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Simple high-performance liquid chromatographic method for the determination of all seven vitamin B_6-related compounds

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Abstract

A simple, sensitive, isocratic high-performance liquid chromatographic method has been developed for the separation of all seven vitamin B_6-related compounds. The separation is accomplished using an ODS column and a mobile phase of 0.15 *M* sodium dihydrogenphosphate, adjusted to pH 2.5 with perchloric acid. The concentration of the compounds is determined with a fluorescence detector (excitation, 290 nm; emission, 389 nm). Isopyridoxal is used as an internal standard. The fluorescence intensity of pyridoxal-5′-phosphate is enhanced by post-column derivatization with sodium bisulfite. All seven compounds are separated in less than 20 min at a flow-rate of 1 ml/min. Applications of this method to yeast cell-free culture media, baker's yeast extract, egg and milk are presented. © 1997 Elsevier Science B.V.

Keywords: Vitamins

1. Introduction

Numerous high-performance liquid chromatographic (HPLC) methods are available for the determination of vitamin B_6 [1,2]. 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, i.e. pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), their corresponding 5′-phosphate esters, PNP, PLP, PMP and the end product of vitamin B_6 metabolism in animals, 4-pyridoxic acid (4-PA) [3–12]. Most of these methods use complicated procedures [3,6,12], ternary or step-gradient elution techniques [4,5,7–10] or are very sensitive to pH changes of the mobile phase [11]. All of these drawbacks limit the number of samples that can be analyzed daily. The method reported here is simple, isocratic and sensitive to all seven vitamin B_6-related compounds. All compounds are eluted in less than 20 min and their quantification is improved by using an internal standard.

2. Experimental

2.1. Reagents

PN, PL, PM, PLP, PMP and baker's yeast (YSC-1) were obtained from Sigma (St. Louis, MO, USA). The synthesis of PNP and that of isopyridoxal (iso-PL), the internal standard, have been reported previously [13,14]. 4-Pyridoxic acid was synthesized according to the method of Heyl [15] and purified by dissolving it in dilute sodium hydroxide solution and reprecipitating it using a dilute hydrochloric acid

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solution. 4-Pyridoxic acid-5′-phosphate (4-PAP) was synthesized as described by Wada and Snell [16]. All other chemicals used were of reagent grade.

2.2. High-performance liquid chromatography

The liquid chromatograph system consisted of a 510 pump (Waters, Milford, MA, USA), a Rheodyne (Cotati, CA, USA) model 7125 injector with 20-μl loop and a model 980 fluorescence detector (Applied Biosystems, Foster City, CA, USA) equipped with a 5-μl flow cell. A 290-nm excitation wavelength was used in conjunction with a 389-nm emission long pass cut-off filter. The detector was connected to a model D-2500 chromato-integrator (Hitachi, San Jose, CA, USA). A 250×4.6 mm Phenosphere 5 μm ODS2 analytical column was used in conjunction with a 3-cm guard column with the same packing material (Phenomenex, Torrance, CA, USA). A 5-cm ODS saturation column preceded the injector. The mobile phase, 0.15 *M* sodium dihydrogenphosphate, in glass-distilled water, was adjusted to pH 2.5 with 70% perchloric acid and passed through a 0.45-μm filter (Millipore, Bedford, MA, USA). For post-column derivatization of PLP, a model RDR-1 reagent delivery/reaction module (Timberline Instruments, Boulder, CO, USA) was used. The post-column reagent was a 1 g/l solution of sodium bisulfite in glass-distilled water, which was delivered at a flow-rate of 0.1 ml/min from a pressurized (with nitrogen gas) 600 ml polypropylene reservoir through a filter and check valve to a mixing tee that was placed ahead of a 2 m×0.5 mm I.D. PTFE mixing coil, woven in a serpentine configuration. The HPLC system was operated at ambient temperature and the column flow-rate was 1 ml/min (pressure ca. 130 bar).

2.3. Standard solutions

Solutions of the six vitamers, 4-PA and the internal standard (iso-PL; 1.25–7.50 μ*M*) in glass distilled water were kept frozen and used within one month.

