. However, since it is advantageous to measure the other vitamers without adding sodium bisulfite in the samples one could inject the same sample before and after the addition of sodium bisulfite, and subtract the area of any peak at the void volume, if there is any present, before the addition of sodium bisulfite. In the same figure one can notice the reduction in fluorescence intensity of the PNP peak after the addition of solid sodium bisulfite.

The dose–response graphs were linear for all the vitamers and for the internal standard 4-dPN at concentrations five times higher than those of the vitamers due to the low fluorescence intensity of 4-dPN under these conditions. The dose–response graph for PLP was that of the PLP–$NaHSO_3$ adduct. The method is sensitive, $1.8 \cdot 10^6$ area counts for PNP (largest) and $9.5 \cdot 10^5$ area counts for PL (smallest) for 25 pmol each with only a 5-μl flow-cell. It is believed that a larger-volume flow-cell could further increase the sensitivity of the method with-

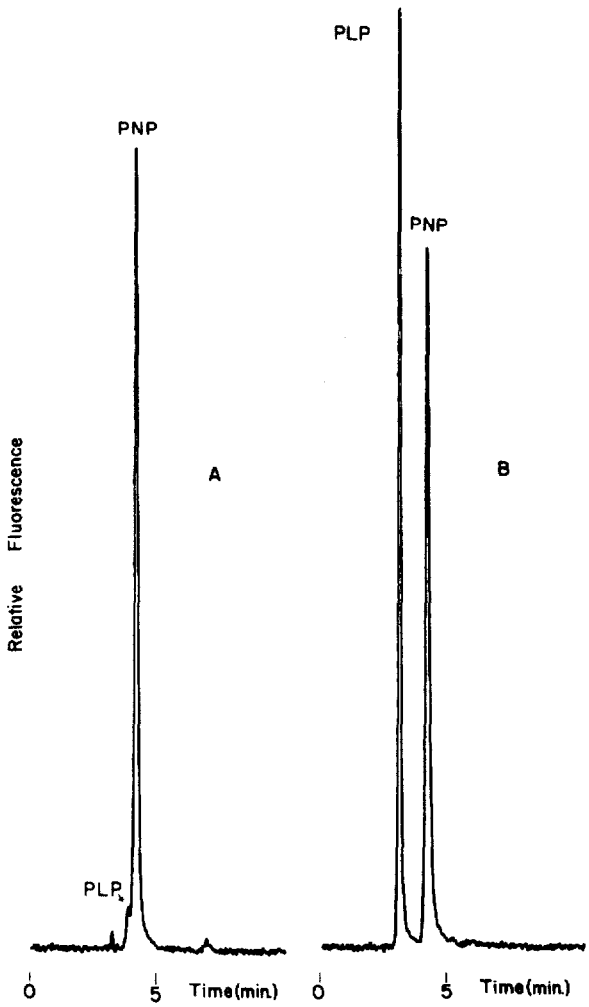

Fig. 2. HPLC tracings of 50 pmol each of PLP and PNP before (A) and after (B) the addition of solid $NaHSO_3$ at 1 mg/ml. Chromatographic conditions same as in Fig. 1.

out sacrificing resolution. No attempt was made to determine the lower limit of detection for the six vitamers. The activation and emission wavelengths were selected for their sensitive response to all vitamers. They are not the optimum settings for all vitamers. The within-run precision of the method is good as can be seen in Table I where the coefficient of variation is presented for five injections of each vitamer at the four levels used.

In Table II are reported the recoveries of the six vitamers at two levels, 25 and 75 pmol. The vitamers were added to culture media that had not been inoculated. Since in the chromatogram of the non-inoculated culture media the fluorescent area is narrow and its intensity small (Fig. 3A), the recoveries of the vitamers are very good especially at the 75-pmol level.

