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APPLICATION OF MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE SEPARATION OF CHIRAL AMINO ACIDS

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SUMMARY

Micro high-performance liquid chromatographic separation of dansyl-D,L-amino acids was examined using copper (II)-L-histidine or copper (II)-L-histidine methyl ester as eluent. Parameters that affect the retention and the resolution of dansyl-D,L-amino acids were examined. Gradient elution was used to separate many pairs of amino acids in a single chromatographic run.

INTRODUCTION

Many papers have reported the separation of chiral amino acids by high-performance liquid chromatography (HPLC)[1–12], in which either chiral stationary phases or mobile phases containing chiral metal complexes are generally employed. The latter approach can separate more pairs of chiral amino acids in a single chromatographic run than can the former approach. Many kinds of chiral metal complex have been proposed. Lam and Karmen[9,10] reported that a system with copper(II)-L-histidine methyl ester as eluent could resolve most of the biologically important amino acids in a single chromatographic run.

Decreased consumption of both stationary and mobile phases is one of the advantages that micro HPLC can offer, and this facilitates the use of expensive stationary phases and reagents. Micro HPLC also has an advantage in the study of operating conditions. This paper describes the application of micro HPLC to the separation of optical isomers of dansyl-amino acids as the mixed chelate copper(II) complexes of L-histidine or L-histidine methyl ester.

EXPERIMENTAL

Apparatus

Microfeeder (Azumadenkikogyo, Tokyo, Japan) equipped with a gas-tight syringe MS-GAN 050 (0.5 ml) (Terumo, Tokyo, Japan) or LKB 2150 HPLC Pump (LKB, Bromma, Sweden) was employed as a pump for the isocratic separation, while Model 590 (Waters, Milford, MA, U.S.A.) and a Micrometric metering pump (LDC/

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Milton Roy, Riviera, FL, U.S.A.) were used for the gradient separation. The gradient elution was carried out using Laboratory-made equipment comprising a mixing chamber and a magnetic stirrer[13]. An FP-110C spectrofluorometer (Jasco: Japan Spectroscopic, Tokyo, Japan) equipped with a small-volume (*ca.* 0.1 μl) flow cell was employed as a detector. The sample was injected with an ML-422 micro-valve injector (0.02 μl; Jasco). Fused-silica tubing (0.26 mm I.D.) and glass-lined stainless-steel tubing (0.3 mm I.D.) were employed as the column material, and ODS-Hypersil (Shandon, Cheshire, U.K.) was selected as the packing material. Preparation procedures were the same as in previous work[14,15].

Reagents

Twenty-one L-amino acids (Kit No. LAA-21), sixteen D-amino acids (Kit No. DAA-16), twenty-four D,L-amino acids (Kit No. DLAA-24) and fifteen dansyl-D,L-amino acids (Kit No. DAN-DL-15) were obtained from Sigma (St. Louis, MO, U.S.A.), as was L-histidine methyl ester. Other reagents were from Wako (Osaka, Japan), unless otherwise noted. Dansylation of amino acids was also carried out in the laboratory at 40°C for 30 min. The pH of the sample solution was adjusted to 9.5–10 with sodium hydroxide and sodium bicarbonate prior to the dansyl reaction. The pH of the mobile phase was adjusted with sodium acetate (2 g/l) and sodium hydroxide.

