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Note

High-performance liquid chromatography of thiazolidinic compounds

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Thiazolidinic derivatives are formed by condensation of aminothiols with aliphatic or aromatic compounds containing a CHO group[1,2]: reactions of pyridoxal-5′-phosphate (PLP) and pyridoxal (PL) with L-cysteine, L-cysteine ethyl ester and homocysteine have been described[3–8].

Aromatic thiazolidinic compounds (TA) could have physiological importance; in fact, several workers have reported the reaction between free aminothiols and enzyme-bound PLP[9–13]; they noted that the TA have no great affinity for the apoenzyme, and are quickly released (this treatment is frequently used to resolve the coenzymic PLP form)[10–14]. Moreover, the synthesis of these derivatives occurs easily, under very mild conditions, (37°C, pH 7, low concentrations) and hence it could occur spontaneously in the cell.

Up to now, the absence of a reliable method has precluded the study of the possible metabolism of TA *in vivo*. The spectrophotometric procedure of Guidotti *et al.*[15] is very useful for the determination of the aliphatic TA but in to our experience it is unsuitable for aromatic derivatives. Recently other workers have published different high-performance liquid chromatographic (HPLC) procedures for the separation of thiazolidinic derivatives. Sen *et al.*[16] report the separation of some compounds found in fried bacon and in other types of meat. Van Doorn *et al.*[17] and, more recently, Ogata and Taguchi[18] have developed HPLC methods for the determination of urinary 2-thiothiazolidine-4-carboxylic acid as an index of carbon disulphide exposure. Their working conditions and thiazolidinic derivatives are very different from ours.

Here we describe two different procedures for the determination of TA obtained by condensation between PLP and L-cysteine (TAL) or D-cysteine (TAD): a spectrophotometric assay using a simple variation of Ellman's reaction[19] and a new HPLC procedure.

EXPERIMENTAL

Chemicals

PLP, L- and D-cysteine, potassium dihydrogenphosphate and potassium hydro-

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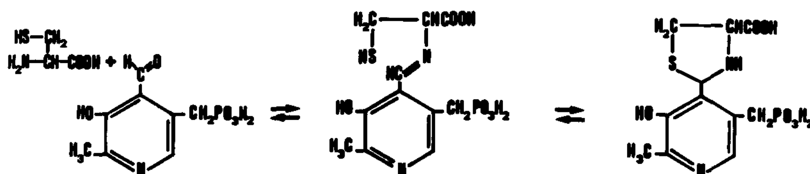

Fig. 1. Formation of thiazolidin-4-carboxylic acid.

genphosphate were obtained from Merck (Darmstadt, F.R.G.). 5',5'-Dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman's reagent) and Norit A were purchased from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC-grade) was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.).

Preparation of thiazolidin-2-(2'-methyl-3'-hydroxy-4'-pyridyl-5'-methylsulphonic)car boxylic acid

This compound was synthesized from PLP and either L- or D-cysteine according to Angeletti and Haertelt[6] (Fig. 1). The compound showed a specific UV spectrum with a absorption maximum at 330 nm ($\varepsilon = 6.41\ mmol^{-1}\ cm^{-1}$). Spectra were obtained with a Shimadzu (Kyoto, Japan) UV-160 spectrophotometer.

Application of Ellman's reaction

The determination of TA was carried out via Ellman's reaction (Fig. 2).

In solution the closed form of TA is in equilibrium with the open form (see Fig. 1) and its free SH groups react with DTNB; the equilibrium is totally shifted toward the formation of Ellman's derivative, which has an absorption maximum at 412 nm. The reaction of TA with DTNB is slower than the reaction of the same reagent with L-cysteine, which is almost istantaneous. In a mixture of L-cysteine and TA, the absorbance at 412 nm (A_{412}) at 0 min gives the amount of L-cysteine present; A_{412} after 40 min is due to TA and L-cysteine, so the TA content is derived from the value of ΔA_{412}.

The reaction was carried out as follows: 70 μl of 10 mM DTNB were added to 10 ml of 0.03 mM thiazolidin-4'-carboxylic acid in 50 mM potassium phosphate buffer (pH 7.5) and readings were immediately taken at 412 nm. After 40 min at 25°C, readings were again taken at the same wavelength.

Preparation and utilization of rat liver supernatant

Rat liver supernatant prepared as shown in Fig. 3 was used. A 2-μmol amount of

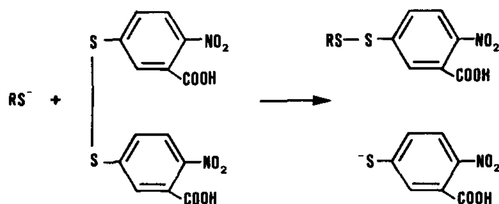

Fig. 2. Formation of 3-carboxy-4-nitrothiophenol derivative.