2.4. Standard curves

Three replicate injections of 20 μl each from the standard solutions were used to construct linear regression lines (peak area versus concentrations). The injected quantities of the compounds (15–150 pmol) were in the linear range of the detector and the correlation coefficients of the straight line graphs of all eight compounds were better than 0.994. Similar correlation coefficients were obtained when the internal standard plot method was used (peak area ratios of each of the compounds over isopyridoxal versus the corresponding ratios of their respective concentrations).

2.5. Sample preparation for HPLC analysis

(a) Yeast cell-free culture media. The growth conditions of the yeast mutant as well as the preparation of the sample injected into the chromatograph have been reported previously [14].

(b) Baker's yeast extract. To about 50 mg of baker's yeast (YSC-1), in a small test tube, 50 μl of 1 m*M* iso-PL and 3 ml of 1 *M* perchloric acid were added. The tube was vortex-mixed for a few seconds and then again after 30 min, then centrifuged for 15 min at 600 *g*. The supernatant was decanted into another tube, its pH was adjusted to 3–4 with a 10 *M* potassium hydroxide solution and it was placed in the refrigerator for a few hours. The precipitated potassium perchloride was centrifuged off and 0.5 ml of the supernatant was filtered using 0.45 μm nylon-66 membrane filters in microfilterfuge tubes (Rainin, Woburn, MA, USA). This filtrate was used for HPLC.

(c) Egg yolk extract. Chicken eggs were bought from the local market. To about 2 g of egg yolk, in a small test tube, was added 100 μl of 1 m*M* iso-PL and 6 ml of 1 *M* perchloric acid. The filtrate to be injected into the HPLC system was prepared the same way as that from the baker's yeast extract, except that the sample used to adjust the pH was taken from the clear middle part of the extract (protein at the bottom of the test tube and fat on top).

(d) Milk extract. Milk, 2% fat, was bought from the local market. To about 2 g of milk, in a small test tube, was added 10 μl of 1 m*M* iso-PL and 1 ml of 1 *M* perchloric acid. The same procedure as the one for the egg yolk extract was used to prepare the filtrate for HPLC analysis.

All experiments were carried out under conditions of subdued light.

3. Results and discussion

To separate the six vitamers of vitamin B_6, we had been using a cation-exchange column with isocratic elution [14,17]. However, under those conditions, 4-PA, which is present in milk and other biological fluids, could not be separated from the vitamers. Therefore, reversed-phase chromatography was tested, since this method has been successfully used by other researchers [4,9,11,12]. Also, due to the greater column stability, reversed-phase chromatography is preferable to cation-exchange chromatography. After trying a few mobile phases, internal standards, variations in pH and molarities, we ended up using 0.15 *M* sodium dihydrogenphosphate adjusted to pH 2.5 with perchloric acid and isopyridoxal as the internal standard to monitor sample preparation and quantitation of the chromatographic system. 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 [3,18,19] or post-column [5,9,11,20] derivatization has been used. In this report, sodium bisulfite [5] in glass distilled water is used for the derivatization of PLP. Under these conditions, there is baseline separation of all eight vitamin B_6-related compounds (Fig. 1A,B). As can be seen, the only vitamin B_6 compound whose fluorescence intensity increases by post-column derivatization is PLP (Fig. 1, B vs. A) since the pH of the mobile phase does not change on mixing with the post-column reagent. The reported [21] enhancement