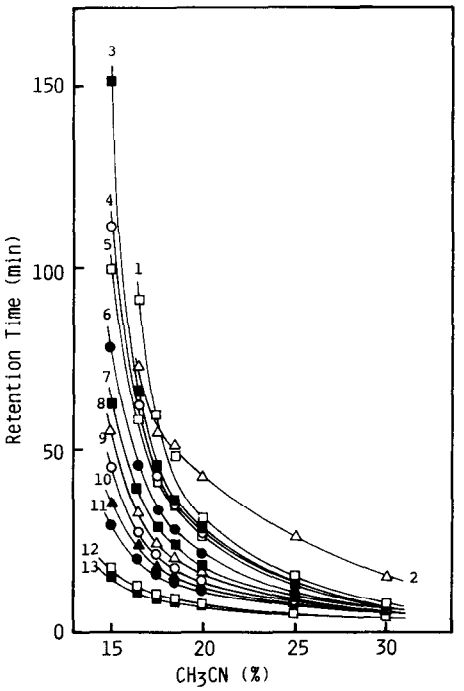


Fig. 1. Relationships between retention time and the mobile phase composition. Column, ODS-Hypersil-5 (5 μm) (100 × 0.26 mm I.D.); mobile phase, acetonitrile–buffer containing 2.5 m*M* copper sulphate, 5.0 m*M* L-histidine and 2 g/l sodium acetate (pH 7.0); flow-rate, 2.1 μl/min; sample, dansyl-amino acids, each *ca.* 13 ng. Curves: 1 = D-Nle; 2 = Dns-NH_2; 3 = L-Nle; 4 = D-Leu; 5 = D-Ile; 6 = L-Leu; 7 = L-Ile; 8 = D-Met; 9 = D-Val; 10 = L-Met; 11 = L-Val; 12 = D-Ala; 13 = L-Ala.

RESULTS AND DISCUSSION

Copper(II)-L-histidine eluent

The stereoselectivity of the isomers of amino acids depends on their complexation with the chiral copper(II) complex in the mobile phase. It has been reported that L-histidine is more selective for resolution of D,L-isomers than L-arginine, L-proline and L-histidine methyl ester[16]. The organic composition of the mobile phase, the concentration of the chiral additive in the mobile phase and the pH of the mobile phase affect the selectivity and the retention of D,L-amino acids.

Fig. 1 shows the relationships between the retention time and the concentration of acetonitrile. The retention time strongly depends on the concentration of acetonitrile, while the effect on the selectivity factor (α) is small. The elution order of dansyl-D,L-amino acids is independent of the concentration of acetonitrile, which facilitates the separation of D,L-isomers by gradient elution. The dependence of the retention time of dansyl amide on the mobile phase composition is different from that of dansyl-amino acids.

The concentration of the chiral additive in the mobile phase affects the selectivity and the capacity factor. Fig. 2 illustrates the dependence of the capacity factor of dansyl-α-amino-*n*-butyric acid (α-AB) on the concentration of the copper-L-his-

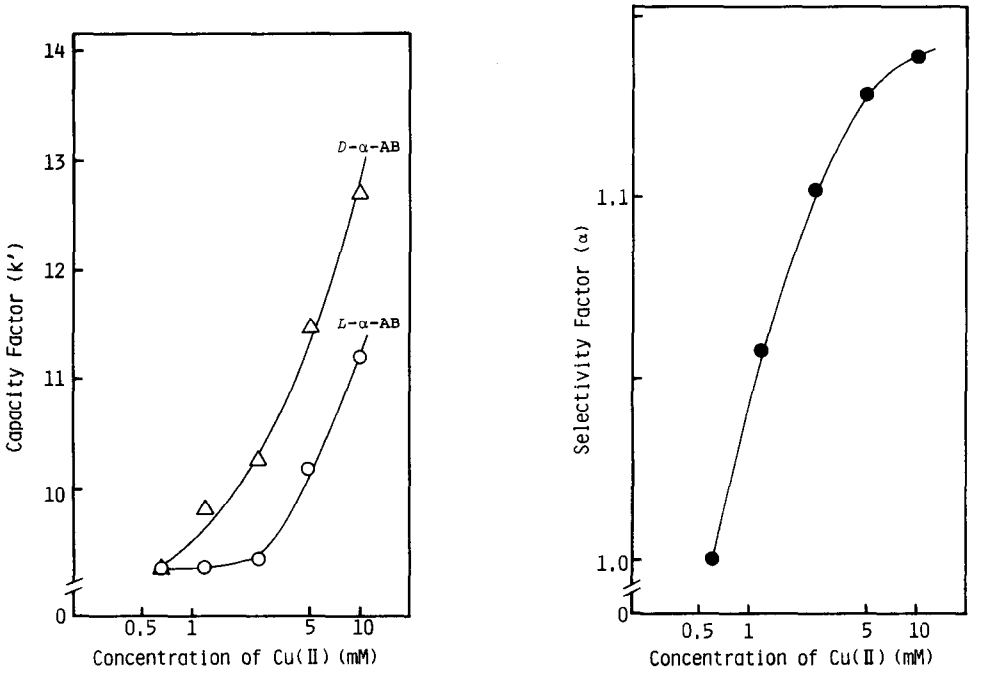


Fig. 2. Dependence of the capacity factor on the concentration of the copper(II)-L-histidine complex. Column, ODS-Hypersil-5 (100 × 0.26 mm I.D.); mobile phase, acetonitrile–sodium acetate buffer (pH 7.0) containing the copper(II)-L-histidine complex (17:83); sample, dansyl-D,L-α-AB (each 100 pmol).