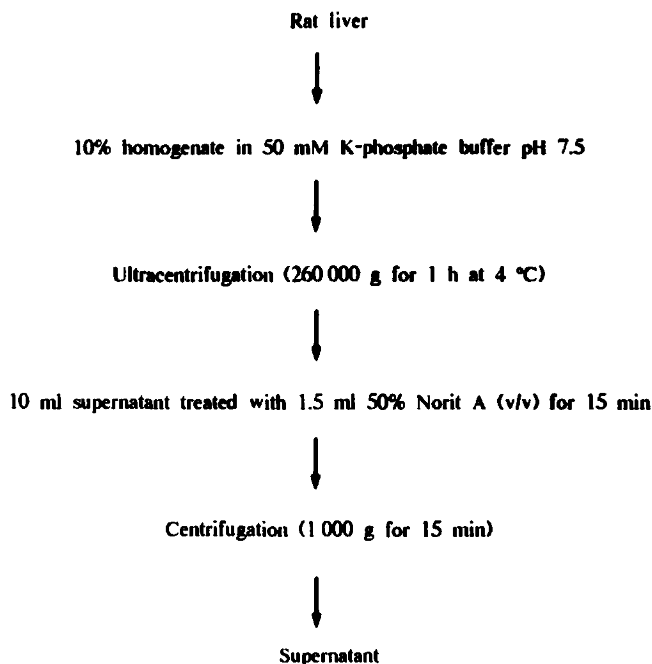

Fig. 3. Preparation of rat liver supernatant.

the thiazolidinic compound 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 50 m*M* potassium phosphate buffer (pH 7.5) until the TA reached final concentration of 0.1 m*M*. The blank was obtained by replacement of the supernatant with 0.5 ml of the same buffer. A 20-μl volume of this solution (2 nmol) was injected into the HPLC system.

Apparatus and chromatographic conditions

A Vista 5500 high-performance liquid chromatograph (Varian, Sunnyvale, CA, U.S.A.) equipped with a variable-wavelength UV detector (Model 2550, Varian) and an electronic integrator (Model 4290, Varian) were used. A ready-to-use prepacked column (250 × 4.6 mm I.D.) of Supelcosil LC-18, 5 μm (Supelco, Bellefonte, PA, U.S.A.), protected by a precolumn (20 × 4.6 mm I.D.) filled with the same packing (Supelguard, Supelco) completed the analytical system.

The mobile phase was 0.01 *M* potassium phosphate buffer (adjusted to pH 5.5 with 0.5 *M* potassium hydroxide)–methanol (95:5, v/v) at flow-rate of 1 ml/min. Detection was performed at 254 nm.

RESULTS AND DISCUSSION

Optimum conditions

A preliminary study involving the separation of cysteine, TA and PLP led us to

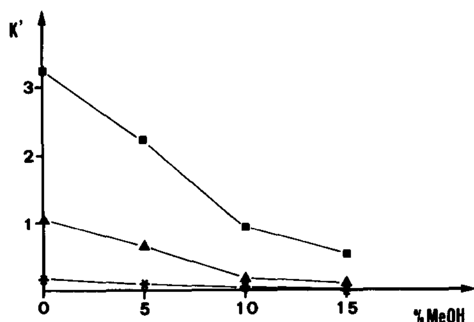

Fig. 4. Effect of methanol concentration on retention (✻) cysteine, (▲) TA and (■) PLP (MeOH = methanol.

consider the effect of different concentrations of organic solvent in the mobile phase (Fig. 4). Fig. 5 shows that a good separation is obtained with isocratic elution.

Cysteine was detected at 204 nm as it has no characteristic peak in the UV spectrum, either in water or in 10 mM potassium phosphate buffer (pH 5.5), but shows a non-specific absorption at low wavelengths (molar absorptivity 0.016 l $mmol^{-1}$ cm^{-1} at 254 nm and 1.027 l $mmol^{-1}$ cm^{-1} at 204 nm) (Fig. 5). The peak eluted at 3.24 min was also submitted to the Ellman reaction, giving a positive result. In subsequent experiments we used detection at 254 nm to minimize the non-specific interferences of buffer and samples at low wavelengths.

Good linearity was obtained for all amounts of TA and PLP used (0.1–20 nmol). The correlation coefficients for TA and PLP were 0.995 ($p < 0.01$) and 0.999 ($p < 0.01$), respectively, and the regression equations of the calibration graphs were $A = 157.52$ $C + 33.86$ and $A = 218.85\,C - 11.56$, respectively, where A = peak area and C (nmol) = amounts of reagent.

