CHROMATOGRAPHIC SEPARATION OF PYRIDOXYL DERIVATIVES OF AMINO ACIDS*

CARLO TURANO, PAOLO FASELLA, PAOLA VECCHINI AND ANNA GIARTOSIO
Istituto di Chimica Biologica, Università di Roma e Centro di Enzimologia del C.N.R., Rome (Italy)

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Much interest has recently been given to pyridoxyl derivatives of amino acids, because it is in this form that coenzyme-binding sites and coenzyme-substrate complexes have been isolated from pyridoxal dependent enzymes.

Even though methods for the synthesis of pyridoxyl-amino acids are known[1], no procedure is yet available for the quick identification of less then micromolar amounts of these compounds. It is the purpose of this paper to describe a two-dimensional chromatographic technique that fulfils this requirement.

The non-phosphorylated form of the pyridoxyl derivatives was chosen for the present work because it is this form which is obtained after hydrolysis of reduced pyridoxal enzymes.

EXPERIMENTAL

Preparation of α-pyridoxyl-amino acids

Twenty milligrams of the amino acid were dissolved in 1.5 ml of hot water. Solutions of dicarboxylic acids were neutralized by the addition of solid $KHCO_3$. The aqueous solution of the amino acid was diluted with an equal volume of methanol, 2 mg of solid pyridoxal hydrochloride were added and the solution was neutralized. The solution acquired a characteristic yellow colour indicating Schiff base formation between the aldehydic group of pyridoxal and the α-amino group of the amino acid[2]. The yellow solution was mixed with 5–10 mg of sodium borohydride. This treatment produced a reduction of the azomethinic double bond and was accompanied by the disappearance of the yellow colour.

The solution was deposited as a 10 cm long band, 7 cm from the end of a sheet of Whatman 3 MM paper, and developed for 20 h by descending chromatography in *n*-butanol–acetic acid–water (40:10:50).

Examination of dried chromatograms in U.V. light revealed the presence of three bright-blue fluorescent spots of decreasing intensity corresponding respectively to the pyridoxyl-amino acid compound, to pyridoxine and to pyridoxamine. A small vertical strip of the chromatogram was cut off and treated with ninhydrin to locate the amino acids.

The band corresponding to the pyridoxyl-amino acid was cut from the chromatogram, eluted with water and the eluate was submitted to two-dimensional paper

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chromatography using collidine–lutidine (1:1) saturated with water for the first and *n*-butanol–acetic acid–water (40:10:50) for the second run.

All pyridoxyl-amino acid compounds tested in this way gave a single spot.

The chromatograms were analyzed in U.V. light and sprayed with ninhydrin to detect free amino groups or with 2,4-dichloroquinone-chlorimide, to detect the phenolic groups of the pyridoxyl derivatives[3].

Preparation of pyridoxyl-lysine derivatives

α,ε-Dipyridoxyl-lysine was prepared (together with α-pyridoxyl-lysine) as follows: 8 mg of lysine dihydrochloride and 24 mg of pyridoxal hydrochloride were dissolved in 0.5 ml of water and neutralized with dilute NaOH. The solution was then evaporated over a gentle heat and to the residue, dissolved in methanol, was added sufficient sodium borohydride to completely discharge the yellow colour. After filtration the solution was applied as a long band on a sheet of Whatman 3 MM paper and analyzed in the usual way.

ε-Pyridoxyl-lysine was prepared as follows: 300 mg of lysine dihydrochloride were dissolved in 2 ml of water and an excess of solid basic copper carbonate was added. The insoluble matter was filtered off on sintered glass and the filtrate was gently heated to dryness in an evaporating basin.

The dry residue was suspended in 5 ml of methanol containing 50 mg of pyridoxal. The suspension was heated at 40° for a few minutes, cooled to room temperature, treated with several aliquots of sodium borohydride and the black copper precipitate was filtered off. The solution was concentrated under vacuum, submitted to preparative chromatography and analyzed as described for the other pyridoxyl-amino acids. The spot corresponding to ε-pyridoxyl-lysine was identified by its fluorescence and by the colour reaction described by OLESEN LARSEN AND KJAER[4] for differentiating α- from ω-amino acids.

α-Pyridoxyl-lysine was prepared as follows: 50 mg of pyridoxal hydrochloride and 100 mg of ε-acetyl-lysine[5] were dissolved in 5 ml 50% ethanol and neutralized with dilute NaOH. The mixture was then dried under vacuum. A suspension of 20 mg sodium borohydride in 2 ml of ethanol was added to the dry residue and, after 10 min, 1 ml of 1 *M* acetic acid. The mixture was dried under vacuum, dissolved in 2 ml of 6 *N* HCl and sealed in a vial. The vial was kept at 120° for 7 h. The excess of acid was then removed under vacuum. The dry residue was dissolved in a minimum of water and analyzed as usual.

RESULTS

The R_F values in different solvents of the pyridoxyl amino acids investigated in the present paper are reproduced in Table I.

A typical two-dimensional chromatogram of a mixture of the derivatives under investigation is reproduced in Fig. 1, where the fluorescent spots which appear upon exposure to U.V. light are shown.

As regards the identification of the lysine derivatives, ε-pyridoxyl-lysine gave the expected positive ninhydrin reaction but showed no ninhydrin reaction after treatment with the OLESEN LARSEN AND KJAER reagent[4], which blocks α-amino groups.