Fig. 3. Dependence of the selectivity factor on the concentration of the copper(II)-L-histidine complex. Operating conditions as in Fig. 2. Selectivity factor is defined as k'(D)/k'(L).

tidine complex. The ratio of the concentration of L-histidine to that of copper sulphate is kept constant (2:1). The capacity factor increases with increasing concentration of the chiral additive. Fig. 3 shows the dependence of the selectivity factor on the concentration of the chiral additive. The selectivity factor also increases with increasing concentration of the chiral additive and seems to level off with further increasing the concentration of the chiral additive.

The pH of the mobile phase affected the chiral separation and peak shape. The capacity factor decreased with increasing pH of the mobile phase. A pH value of *ca.* 7 gave symmetric chromatographic peaks and good selectivity.

Figs. 4 and 5 show isocratic and gradient separations of dansyl-D,L-amino acids on an ODS column (284 × 0.3 mm I.D.). D-Val and L-Met are separated by gradient elution, but they are not separated in the isocratic mode. The analysis time is less for the gradient separation. Six pairs of dansyl-D,L-amino acids are separated.

Copper(II)-L-histidine methyl ester eluent

Although the copper(II)-L-histidine methyl ester eluent is less selective than

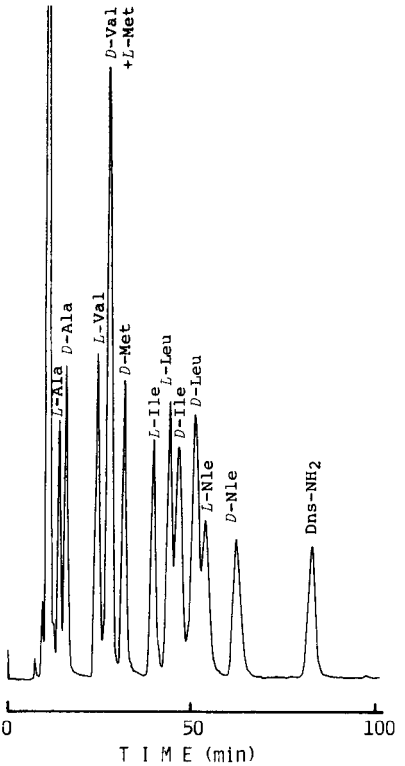


Fig. 4. Isocratic separation of dansyl-D,L-amino acids with copper(II)-L-histidine as eluent. Column, ODS-Hypersil-5 (284 × 0.3 mm I.D.); mobile phase, acetonitrile–buffer containing 2.5 m*M* copper sulphate, 5.0 m*M* L-histidine and 2 g/l sodium acetate (pH 7.0) (22.5:77.5); flow-rate, 10 μl/min; wavelengths of detection, 365 nm (excitation) and 522 nm (emission); samples *ca.* 13 ng each.

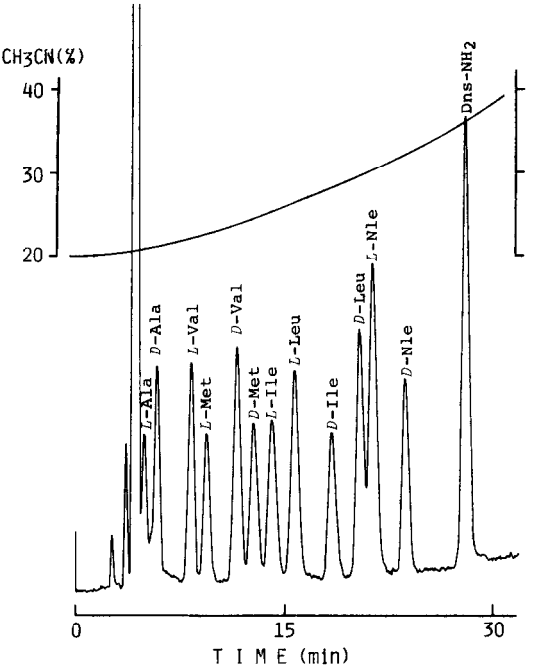


Fig. 5. Gradient separation of dansyl-D,L-amino acids with copper(II)-L-histidine as eluent. Operating conditions as in Fig. 4 except for the mobile phase composition, which was as indicated.

