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Note

Enantiomeric separation of Dns-amino acids by reversed-phase thin-layer chromatography

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The advantages of thin-layer chromatography (TLC) arise from the simplicity by which the chromatographic parameters can be adjusted and the ease with which the success of the separations can be evaluated. The use of gradients in TLC, *e.g.*, a temperature gradient or an elution gradient, has been demonstrated for the separation of complex mixtures. Liteanu and Gocan[1,2] described for the first time the separation of some metal ions using paper chromatography with a temperature gradient. Later, Hodisan and Liteanu[3] and Grinberg and co-workers[4,5] studied the influence of a temperature gradient on the TLC separation of amino acids on cellulose and of aflatoxins on silica gel. The temperature gradient corresponds to a decrease in the height equivalent to a theoretical plate, resulting in improved separations. Hence this technique permits separations that often cannot be achieved under ambient conditions.

One of the first theoretical papers on the elution gradient mode in TLC was written by Snyder and Saunders[6]. More recently, Golkiewicz and co-workers[7,8] used this technique to improve the separation of several classes of compounds.

The introduction of chemically bonded C_{18} reversed-phase TLC (RP-TLC) widened the scope of TLC[9]. The use of isocratic conditions in the reversed-phase mode is not adequate for resolving a mixture containing compounds not very different in hydrophobicity. This difficulty can be overcome by using gradient elution. Thus, Sander and Field[10] reported the RP-TLC resolution of a four-component mixture, which had been difficult to resolve under isocratic conditions, using an apparatus for solvent gradient elution.

The separation of enantiomers by high-performance liquid chromatography (HPLC) with chiral additives in the mobile phase is a relatively recent method. Since Le-Page *et al.*[11] separated Dns-amino acids into enantiomers and Gil-Av and co-workers[12,13] separated free amino acids into their enantiomers, a number of papers reporting separations of enantiomers with chiral additives have appeared[14–17].

Recently, Wainer *et al.*[18] described the RP-TLC separation of 2,2,2-trifluoro-1-(9-anthryl)ethanol into enantiomers and Weinstein[19] reported the separation of Dns-amino acids of proteins. The separation of Dns-amino acids into D- and L-enantiomers was performed on commercial plates, treated with a copper complex of N,N-di-*n*-propyl-L-alanine (DPA)[7]. All the protein amino acids except proline were resolved. The method cannot be applied, however, to a mixture of all the Dns-deriv-

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atized protein amino acids because of overlapping of some spots of the multi-component mixture. As it is desirable to analyse complex mixtures in many practical applications, especially in biological samples, we have introduced a two-dimensional RP-TLC technique. In the first dimension, the Dns-amino acids are separated into components in a non-chiral mode. To achieve a satisfactory separation we used a convex gradient elution with aqueous sodium acetate buffers and varying concentrations of acetonitrile. For the second dimension we treated the plates with the chiral copper complex of N,N-di-*n*-propyl-L-alanine and developed them in aqueous acetonitrile–sodium acetate buffers. To optimize the separation of each Dns-amino acid into its enantiomers, a temperature gradient was applied.

EXPERIMENTAL

RP-18 RP-TLC plates (10 × 20 cm) were purchased from Merck (Darmstadt, F.R.G.). The Dns-amino acids were purchased from Sigma (St. Louis, MO, U.S.A.) or were prepared according to ref. 20.

The elution and temperature gradients were performed in special chromatographic chambers, shown in Figs. 1 and 2.

For the elution gradient, the mobile phase was 0.3 *M* sodium acetate in water–acetonitrile (80:20) (adjusted to an apparent pH of 6.3 with glacial acetic acid) (A), to which was added 0.3 *M* sodium acetate in water–acetonitrile (70:30) (apparent pH 6.8) at a flow-rate 0.5 ml/min, to give a final acetonitrile concentration of 38% or 47%. A convex gradient elution was obtained.

The mobile phase in the chiral mode was 8 m*M* N,N-di-*n*-propyl-L-alanine (DPA) and 4 m*M* copper(II) acetate disolved in 0.3 M sodium acetate in water–acetonitrile (70:30) (apparent pH 7).

The plates were developed in a temperature gradient mode. DPA was prepared as described in refs. 16, 21 and 22 (it will be available from J. T. Baker, Phillipsburgh, NJ, U.S.A., in the near future).