In Fig. 3B is depicted the chromatogram obtained from cell-free culture media

TABLE I

WITHIN-RUN PRECISION OF METHOD FOR ANALYSES OF REFERENCE SOLUTIONS ($n=5$)

Vitamer	Coefficient of variation (%)			
	25 pmol	50 pmol	75 pmol	100 pmol
PLP	3.9	2.2	0.9	1.6
PNP	2.9	3.5	2.4	0.7
PMP	2.9	3.3	2.4	0.4
PL	4.6	3.8	2.8	0.8
PN	5.1	3.3	3.0	2.0
PM	3.6	3.2	3.3	1.0
4-dPN	4.9	3.1	3.0	0.4

of yeast mutant P-131 after it was grown for five days. None of the peaks preceding PL correspond to the retention times of the phosphorylated vitamers, PNP or PMP. When a similar aliquot was injected after the addition of sodium bisulfite a new peak appeared close to void volume but its retention time was longer than that of PLP. Apparently, in the culture media of the mutant there is present a carbonyl-containing compound which forms an adduct with sodium bisulfite and fluoresces under these conditions. It has been shown before [8] that the culture media of the mutant P-131 do not contain the phosphorylated vitamers. The main vitamer secreted in the media is PN (79.1%) along with small quantities of PL (10.5%) and PM (10.4%). The HPLC profile of the cell-free culture media is very similar to the one reported with column chromatography using ion-exchange resin [8]. When mutant P-131 was grown in the culture media reported previously [8], i.e. Pyridoxine-Y (Difco Labs., Detroit, MI, U.S.A.) the chromatogram showed quite a few very large peaks eluting before PL. Most of those peaks are due to compounds present in the Pyridoxine-Y media since they exist in non-inoculated media.

In Table III are reported the individual and total vitamin B_6 quantities in cell-

TABLE II

RECOVERY OF VITAMERS ADDED TO NON-INOCULATED CULTURE MEDIA

Vitamer	Recovery (mean ± S.D., $n=3$) (%)	
	25 pmol per 20 μl culture media	75 pmol per 20 μl culture media
PLP	102.1 ± 4.8	101.3 ± 2.7
PNP	99.3 ± 4.2	102.1 ± 2.5
PMP	101.4 ± 3.8	99.4 ± 2.3
PL	100.1 ± 4.2	99.2 ± 2.9
PN	98.2 ± 3.9	101.2 ± 3.1
PM	97.3 ± 4.6	98.6 ± 3.2

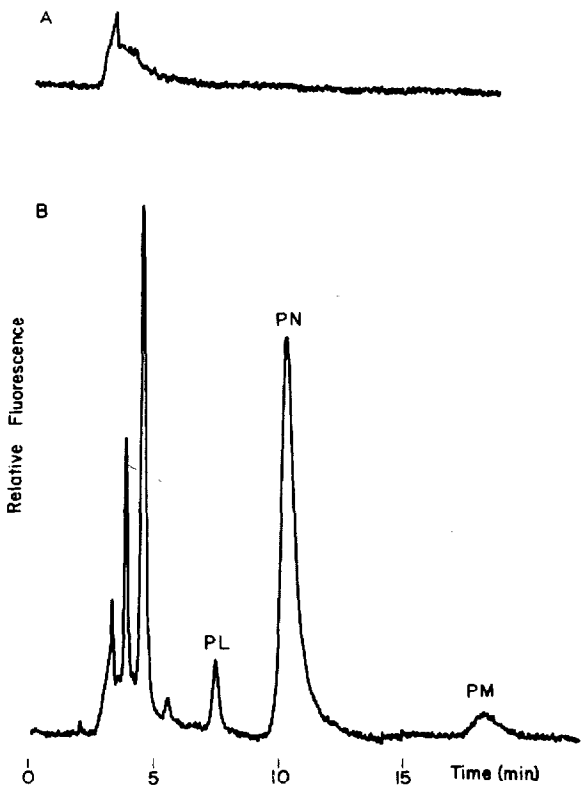

Fig. 3. HPLC tracings of yeast culture media that had not been inoculated (A) and cell-free culture media of yeast mutant P-131 (B). Chromatographic conditions are the same as in Fig. 1.

free culture media of yeast mutant P-131 as calculated by the HPLC and by the microbiological assay methods. The correlation is very good.

Reliable determination of the vitamers in biological systems, especially if clean-up steps are involved, necessitates the use of an internal standard with a chemical structure similar to that of the vitamers. Two vitamin B_6 analogues, 4-dPN and isoPL, fulfill this requirement and can be used as internal standards with this method. The analogue 4-dPN has been used before as internal standard [5] and

TABLE III

INDIVIDUAL AND TOTAL VITAMIN B_6 IN CELL-FREE CULTURE MEDIA OF MUTANT P-131 CALCULATED BY HPLC AND MICROBIOLOGICAL METHODS