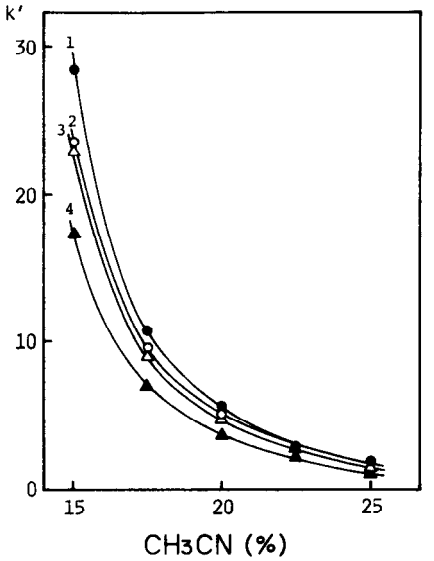
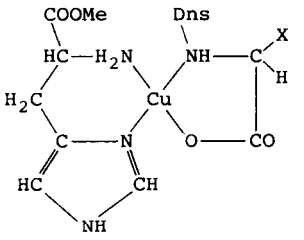


Fig. 6. Dependence of the capacity factors of D,L-Asp and D,L-Glu on the mobile phase composition. Column, ODS-Hypersil-5 (100 × 0.26 mm I.D.); mobile phase, acetonitrile–buffer containing 2.5 m*M* copper sulphate, 5.0 m*M* L-histidine methyl ester and 2 g/l sodium acetate (pH 7.0); samples, 100 pmol each. Curves: 1 = L-Asp; 2 = D-Asp; 3 = D-Glu; 4 = L-Glu.

copper(II)-L-histidine, the former offers better separations of the different amino acids[10]. Parameters that affect the selectivity factor and the capacity factor of dansyl-D,L-amino acids were examined. Fig. 6 illustrates the dependence of the capacity

L-Asp(five-membered ring)



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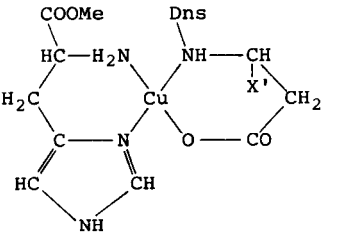
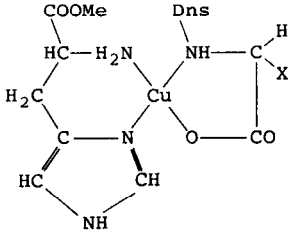
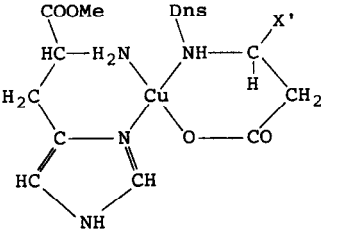


D-Asp(five-membered ring)



D-Asp(six-membered ring)



X=CH_2COO^- X'=COO^-

Fig. 7. Structures of copper(II)-L-histidine methyl ester Asp complexes.

factors of the dansyl derivatives of D,L-Asp and D,L-Glu on the mobile phase composition. Although Glu is more hydrophobic than Asp, the latter is retained longer. Furthermore, the elution order of D,L-isomers of Asp is different from that of D,L-Glu. This may be because dansyl-Asp has two carboxyl groups and it forms a six-membered ring complex with copper(II) as well as a five-membered ring complex, as shown in Fig. 7. On the other hand, dansyl-Glu forms with a five-membered ring complex, as do other dansyl-amino acids. It can be assumed that the six-membered ring complexes of dansyl-Asp are retained longer than the five-membered ring complexes of dansyl-Glu.

Fig. 8 shows that each isomer of dansyl-Asp gives two peaks. It is found that the selectivity between briefly retained peaks and longer retained peaks is different. The former peaks may be due to the five-membered ring complexes, and the latter peaks may be due to the six-membered ring complexes. The difference in selectivity can be explained by the fact that the co-ordination direction of the substituent (represented by X and X′ in Fig. 1) on the α-carbon of each corresponding isomer is opposite, which leads to different stabilities of the complexes. In addition, the data for Asp in Fig. 6 are based on the longer retained peaks shown in Fig. 8.

