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Short Communication

High-performance liquid chromatography of thiazolidinic compounds obtained by condensation of pyridoxal 5′-phosphate or pyridoxal with aminothiols (L- or D-cysteine, cysteamine, L-cysteine ethyl ester)

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ABSTRACT

We investigated six thiazolidine 4-carboxylic acids of biological interest, obtained by condensation of pyridoxal 5′-phosphate or pyridoxal with L- or D-cysteine, cysteamine or L-cysteine ethyl ester. A reversed-phase high-performance liquid chromatographic method, using a C_{18} column for their separation, was developed by sequential optimization of the pH and the gradient of the mobile phase. Resolution of the compounds was obtained with an analysis time of less than 20 min.

INTRODUCTION

Thiazolidine compounds are formed by condensation of either aliphatic or aromatic moieties, containing a –CHO group, with different aminothiols [1,2]. We obtained several thiazolidine 4-carboxylic acids (TAs) by condensation [3–8] of pyridoxal 5′-phosphate (PLP) or pyridoxal (PL) (the other aldehyde form of vitamin B_6), with L- of D-cysteine, cysteamine or L-cysteine ethyl ester (Fig. 1, compounds I–VI).

Interest in these compounds arose following the observation that several aminothiols, such as L- or D-cysteine, cysteamine, and L-cysteine ethyl ester, exert a high degree of inhibition on certain PLP-dependent enzymes, such as rat liver L-threonine deaminase, the properties of which we have extensively studied [9–11]. Inhibition is due to the formation of a thiazolidinic ring, from condensation of L-cysteine and the enzyme-bound PLP.

Since the thiazolidine compounds are easily formed under physiological conditions (pH 7 and 37°C), it seems likely that they also form *in vivo*. If so, it is important to identify their biological role in the cell. To solve this problem, a procedure was needed for the isolation and determination of TAs, which would also be valid in the presence of tissue extract. Accordingly we developed the selective high-performance liquid chromatographic (HPLC) procedure, described in this paper.

EXPERIMENTAL

Chemicals

PLP, PL, L- and D-cysteine, cysteamine, L-cysteine ethyl ester, potassium dihydrogenphosphate

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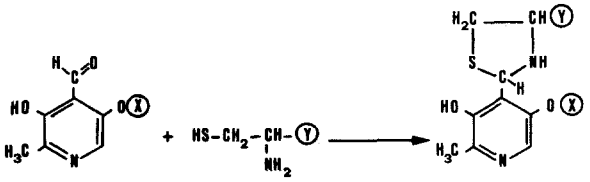

I	Y = C OOH	X = PO_3H_2
II	Y = H	X = PO_3H_2
III	Y = C OOC_2H_5	X = PO_3H_2
IV	Y = C OOH	X = H
V	Y = H	X = H
VI	Y = C OOC_2H_5	X = H

Fig. 1. Formation of thiazolidine 4-carboxylic acids through condensation of PLP and L-cysteine (I), cysteamine (II), and L-cysteine ethyl ester (III); or PL and L-cysteine (IV), cysteamine (V), and L-cysteine ethyl ester (VI).

and potassium monohydrogenphosphate were obtained from Merck (Darmstadt, Germany). Norit A was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanol (HPLC grade) was obtained from Baker (Phillipsburg, NJ, USA).

Preparation of thiazolidine 4-carboxylic acids (I–VI)

Compounds I–VI were synthetized from PLP or PL with L- of D-cysteine, cysteamine, or L-cysteine ethyl ester, by known methods [3,4,6]. Their purity was tested through elemental analysis, and IR and NMR spectra.

IR analysis was performed using a Perkin-Elmer (Garden Grove, CA, USA) Model 782 spectrometer; NMR spectra were obtained using a Perkin-Elmer Model R600 instrument.