The plates were equilibrated before application of the Dns-amino acids by development in the mobile phase A. Samples were applied on the plates from an aqueous solution of the Dns-amino acid using glass capillaries. The mixture was

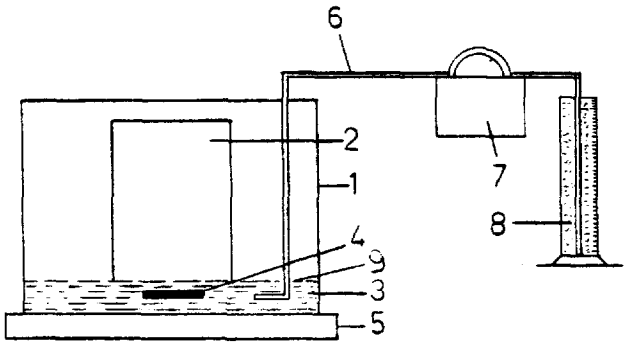

Fig. 1. Chromatographic system for elution gradient. 1 = Chamber; 2 = TLC plate; 3 = mixing chamber; 4 = magnetic bar; 5 = magnetic stirrer; 6 = PTFE tube; 7 = peristaltic pump; 8 = solvent reservoir for the elution gradient; 9 = PTFE plate with holes.

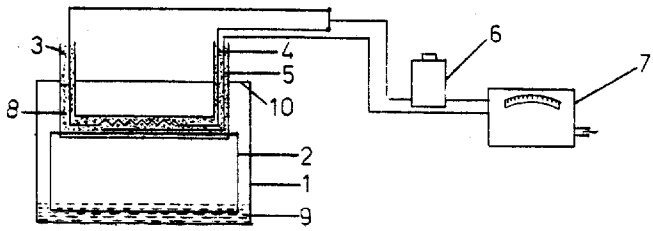

Fig. 2. Chromatographic system for temperature gradient. 1 = Chamber; 2 = TLC plate; 3 = U-shaped tube; 4 = heating element; 5 = thermocouple; 6 = adjustable autotransformer; 7 = temperature controller; 8 = silicone oil; 9 = mobile phase; 10 = lid.

spotted 2.5 cm from the bottom of the plates and 1 cm from the lateral edge. The plates were immersed about 0.3 cm in the mobile phase and developed. Detection of the individual Dns-amino acids was effected under UV light at 366 nm.

After the separation of the Dns-amino acids, the plates were dried with a fan, the strip of the chromatographic plates containing the amino acids was covered with a glass plate and the remaining exposed plate was sprayed with DPA and copper(II) acetate (8:4 m*M*) dissolved in water–acetonitrile (5:95). The plates were developed in the second dimension using a temperature gradient.

TABLE I

hR_F VALUES* OF Dns-AMINO ACIDS SEPARATED WITH A CONVEX CONTINUOUS ELUTION GRADIENT

DnsAA	*Final concentration in acetonitrile (%)*	
	38	*47*
Asp	53.1	62.9
Ser	29.6	42.9
Gly	23.4	37.0
Phe	7.0	11.7
Cyst Ac**	53.9	63.5
Glu	48.4	59.4
His	22.5	38.8
Tyr	3.9	5.8
Thr	24.2	34.7
Ala	19.5	29.4
Met	11.7	17.6
Lys	8.5	13.5
Arg	20.3	30.0
Val	12.5	20.5
Ileu	7.0	15.8
Pro	16.4	25.2
Leu	7.8	16.8
Trp	5.4	12.3
Dns-OH***	35.1	47.0

* hR_F is defined as 100 R_F.
** Cyst Ac = cysteic acid.
*** Dns-OH = dansylic acid.