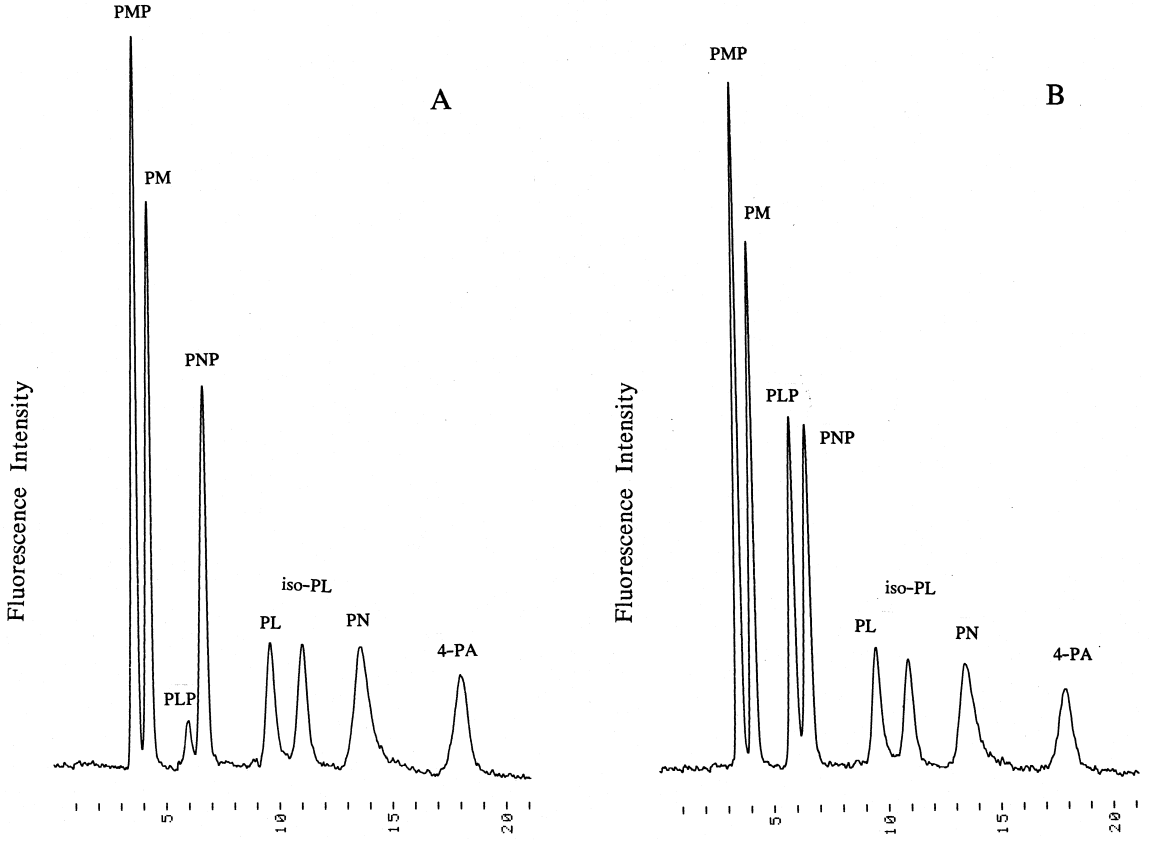

Fig. 1. HPLC tracing of standards of vitamin B_6-related compounds. Quantities injected (pmol): PMP (15), PM (15), PLP (30), PL (30), iso-PL (30), PN (30) and 4-PA (6), without (A) and with (B) post-column derivatization. The time scale is given in min.

of the fluorescence intensity of 4-PA when 0.5 g of sodium bisulfite was added to a liter of mobile phase was not observed under our post-column derivatization conditions.

The fact that the fluorescence intensity of PLP increases following post-column derivatization could be used to verify its presence and to calculate its quantity, by injecting the sample into the HPLC system twice, i.e. without and with the post-column reagent, Fig. 2B vs. Fig. 2A. From this chromatogram it is apparent that the main vitamer in raw chicken egg yolk is PLP (96.1%), with very small quantities of PMP (1.5%) and PM (2.4%).

In Fig. 3, the chromatogram obtained from commercial 2% pasteurized milk without (A) and with (B) post-column derivatization is depicted. The presence of the PLP peak can be seen in Fig. 3B. There is a very small peak close to the retention time of PNP, but it is doubtful that this peak is indeed PNP.

In Fig. 4, the chromatogram obtained from cell-free culture media of the yeast mutant after it was grown for five days is presented. The only vitamers present were PM, PL and PN. As has been reported before [14], none of the phosphorylated forms were present.