Aqueous solutions of the compounds showed a specific UV spectrum with an absorption maximum at 330 nm with ε = 6.41 (I), 6.98 (II), 4.05 (III), 6.08 (IV), 1.49 (V), 5.92 (VI) l $mmol^{-1}$ cm^{-1}. The spectra were obtained using a Shimadzu (Kyoto, Japan) UV-160 spectrophotometer.

Preparation and use of rat liver supernatant

Rat liver supernatant was prepared as previously reported [12]. Male albino rats, 9 weeks old, 250 g body weight, were decapitated and their 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. A 10-ml volume of the supernatant was treated with 1.5 ml of 50% Norit A suspension (v/v) according to Hershko *et al.* [13], for 15 min and centrifuged at 1000 *g* for 15 min.

A 0.5-ml volume of 4 m*M* solution of compounds I–VI was added to 0.5 ml of supernatant, immediately deproteinized with 2 *M* hydrochloric acid (0.5 *M* final concentration), centrifuged at 8000 *g* and diluted with a 50 m*M* potassium phosphate buffer (pH 7.5) to 1 m*M* final concentration of TAs. The blank was obtained by replacement of the supernatant with 0.5 ml of the same buffer, and 20 μl of this solution (20 nmol of each) was submitted to HPLC analysis.

Apparatus and chromatographic conditions

We used a Beckman (San Ramon, CA, USA) System Gold high-performance liquid chromatograph equipped with a 126 programmable solvent module, a scanning detector module 167 and a Beckman Ultrasfere XL C_{18} column (70 × 4.6 mm I.D., 3-μm particle size) protected by a precolumn.

The mobile phase was a mixture of 0.05 *M* potassium phosphate buffer [adjusted to pH 5.5 with 0.5 *M* potassium hydroxide (buffer A)], 0.01 *M* potassium phosphate buffer pH 5.5 (buffer B) and methanol. The programme for the mobile phase gradient is given in Table I. The flow-rate was 1 ml/min and the detection wavelength was 254 nm.

RESULTS AND DISCUSSION

Optimal conditions

Under these conditions we ignored the free aminothiols (L- and D-cysteine, cysteamine, and L-cysteine ethyl ester) because they did not interfere with the chromatography.

We carried out a preliminary study to obtain the best separation of TAs from the starting products (PLP and PL): the effects of different pH values of the mobile phase are reported in Fig. 2. The pH chosen for the chromatography was 5.5. Moreover, we varied the relative proportions of buffers A and B and methanol in order to obtain the best separation of the six TAs, PLP and PL, as shown in Fig. 3.

Good linearity was obtained for TAs I, II, IV and VI, PLP and PL in the range 0.3–20 nmol and for TAs III and V at 1–40 nmol and 5–40 nmol, respec-

TABLE I

GRADIENT PROGRAMME FOR MOBILE PHASE COMPOSITION

Time (min)	Mobile phase (%, v/v)[a]			Duration (min)
	Buffer A	Buffer B	Methanol	
0 (start)	97	–	3	3
3	–	97	3	2
5	–	60	40	3
16	–	97	3	2
18	97	–	3	2
25 (stop)	97	–	3	–

[a] Buffer A: 0.05 *M* potassium phosphate buffer (adjusted to pH 5.5 with 0.5 *M* potassium hydroxide); buffer B: 0.01 *M* potassium phosphate buffer (adjusted to pH 5.5 with 0.5 *M* potassium hydroxide).