Vitamer	Concentration (mean ± S.D., $n=3$) (nmol/ml)	
	HPLC	Microbiological
PL	0.76 ± 0.06	
PN	5.73 ± 0.12	
PM	0.75 ± 0.07	
Total	7.24 ± 0.15	6.90 ± 0.55

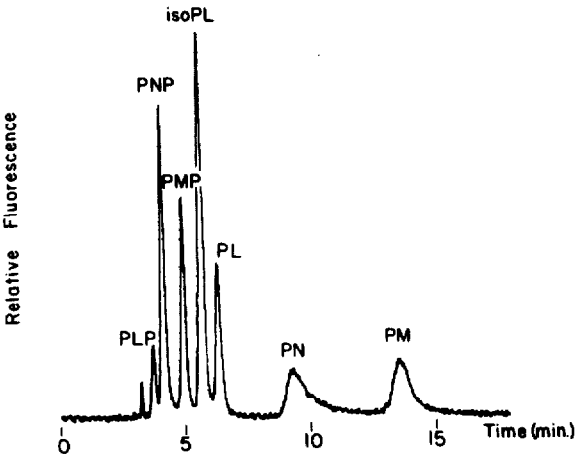

Fig. 4. HPLC tracing of B_6 vitamers and isoPL on an "aged" Partisil 10SCX (250×4.6 mm) column at 0.05 a.u.f.s. Chart speed was 0.635 cm/min. Amounts injected: 25 pmol of each of the five vitamers, 250 pmol of PLP and 50 pmol of isoPL. Mobile phase was 0.1 *M* $NH_4H_2PO_4$ at a flow-rate of 1 ml/min.

has the advantage that it is commercially available. The other analogue, isoPL, can be easily synthesized [7].

After a few months' use of the column under the described conditions one could change the mobile phase to 0.1 *M* $NH_4H_2PO_4$ in which case all six vitamers are eluted in less than 15 min as shown in Fig. 4. Additional advantages with this mobile phase are first that the last peak (PM) becomes narrower, thus improving the precision of integration, and second that the resolution between PLP and PNP is improved, desirable in case post-column derivatization is used. A drawback of this mobile phase is the fact that the internal standard 4-dPN co-elutes with PM and that the fluorescence intensity of each of the six vitamers is reduced a little. However, the problem with the internal standard can be overcome by using isoPL as the new internal standard as shown in Fig. 4.

In conclusion, the described method for the separation of the six vitamers of vitamin B_6 is simple and fast permitting the injection of a new sample every 20 min. An additional advantage of the method is that it improves as the column "ages" by changing the pH of the mobile phase. This method can be used for our yeast culture media without any clean-up steps. Experimentation will show whether or not it may be used, without clean-up steps, in other biological systems.

ACKNOWLEDGEMENT

This study was a part of Project No. 50-0330 of the Agricultural Experiment Station, College of Agriculture, University of Illinois at Urbana-Champaign.

REFERENCES

1 S.P. Coburn, in D. Dolphin, R. Poulson and O. Avramović (Editors), Vitamin B_6 Pyridoxal Phosphate, Chemical, Biochemical and Medical Aspects, Part A, Wiley, New York, 1986, Ch. 15, p. 497.

2 G.P. Tryfiates and S. Sattsangi, J. Chromatogr., 227 (1982) 181.
3 J.T. Vanderslice and C.E. Maire, J. Chromatogr., 196 (1980) 176.
4 S.P. Coburn and J.D. Mahuren, Anal. Biochem., 129 (1983) 310.
5 J.F. Gregory, III and D. Feldstein, J. Agric. Food Chem., 33 (1985) 359.
6 C.J. Argoudelis, J. Agric. Food Chem., 34 (1986) 995.
7 P.D. Sattsangi and C.J. Argoudelis, J. Org. Chem., 33 (1968) 1337.
8 R.S. Pardini and C.J. Argoudelis, J. Bacteriol., 96 (1968) 672.
9 C.J. Argoudelis, Biochem. Pharm., 27 (1978) 1145.
10 E.W. Toepfer and J. Lehmann, J. Assoc. Offic. Agric. Chem., 44 (1961) 426.
11 C.J. Argoudelis, J. Chromatogr., 303 (1984) 256.
12 C.J. Argoudelis, presented at the 193rd National Meeting of the American Chemical Society, Denver, CO., April 5–10, 1987, Paper No. 10.
13 J.T. Vanderslice, C.E. Maire and G.R. Beecher, Am. J. Clin. Nutr., 34 (1981) 947.