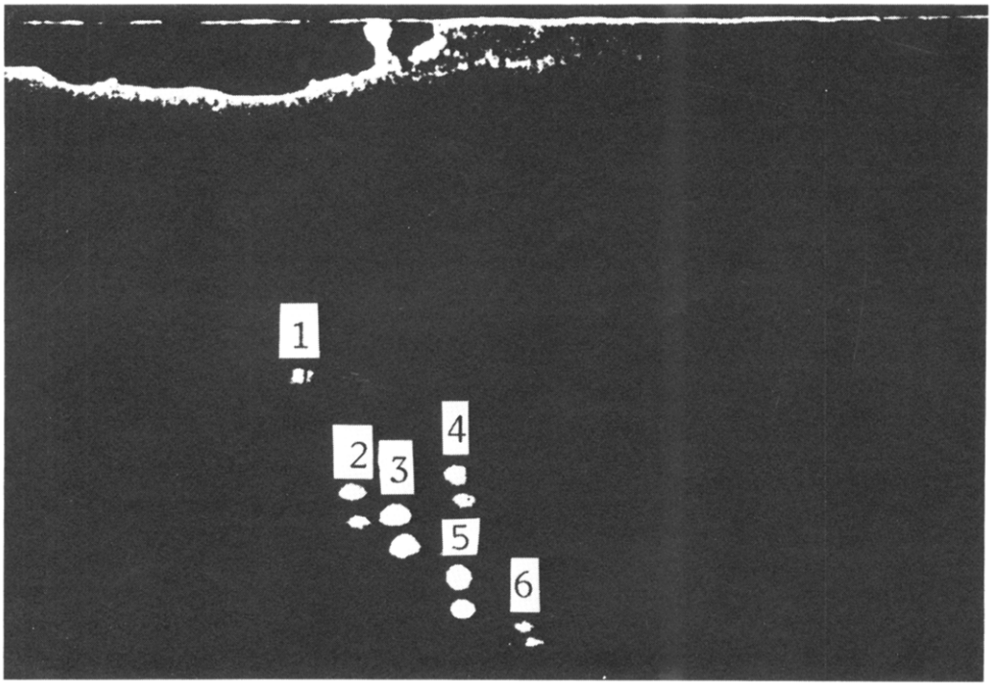

Fig. 3. Enantiomeric separation of Dns-amino acids by RP-TLC using a temperature gradient. 1 = Dns-OH; 2 = Asp; 3 = Ser; 4 = Met; 5 = Ala; 6 = Phe.

RESULTS AND DISCUSSION

The R_F values of the Dns-amino acids in the first dimension are presented in Table I. In order to identify the spots, individual Dns-amino acids were used as markers and were developed in parallel with the mixtures of all the 18 Dns-amino acids. As can be seen in Table I, at a final concentration of acetonitrile of 38% Ile is not separated from Phe and some of the Dns-amino acids are not completely displaced from the start (hR_F below 11). On addition of more organic solvent (final acetonitrile concentration 47%), their separation is achieved. Trp and Phe are only

TABLE II

RESOLUTION COEFFICIENTS (α) OF SOME DL-Dns-AMINO ACIDS SEPARATED BY RP-TLC WITH A CHIRAL PHASE AND A TEMPERATURE GRADIENT (6.2°C/cm)

Resolution coefficients (α) defined according to Perry[23]. The chiral phase is copper(II) acetate–DPA (4:8 m*M*).

Dns-Amino acid	α
Asp	1.3
Ser	1.4
Ala	2.1
Met	1.3
Phe	2.0

partially resolved. The low R_F values recorded for some Dns-amino acids (Tyr, Phe, Trp) can be explained by strong hydrophobic interactions with the stationary phase.

It has already been shown[19] that it is possible to separate each Dns-amino acid into enantiomers by RP-TLC using the chiral copper complex of DPA. In our experiments only 8 cm of the plates were left for the second dimension, which is not sufficient for a good separation. In order to circumvent this problem, we used a temperature gradient of 6.25°C/cm, which was found to be efficient. With this gradient, the amount of mobile phase evaporated in the heated part of the plate is enough to simulate a theoretical supplementary column for the resolution of the Dns-amino acids into enantiomers. The resolution in the second dimension required 150 min, the D-enantiomers moving faster than the L-enantiomers.

An example of the chiral separation in the second dimension is illustrated in Fig. 3. Table II lists the α values of the separated enantiomers.

The method described here for the resolution of Dns-amino acids has all the advantages and versatility of TLC. The combination of an elution gradient in one dimension in order to optimize the separation of the different Dns-amino acids and of a temperature gradient in the second dimension for the separation of enantiomers makes this technique applicable to multi-component mixtures, *e.g.*, amino acids in a protein hydrolysate. Quantitative results can be obtained by using densitometry.

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