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Short Communication

High-performance liquid chromatography of two derivatives of vitamin B_6, the carbamoyl derivatives of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate

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ABSTRACT

We present the results of a study of the high-performance liquid chromatography of two vitamin B_6 derivatives: the carbamoyl derivatives of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate. These compounds, obtained by condensation of either pyridoxal 5′-phosphate or pyridoxamine 5′-phosphate with either carbamoyl phosphate or potassium cyanate, were separated on an octadecyl silica column with a mobile phase consisting of 0.01 *M* potassium phosphate (pH 5.0)–methanol (98:2); detection was at 254 nm. The method was sensitive, fast and precise.

INTRODUCTION

. In previous studies [1–4] we have observed that L-threonine deaminase (EC 4.2.1.16), a pyridoxal 5′-phosphate (PLP)-dependent enzyme, is inhibited by both carbamoyl phosphate (CP) and potassium cyanate, a decomposition product of carbamoyl phosphate [5]. We have demonstrated [6] (1) that CP and potassium cyanate inhibit the enzyme essentially by an interaction with the coenzyme, and (2) that this chemical reaction occurs more easily with free PLP. In fact, when CP was added to incubation mixtures containing the holoenzyme, the inhibition was appreciable only at the final 50 m*M* (K_1 = 42 m*M*) concentration of the inhibitor. On the other hand, when the dialyzed enzyme was incubated with increasing amounts of PLP, the inhibition was highest at a final inhibitor concentration of only 0.1 m*M* (K_1 = 30 μM). When free PLP was added to incubation mixtures containing the holoenzyme, activity was enhanced, but the activation was no longer evident when the PLP was preincubated with CP. Moreover, when free PLP was preincubated either with CP or

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potassium cyanate, it failed to restore the activity of the dialyzed enzyme. This indicates that inhibition affected the association reaction

apoenzyme + PLP → holoenzyme

The occurrence of a chemical reaction between free PLP and either CP or potassium cyanate was confirmed when free PLP was incubated with either CP or potassium cyanate under physiological conditions (pH 7) and even at pH 5. Characteristic changes in PLP spectra were observed, and a new compound was crystallized which, by means of chemical tests and spectra (UV, IR and NMR), was identified as 3,4-dihydro-2H-pyrido[3,4-*e*]1-1,3-oxazin-2-one, which we have called "carbamoyl pyridoxal 5'-phospate" (C-PLP). Its structure is shown in Fig. 1.

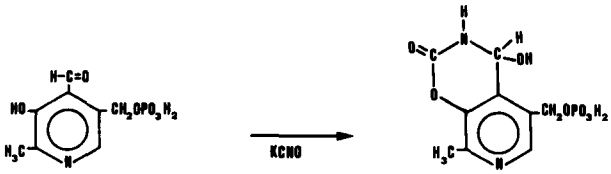

Fig. 1. Formation of 4'-carbamoyl pyridoxal 5'-phosphate.

Under analogous conditions, we obtained another derivative of vitamin B_6: 4'-carbamoyl pyridoxamine 5'-phosphate (C-PMP) (unpublished results). Its structure is shown in Fig. 2.

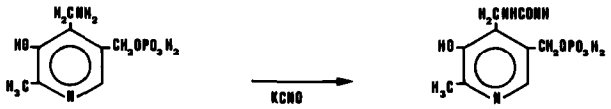

Fig. 2. Formation of 4'-carbamoyl pyridoxamine 5'-phosphate.

We were interested in investigating the biological properties of these new compounds, and for this purpose a satisfactory procedure of separation and determination of C-PLP, C-PMP, pyridoxamine 5'-phosphate (PMP) and PLP, also suitable for tissue extracts, was imperative. This procedure is presented in the Experimental section.

EXPERIMENTAL

Chemicals

PLP, PMP, potassium cyanate, potassium dihydrogenphosphate, and dipotassium hydrogenphosphate were obtained from Merck (Darmstadt, Germany). CP and Norit A were purchased from Sigma (St. Louis, MO, USA). Methanol [high-performance liquid chromatography (HPLC) grade] was obtained from Baker (Phillipsburg, NJ, USA).

Preparation of C-PLP and C-PMP

In preliminary experiments, we found that the reaction either between PLP and CP or between PMP and CP produced two derivatives, C-PLP and C-PMP, respectively, which could be obtained more rapidly by using potassium cyanate; therefore, all subsequent syntheses were carried out with potassium cyanate instead of CP.

A 1-g sample of PLP or 1.12 g of PMP was dissolved in a few milliliters of water and mixed slowly with 0.6 g of potassium cyanate: the condensation product precipitated at pH 4.0. Recovery was 1.05 g of C-PLP (90%) and 0.86 g of C-PMP (80%).