When lysine was reacted with an excess of pyridoxal (see preparation of α,ε-dipyridoxyl-lysine), two pyridoxyl derivatives were obtained, one of which, with R_F

TABLE I

R_F VALUES OF PYRIDOXYL-AMINO ACIDS IN VARIOUS SOLVENTS

Pyridoxyl-amino acid	*R_F values*	
	in collidine–lutidine (1 : 1) saturated with water	*in n-butanol–acetic acid–water (40 : 10 : 50)*
Alanine	0.56	0.39
Arginine	0.28	0.06
Aspartic acid	0.41	0.19
Phenylalanine	0.57	0.62
Glycine	0.54	0.22
Glutamic acid	0.30	0.32
Histidine	0.54	0.07
Leucine	0.72	0.52
Lysine (ε-pyridoxyl)	0.45	0.06
Lysine (α-pyridoxyl)	0.24	0.13
Lysine (α,ε-dipyridoxyl)	0.47	0.09
Methionine	0.70	0.40
Serine	0.59	0.20
Threonine	0.77	0.31
Tyrosine	0.83	0.38
Valine	0.74	0.49
Pyridoxine	0.88	0.63
Pyridoxamine	0.52	0.45

All values were obtained on Whatman No. 4 paper by descending chromatography.

values of 0.47 and 0.09 in collidine–lutidine and butanol–acetic acid respectively, was ninhydrin negative and was identified as α,ε-dipyridoxyl-lysine. The other derivative, with R_F values of 0.25 and 0.13 respectively, was ninhydrin positive, even after treatment with the OLESEN LARSEN AND KJAER reagent[4], and was identified as α-pyridoxyl-lysine; the same spot was obtained starting the preparation with ε-acetyl-lysine (see EXPERIMENTAL).

DISCUSSION

The chromatographic procedure described in this paper provides a simple and quick way of identifying 0.01 micromolar amounts of 16 pyridoxyl-amino acids.

The method could therefore prove useful in the investigation of the apoenzyme-coenzyme and enzyme-substrate complexes of pyridoxyl phosphate dependent enzymes. Application of this method to glutamic-aspartic transaminase from pig heart will be described elsewhere.

It is interesting to observe that owing to the presence of the pyridoxyl group in the molecules of the amino acid derivatives, the R_F values are, as was to be expected, slightly higher then those of the corresponding amino acids in the same solvents.

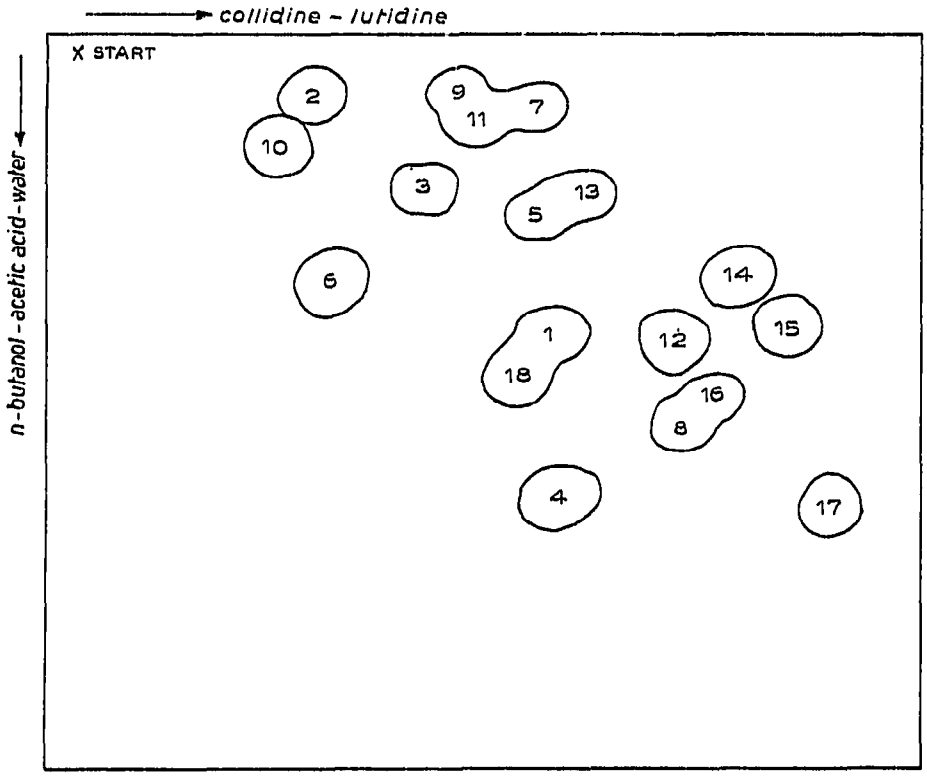

Fig. 1. The fluorescent spots appearing on a typical chromatogram of a mixture of the pyridoxyl-derivatives of the following amino acids: 1 = alanine; 2 = arginine; 3 = aspartic acid; 4 = phenylalanine; 5 = glycine; 6 = glutamic acid; 7 = histidine; 8 = leucine; 9 = lysine (ε-pyridoxyl); 10 = lysine (α-pyridoxyl); 11 = lysine (α,ε-dipyridoxyl); 12 = methionine; 13 = serine; 14 = threonine; 15 = tyrosine; 16 = valine; 17 = pyridoxine; 18 = pyridoxamine.

SUMMARY

The preparation of sixteen pyridoxyl amino acid compounds is described, together with their separation by means of a two-dimensional chromatographic system.

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