In Fig. 5, the chromatogram obtained from the cell extract of the yeast YSC-1 without (A) and with (B) post-column derivatization is depicted. The size of the peak with a retention time close to that of PLP is the same in both cases, which indicates that it is not PLP. The quantities of PMP and PM are, as expected, fairly large. Due to the presence of interfering peaks under these conditions, it was thought that increasing the excitation wavelength to 325–330 nm, as has been used by others [5,11,22], might eliminate

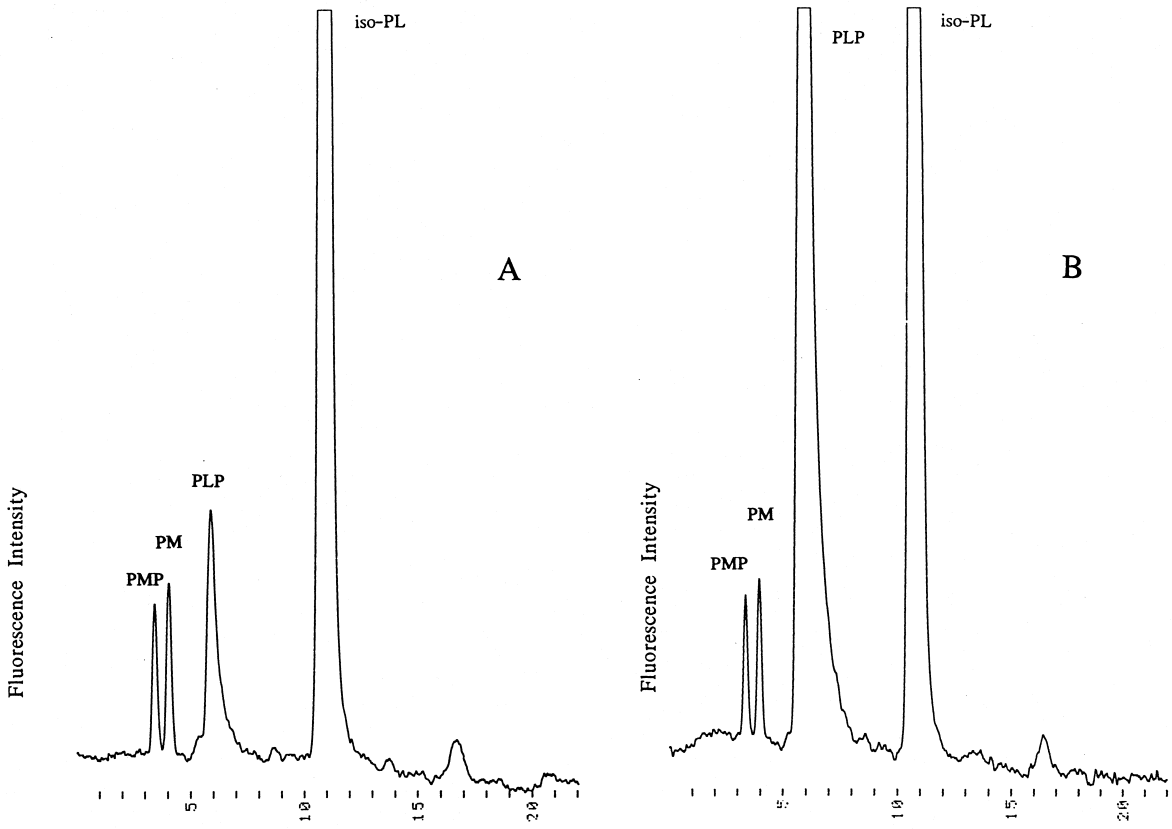

Fig. 2. HPLC tracing of an extract from raw chicken egg yolk, without (A) and with (B) post-column derivatization.