As previously demonstrated [7], desiccated products were very stable; in solution both C-PLP and C-PMP were in equilibrium with their starting compounds: CP or potassium cyanate, PLP or PMP. Their equilibrium differed according to the pH and temperature of the solution.

Preparation and utilization of rat liver supernatant

We used a rat liver supernatant prepared as previously reported [8]. Male albino rats, 9 weeks old, 250 g bodyweight, were decapitated and the livers rapidly removed. A 10% homogenate (in 50 m*M* potassium phosphate, pH 7.5) was prepared and centrifuged at 260 000 *g* for 1 h at 4°C; 10 ml of supernatant were treated with 1.5 ml of 50% Norit A (v/v) for 15 min and centrifuged at 1000 *g* for 15 min.

A 2-μmol sample of the C-PLP or C-PMP was added to 0.5 ml of supernatant, immediately deproteinized with 2 *M* hydrochloridric acid (0.5 *M* final concentration), centrifuged at 8000 *g* and diluted with a 50 m*M* potassium phosphate buffer (pH 7.5) until the C-PLP or C-PMP reached a final concentration of 0.1 m*M*. The blank was obtained by replacement of the supernatant with 0.5 ml of the same buffer, and 20 μl of this solution (2 nmol) were injected into HPLC system.

Apparatus and chromatographic conditions

A Vista 5500 high-performance liquid chromatograph (Varian, Sunnyvale, CA, USA) equipped with a Model 2550 variable-wavelength UV detector (Varian) and Model 4290 electronic integrator (Varian) was used. A ready-for-use, prepacked (250 × 4.6 mm) Supelcosil LC-18, 5-μm column (Supelco, Bellefonte, PA, USA), protected by a precolumn (20 × 4.6 mm) filled with the same packing (Supelguard, Supelco), completed the analytical system. The mobile phase consisted of 0.01 *M* potassium phosphate buffer (adjusted to pH 5.0 with 0.5 *M* potassium hydroxide) and 2% methanol, at a flow-rate of 1 ml/min. Detection was performed at 254 nm.

RESULTS AND DISCUSSION

After HPLC separation, the compounds were detected at 254 nm, to minimize the non-specific interferences either of the buffer or of the samples at lower wavelengths. Moreover, at this wavelength, CP and potassium cyanate did not show any significant absorption (molar absorptivity of potassium cyanate 0.001 $mmol^{-1}\ cm^{-1}$ and CP 0.059 $mmol^{-1}\ cm^{-1}$). These compounds can be detected at 204 nm (molar absorptivity of potassium cyanate 0.180 $mmol^{-1}\ cm^{-1}$ and CP 0.211 $mmol^{-1}\ cm^{-1}$ at 204 nm), with retention times for potassium cyanate and CP of 2.74 and 2.36 min, respectively.

A preliminary study involving the separation of PMP, C-PMP, PLP and C-PLP

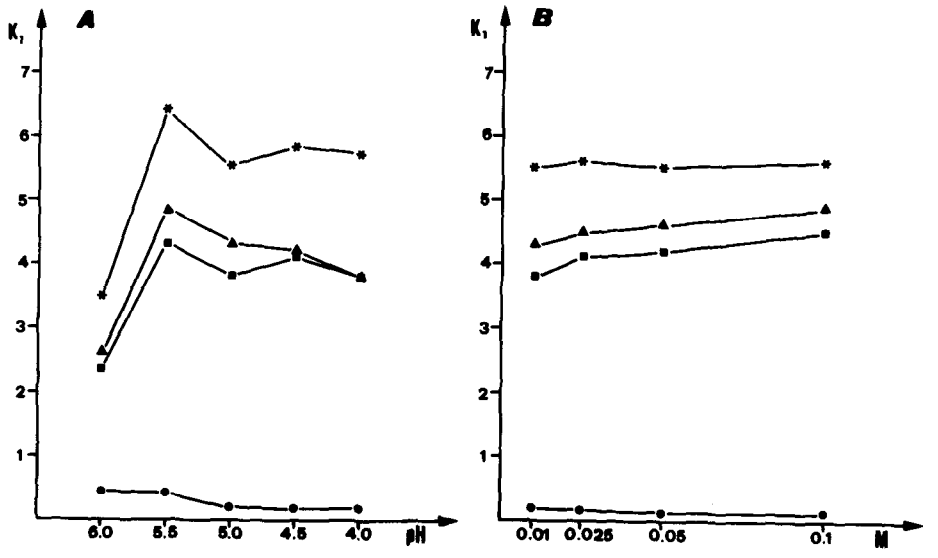

Fig. 3. Effect of (A) pH and (B) ionic strength buffer at pH 5.0 on K_1 value of (●) PMP, (▲) C-PLP, (■) PLP and (*) C-PMP.