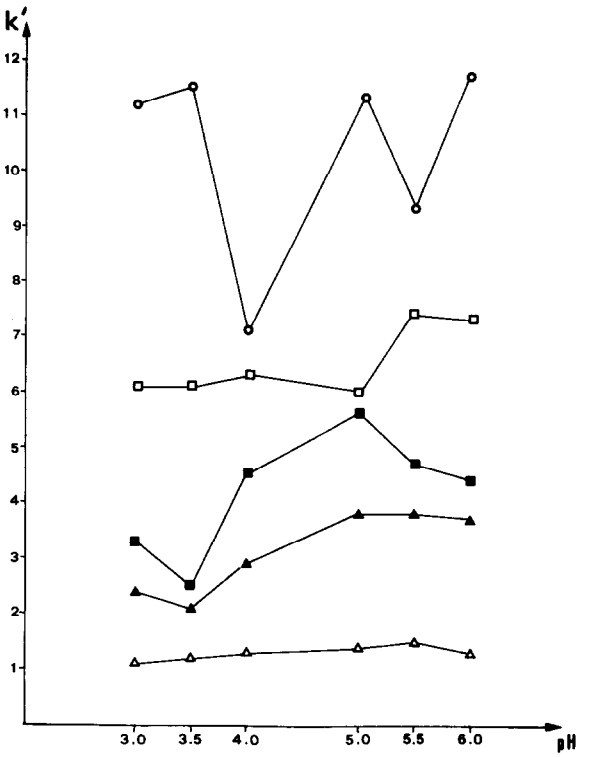

Fig. 2. Effect of pH on capacity factors (k') values of (■) PLP, (▲) PL, (△) I, (□) II, (○) IV. The data relating to compounds III, V and VI are not shown because these compounds were not eluted under the experimental conditions used, but only after addition of methanol to the mobile phase.

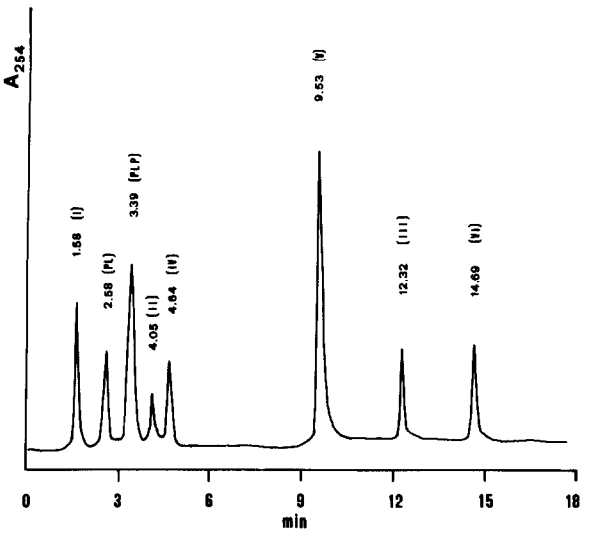

Fig. 3. Separation of PLP, PL, I, II, III, IV, V and VI. Injected amount: 20 nmol of each standard.

tively. Correlation coefficients and regression equations are reported in Table II.

The overall between-run and between-day precisions of the retention times and peak areas were studied, and the results are presented in Table III.

Behaviour of TAs in the presence of tissue extracts

Rat liver supernatant was added to the solution of these compounds, as indicated in Experimental. As shown in Fig. 4, their chromatographic behaviour did not change. These data demonstrate that there were no biological compounds in the supernatant affecting the determination of TAs, and that

TABLE II

CORRELATION COEFFICIENTS AND REGRESSION EQUATIONS OF PLP, PL, I, II, III, IV, V AND VI

Compound	Regression equation	Correlation coefficient (r)
PL	$y = 5.59x - 1.58$	0.9994
PLP	$y = 3.27x + 1.05$	0.9993
I	$y = 3.28x - 0.51$	0.9999
II	$y = 3.78x - 0.70$	0.9999
III	$y = 0.19x + 0.10$	0.9989
IV	$y = 3.26x - 0.84$	0.9998
V	$y = 1.09x - 1.03$	0.9992
VI	$y = 2.70x - 0.17$	0.9999