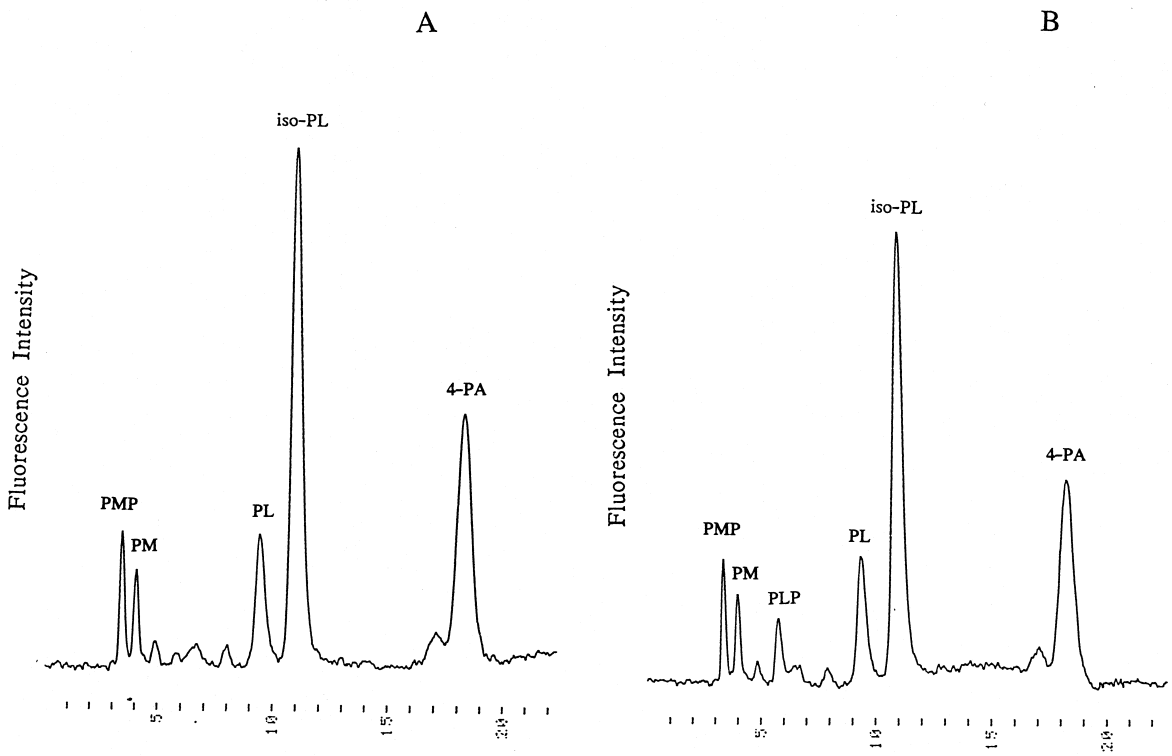

Fig. 3. HPLC tracing of an extract from 2% pasteurized milk, without (A) and with (B) post-column derivatization.

those peaks. Since our mobile phase is very acidic, the post-column reagent had to be dissolved in a strongly basic solution in order for the effluent, after being mixed with the mobile phase, to have a pH close to neutral because, at neutral pH, the absorption maximum of PMP, PNP, PM and PN is in the area of 320–330 nm [23]. To accomplish this, the post-column reagent, sodium bisulfite, was dissolved in 1 *M* tripotassium phosphate (1 mg/ml) and, thus, the pH of the effluent was 6.6. However, in our system, when the excitation wavelength was set at 325 or 330 nm, the fluorescence intensities of PMP, PNP, PM, PN and 4-PA were approximately one half to one third of what they were at an excitation wavelength of 290 nm and without post-column derivatization. The fluorescence intensities of PLP, PL and iso-PL were much smaller, since their absorption maxima, at neutral pH, are not in the area of 320–330 nm. The only sample from those examined in this report whose chromatographic profile had the fluorescence intensities of some peaks reduced appreciably when the excitation wavelength was set at 330 nm was that of the YSC-1 yeast extract (Fig. 6). In all other samples, the chromato-

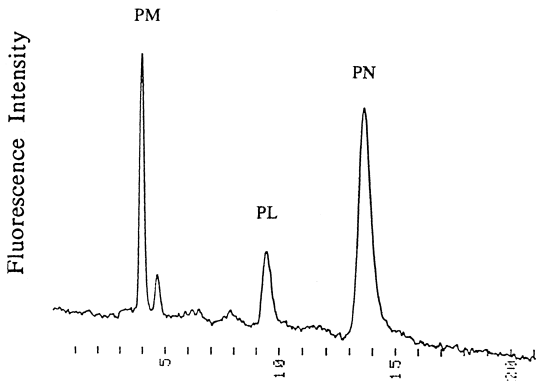

Fig. 4. HPLC tracing of the cell-free culture media of the yeast mutant after it was grown for five days. Post-column derivatization was used.