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

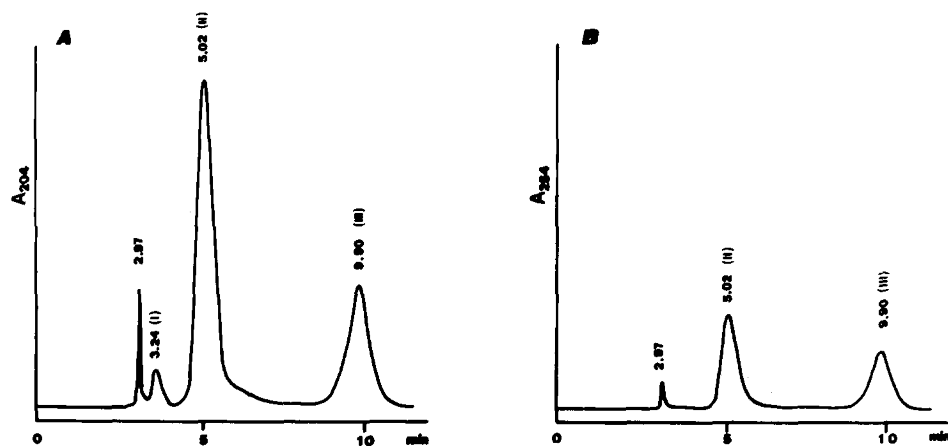

Fig. 5. Separation of (I) cysteine, (II) TA and (III) PLP with detection at (A) 204 and (B) 254 nm. Injection volume: 20 μl of solution 1 mM in each standard.

TABLE I

REPRODUCIBILITY AND ACCURACY OF RETENTION TIMES AND PEAK AREAS OF TA AND PLP

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	TA	4.99	0.03	0.60	3202.5	68.3	0.21
(within 1 day)	PLP	10.2	0.17	1.66	4297.4	45.0	1.04
Between-day precision	TA	4.99	0.02	0.40	3236.2	48.5	1.50
(7 days)	PLP	10.21	0.04	0.39	4346.1	44.2	1.02

Effect of addition of rat liver supernatant to TA solution

We wanted to ascertain whether the TAL was modified in the presence of rat liver supernatant. The supernatant was added to TAL, as described under Experimental. The elution patterns were not much influenced by the addition of supernatant, as shown in Fig. 6, where the area of peak I in B is more than 90% of peak I in A. The same experiment was repeated for different final concentrations of TAL (2, 1.5 and 0.5 m*M*), giving good proportionality and recovery.

TAD was also measured under all of the above conditions. The compound showed the same retention time as TAL and the same behaviour in both the absence and presence of rat liver supernatant.

Ellman's reaction

The thiazolidinic compound was submitted to the Ellman reaction: suitable aliquots were treated as indicated under Experimental and readings were taken at different times (0 and 40 min); the colour was stable after 40 min. Linearity

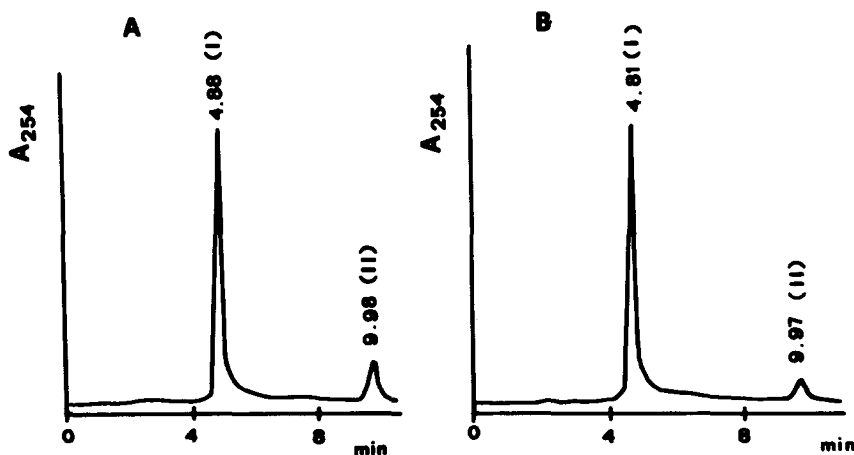

Fig. 6. Chromatography of TAL in the presence of tissue extracts: 2 nmol of (I) TAL in (A) the absence and (B) the presence of rat liver supernatant; (II) PLP derived by spontaneous decomposition of TAL.

(absorbance *versus* concentration) was tested on standards in buffered solutions and the regression equation of the calibration graph was $A_{412} = 0.02\,C + 0.025$, $r = 0.998$, $p < 0.01$. The linearity was excellent between $C = 6$ and 30 nmol.

The spectrophotometric procedure showed a low sensitivity (more than 6 nmol) and long times of execution (40 min), and it could not be applied to the TA compounds in presence of rat liver supernatant owing to severe tissue interference (not yet analysed).

From the results it is evident that the thiazolidinic derivatives of both L- and D-cysteine, can be easily separated from PLP by HPLC. The procedure can easily be applied to tissue extracts, and in this way it should be possible to ascertain whether, and in which tissue or organisms, TA compounds are either synthesized or degraded.

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