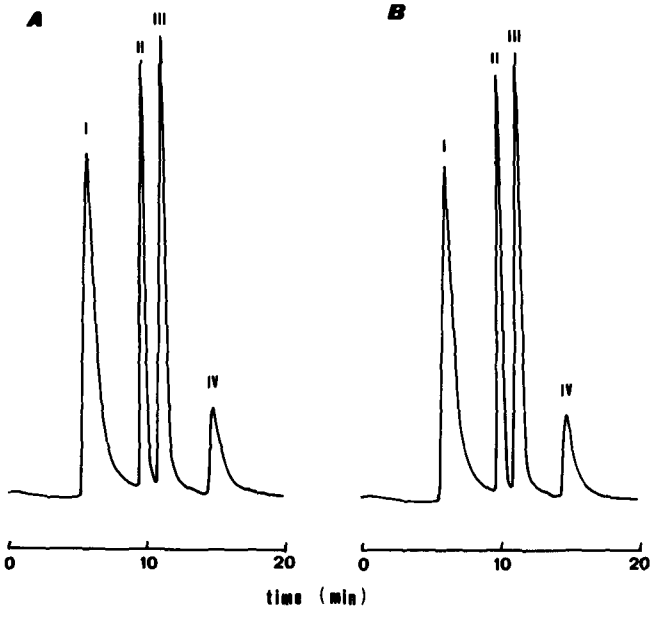

Fig. 4. Separation of (I) PMP, (II) C-PLP, (III) PLP and (IV) C-PMP (A) in the absence and (B) in the presence of rat liver supernatant. Injection volume: 20 μl of 1 mM solution of each standard.

TABLE I

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS FOR PMP, C-PLP, PLP, PMP

Compound	Regression equation[a]	Correlation coefficient
PMP	$A = 282617\ C - 35922$	0.998
C-PLP	$A = 115818\ C - 5671$	0.996
PLP	$A = 144900\ C + 237$	0.999
C-PMP	$A = 61632\ C - 14854$	0.998

[a] A = peak area; C (nmol) = amount of reagent.

led us to study the effect of (A) different pH and (B) ionic strength buffer at pH 5.0 in the mobile phase (Fig. 3).

Fig. 4 shows a satisfactory separation of the four compounds obtained by isocratic elution at pH 5.0. Linearity was obtained for all amounts of PMP, C-PMP, PLP and C-PLP used in this study (2–20 nmol). The minimum amount of these compounds detectable in our mixtures was 0.5 nmol.

Correlation coefficients and the regression equations of the calibration curve are reported in Table I.

The overall precision of the retention times and peak areas was assessed by determination of run-to-run and day-to-day precision (Table II).

Effect of rat liver extract on C-PLP and C-PMP solutions

We also wanted to ascertain if the determination of these substances was possible in presence of rat liver extracts. We prepared a rat liver supernatant, to which we added C-PLP and C-PMP: the final solutions were chromatographed as reported in the Experimental section.

The elution patterns were not influenced by the addition of the supernatant, as

TABLE II

REPRODUCIBILITY AND ACCURACY OF RETENTION TIMES AND PEAK AREAS OF PMP, C-PLP, PLP, C-PMP

Compound	Retention time (min)	S.D. ($n=5$) (min)	C.V. (%)	Area (arbitrary units)	S.D. ($n=5$)	C.V. (%)
Run-to-run precision (within 1 day)						
PMP	5.74	0.02	0.35	1393947	56769	4.07
C-PLP	9.90	0.06	0.61	552373	6054	1.10
PLP	11.37	0.05	0.44	729938	4181	0.57
C-PMP	15.48	0.02	0.13	275147	9297	3.38
Day-to-day precision (7 days)						
PMP	5.58	0.13	2.33	1370303	52561	3.84
C-PLP	9.99	0.15	1.50	531595	22382	4.21
PLP	11.42	0.14	1.23	723601	18757	2.59
C-PMP	15.25	0.17	1.11	294121	13971	4.75

demonstrated by Fig. 4A and B, which shows that the area of peaks of C-PLP (II, Fig. 4B) and C-PMP (IV, Fig. 4B) were almost identical under differing conditions.

The experiment was repeated with different final concentrations of C-PLP and C-PMP (2, 1.5 and 0.5 m*M*; 20 μl of these solutions were injected into the HPLC system) with good proportionality and recovery.

We conclude that tissue extracts do not interfere with chromatographic runs and permit the determination of these substances also under various biological conditions.

CONCLUSIONS

Our results show that the carbamoyl derivatives of PLP and PMP can easily be separated from each other and from their starting compounds through HPLC. In certain conditions, the tested compounds were stable. The procedure is easily reproducible even when two compounds are added to tissue extracts and it is expected that it will be useful in clarifying the biological role of C-PLP and C-PMP.

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