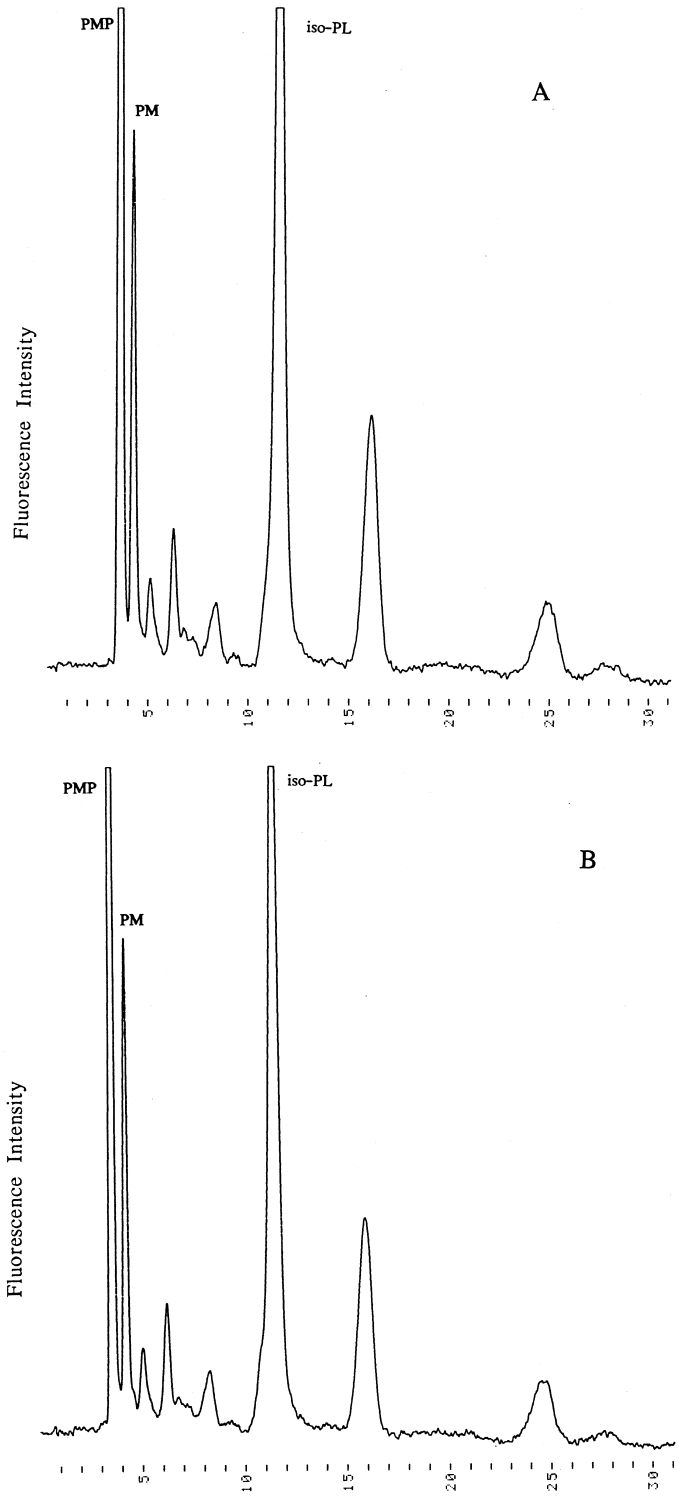

Fig. 5. HPLC tracing of a cell extract of the yeast YSC-1, without (A) and with (B) post-column derivatization.