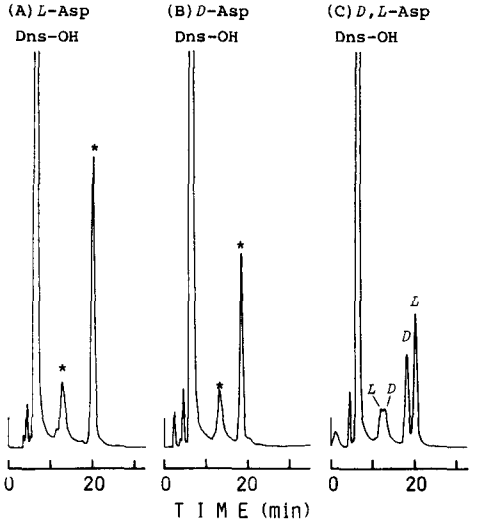


Fig. 8. Separation of dansyl-Asp. Column, ODS-Hypersil-3 (3 μm) (122 × 0.26 mm I.D.); mobile phase, acetonitrile–buffer containing 2.5 m*M* copper sulphate, 5.0 m*M* L-histidine methyl ester and 2 g/l sodium acetate (pH 4.6) (2:8); flow-rate, 2.1 μl/min; wavelengths of detection as in Fig. 4.

Fig. 9 shows the effect of the concentration of the chiral additive in the mobile phase on the selectivity factor and the capacity factor. The ratio of the concentration of L-histidine methyl ester to that of copper sulphate was kept constant (2:1). The capacity factor increases with increasing concentration of the chiral additive, whereas the dependence of the selectivity factor on the concentration of the chiral additive is slight in the regions examined.

Fig. 10 shows the effect of the pH of the mobile phase on the capacity factors of acidic amino acids. D,L-Isomers of dansyl-Asp can be separated in the lower pH

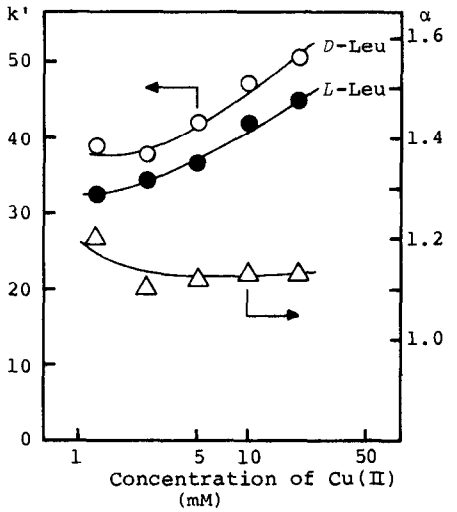


Fig. 9. Effect of the concentration of the chiral additive in the mobile phase on the selectivity and the capacity factor. Column, ODS-Hypersil-5 (100 × 0.26 mm I.D.); mobile phase, acetonitrile–sodium acetate buffer containing copper-L-histidine methyl ester complex (2:8).

region, and D,L-isomers of dansyl-Glu can be separated at pH values higher than 6.5. Most of neutral amino acids, such as Val, Nval, Leu, Nle, Ile, Phe, etc., have a similar tendency to Glu. This result indicates that the selectivity and the retention can be also controlled by the pH of the mobile phase.

Fig. 11 shows the isocratic separation of a mixture of several dansyl-D,L-amino acids. Optical isomers of Thr and Ala could not be resolved. Dansyl-D,L-Ile mixture (Kit No. DAN-DL-15) gave four peaks, which may be due to two asymmetric atoms of Ile. Dansyl-L- and dansyl-D-isomers prepared in our laboratory gave a single peak, represented by "L" or "D".

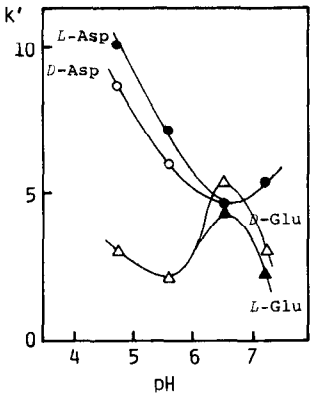


Fig. 10. Effect of the pH of the mobile phase on the capacity factor. Column, ODS-Hypersil-5 (100 × 0.26 mm I.D.); mobile phase, acetonitrile–buffer (as in Fig. 6) (2:8); samples, 100 pmol each.