TABLE III

PRECISION OF RETENTION TIME AND PEAK AREAS OF PLP, PL, I, II, III, IV, V AND VI

Parameter	Compound	Retention time (min)	S.D. ($n=5$)	Relative S.D. (%)	Peak area (arbitrary units)	S.D. ($n=5$)	Relative S.D. (%)
Between-run precision (within 1 day)	I	1.50	0.02	1.13	6.29	0.04	0.63
	PL	2.46	0.01	0.24	10.76	0.13	1.21
	PLP	3.12	0.03	0.96	7.31	0.19	2.59
	II	4.05	0.01	0.15	6.37	0.04	0.63
	IV	4.48	0.01	0.24	12.13	0.07	0.62
	V	9.71	0.01	0.06	3.18	0.04	1.26
	III	12.27	0.00	0.00	20.31	0.06	0.29
	VI	14.46	0.00	0.00	12.17	0.15	1.23
Between-day precision (7 days)	I	1.48	0.05	3.31	5.86	0.32	5.46
	PL	2.48	0.03	1.17	10.20	0.42	4.12
	PLP	3.11	0.07	2.25	7.33	0.26	3.55
	II	4.05	0.05	1.16	6.50	0.43	6.61
	IV	4.54	0.07	1.49	13.60	2.34	17.20
	V	9.67	0.07	0.70	3.65	0.31	8.49
	III	12.18	0.05	0.40	20.16	1.14	5.65
	VI	14.56	0.10	0.69	13.95	0.76	5.44

it can be carried our in the presence of tissue extracts.

Our results show that TAs I–VI can be easily separated from each other and from their precursors (PLP or PL) by HPLC. The same procedure can be used when tissue extracts are added to the assay mixture. Only compound IV showed irregular behaviour during HPLC separation, as evidenced by the high S.D. value (Table III): this was not due to contamination, since its purity had been carefully controlled, as reported in Experimental. In spite of this however, compound IV can be easily separated from the others and determined in the presence of tissue extracts.

We conclude that our procedure facilitates investigation of the biological role of all these com-

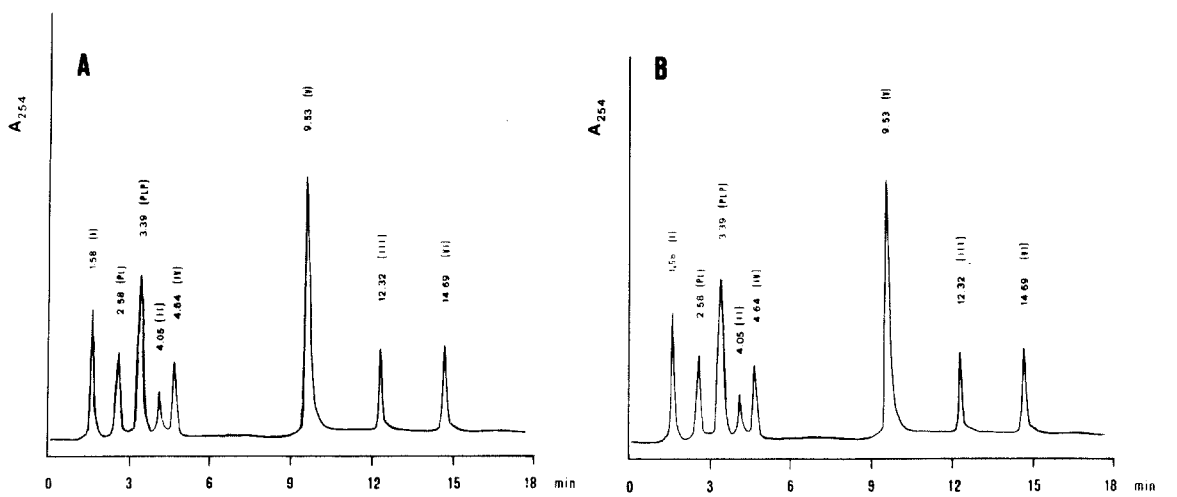

Fig. 4. Behaviour of TAs in (A) the absence and (B) the presence of tissue extract.

pounds, and in particular can be used to ascertain:

(1) if they are present in cellular extracts or in biological fluids;

(2) if they undergo quantitative fluctuations under different conditions;

(3) if they are synthesized from the cell or undergo metabolic transformations;

(4) if they interfere only in the PLP-dependent reactions or also in other enzymic transformations.

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