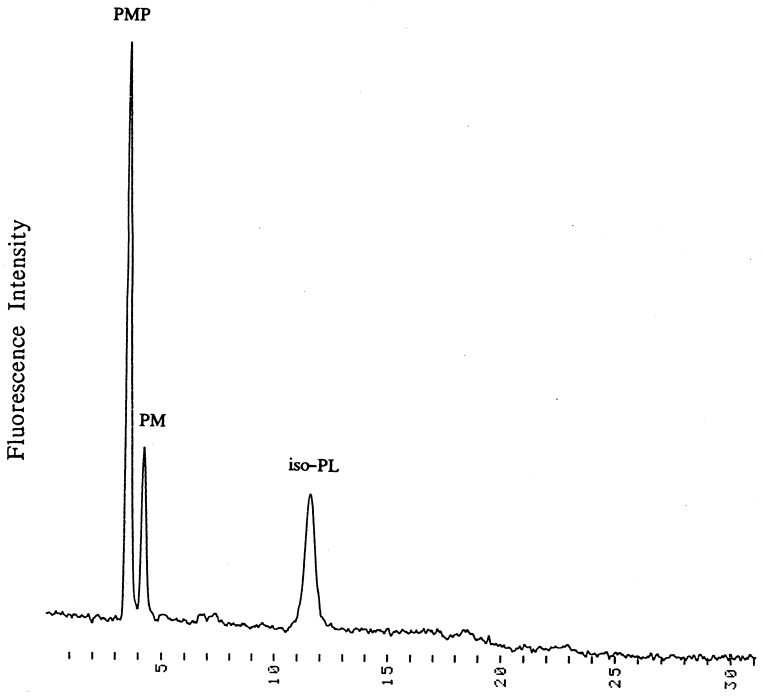

Fig. 6. HPLC tracing of a cell extract (the same as in Fig. 5) but at an excitation wavelength 330 nm and the post-column reagent was dissolved in 1 *M* tripotassium phosphate.

graphic profile was almost the same at both excitation wavelengths; the only difference being that the peaks were much smaller, especially that of iso-PL, at the 330 nm excitation wavelength, compared to that at 290 nm. However, excitation at 330 nm might be useful in cases where interfering peaks appear with retention times close to those of vitamin B_6-related compounds.

It has been reported that frozen solution of mixtures of vitamin B_6 vitamers in distilled water are not stable, while individual vitamers are stable for many months [12,24]. The loss of PMP and PM in the mixture has been explained as being due to their decomposition to PLP or PL [12]. Shephard and Labadarios [24] reported appreciable losses of PLP and PMP in a frozen solution of this mixture. We usually keep two sets of mixtures of vitamer solutions in glass-distilled water, frozen at −20°C; one set is a 5 μ*M* mixture of PMP and PM and the other is a 5 μ*M* mixture of PLP, PNP, PL, PN and 1 μ*M* 4-PA. An aliquot from the mixture of PMP and PM injected into the HPLC system after three or six months showed that the concentrations of PMP and PM did not change and that no new peaks appeared. However, an aliquot from the other mixture of vitamers injected into the HPLC system after three months showed (Fig. 7) that the fluorescence intensities of PLP and PL were reduced while that of 4-PA was increased and a new peak appeared which had the same retention time as that of 4-PAP. Therefore, the reported [12] increase in the concentration of PLP could have been due to the formation of 4-PAP, since that was what was measured as PLP by that method and it was not due to the decomposition of PMP to PLP.

In Table 1 the concentrations of vitamin B_6-related compounds (in nmoles/g) are reported. Two aliquots from the same sample were analyzed. These values are mostly in agreement with those reported by others [5,12,22,25], except for the baker's yeast sample in which we did not find PLP, PNP, PL and PN, as has been reported previously [12]. The

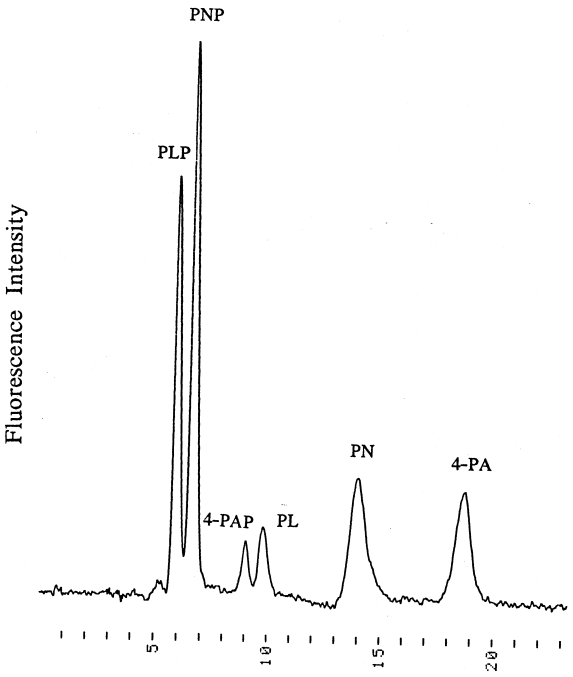

Fig. 7. HPLC tracing of a mixture of PLP, PNP, PL, PN and 4-PA, which had been kept frozen at −20°C for three months. This mixture was the same one as that used for Fig. 1.