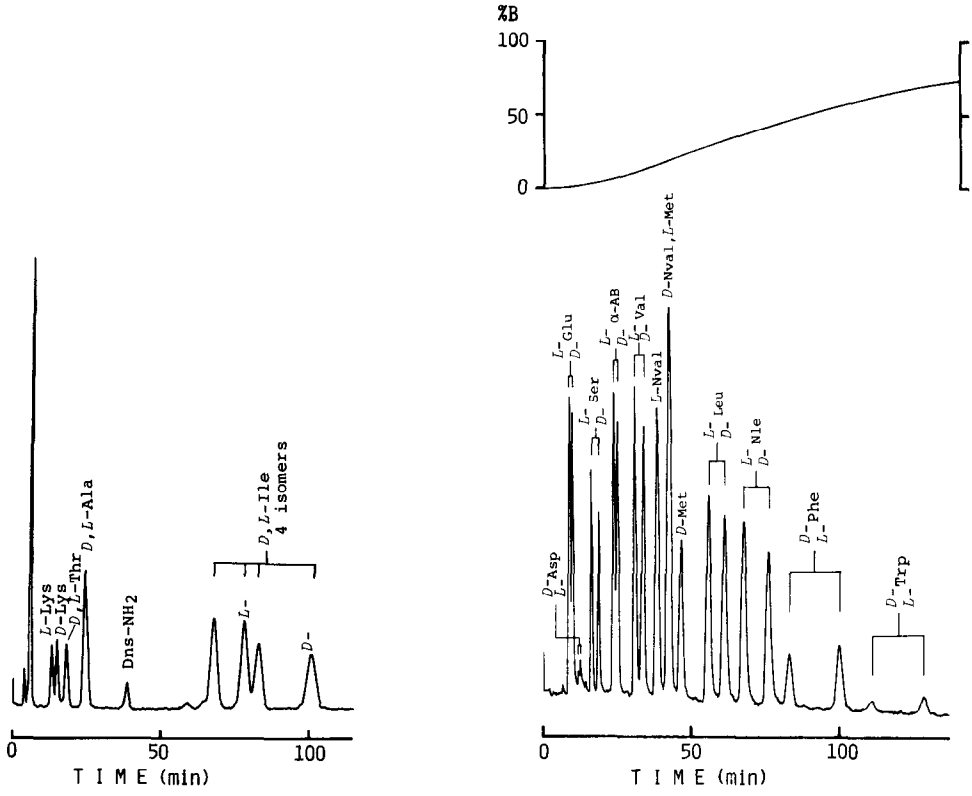


Fig. 11. Isocratic separation of dansyl-D,L-amino acids with copper(II)-L-histidine methyl ester as eluent. Column, ODS-Hypersil-3 (110 × 0.26 mm I.D.); mobile phase, acetonitrile–buffer (as in Fig. 6) (21:79); flow-rate, 2.1 μl/min; samples, *ca.* 18 ng each; wavelengths of detection as in Fig. 4.

Fig. 12. Gradient separation of dansyl-D,L-amino acids with copper(II)-L-histidine methyl ester as eluent. Column, ODS-Hypersil-3 (144 × 0.3 mm I.D.). Mobile phases: A, acetonitrile–sodium acetate buffer containing 5 m*M* copper sulphate and 10 m*M* L-histidine methyl ester (pH 6.66) (20:80); B, acetonitrile–sodium acetate buffer containing 6 m*M* copper sulphate and 12 m*M* L-histidine methyl ester (pH 5.50) (27.5:72.5), composition as indicated. Samples 50 pmol each; wavelengths of detection as in Fig. 4.

Fig. 12 shows the gradient separation of eleven pairs of dansyl-D,L-amino acids on an ODS column (143 × 0.3 mm I.D.). The D-isomers are generally retained longer, except those of Asp, Phe and Trp.

CONCLUSION

Micro HPLC is a convenient method for investigating the optimum conditions for the separation of chiral isomers of dansyl-amino acids. Many pairs of dansyl-D,L-amino acids were separated by gradient elution with copper(II)-L-histidine methyl ester as eluent.

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