discrepancy could be due to the particular batch of commercial yeast (YSC-1) or to the way it was prepared (drying).

Perchloric acid was used to remove the proteins and extract the vitamers. To accomplish this, others have used trichloroacetic acid, metaphosphoric acid or sulfosalicylic acid, which makes sample preparation for injection into the HPLC system more time-consuming. Perchloric acid has been implied [22] to be a better extraction agent of the vitamers, for animal tissues, than sulfosalicylic acid. Other researchers [12] have also confirmed that extraction of various food samples with 1 *M* perchloric acid gave the most reliable data for the vitamers. Recoveries of the vitamers when perchloric acid was used as the extracting agent are fairly good [11,12,21,22] and, of course, depend, among other things, on the vitamer and the matrix, e.g. for plasma, recoveries for all seven vitamin B_6-related compounds were in the range 85.1–98.2% [11], for raw liver they were in the range 89.2–101.8% and for pasteurized milk, they were in the range 92.4–101.4% [22].

Reliable quantitation of the vitamers in biological systems, especially if proteins are denatured during extraction, necessitates the use of an internal standard with a chemical structure similar to that of the vitamers to correct for any volume changes during sample preparation and for day-to-day variations in the system's components. Unfortunately, 4-deoxypyridoxine, which is commercially available, elutes under these conditions far from the last-eluting compound, 4-PA. Another disadvantage of 4-deoxypyridoxine is its low fluorescence quantum yield. Isopyridoxal, on the other hand, has a retention time between that of PL and PN and it has a similar fluorescence quantum yield to that of the other vitamin B_6 compounds. There were no peaks close to the retention time of iso-PL in the chromatograms of the samples reported here when the internal standard was not added.

For biological samples whose vitamin B_6 vitamer content is small (e.g. blood) and might not be measured by this method as reported here, it is believed that increasing the injection volume or using a larger volume flow cell might increase the sensitivity without decreasing the resolution.

As the column ages, the resolution between PLP and PNP is not as good, but this should not be much of a problem since it has been reported [3,5] that

Table 1
Content (nmoles/g) of vitamin B_6-related compounds in some biological samples

Sample[a]	PMP	PM	PLP	PL	PN	4-PA
Chicken egg yolk (raw)	0.33±0.02	0.51±0.02	20.7±0.57	[b]	[b]	[b]
Milk (2%)	0.16±0.02	0.14±0.02	0.19±0.02	1.67±0.08	[b]	0.66±0.07
Yeast (YSC-1)	137±12	44±6	[b]	[b]	[b]	[b]
Yeast (mutant) cell-free culture medium	[b]	0.62	[b]	1.40	4.36	[b]

[a] Two aliquots from the same sample were analyzed.
[b] Not detected or trace amount.

PNP has a minor significance as a naturally occurring vitamer. Therefore, its concentration will not interfere with the integration of the PLP peak. It must be mentioned, however, that the presence of PNP has been reported in foods [12] and in human plasma when subjects were given more than 100 mg of PN [26]. If resolution between PLP and PNP standards becomes a problem, then washing the column with water, methanol, chloroform, methanol, water, in that sequence, restores the resolution of these two compounds to almost the original value.

The post-column reagent, sodium bisulfite, is dissolved in glass-distilled water and is stable for at least three months; whereas, if it is dissolved in alkaline solution, it needs to be added just before use [5]. Only one excitation wavelength is used for all of the compounds, the mobile phase is inorganic, fairly acidic and there is no problem with microbial growth, the elution is isocratic and thus column regeneration is not required after each run, which makes automation easy.

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