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MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 5-DIMETHYLAMINONAPHTHALENESULPHONYL-AMINO ACIDS

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SUMMARY

Micro high-performance liquid chromatographic separations of 5-dimethylaminonaphthalenesulphonyl (Dns)-amino acids was examined. Both isocratic and gradient separations were investigated in the reversed-phase mode. The detection limit of Dns-amino acids was less than 0.3 pmol at a signal-to-noise ratio of 2 with UV spectrophotometric detection. The system was applied to the analysis of amino acids in soy sauce or in sake.

INTRODUCTION

Micro high-performance liquid chromatography (micro HPLC) has some advantages over conventional HPLC due to the small consumption of both mobile and stationary phases and the low solute dispersion. Micro HPLC thus facilitates the use of exotic, expensive or hazardous phases and has the ability to increase the mass sensitivity owing to the low solute dispersion. The low flow-rates possible in micro HPLC are also convenient when coupling a liquid chromatograph to a mass spectrometer.

Liquid chromatographic analysis of amino acids has been investigated by many researchers. Derivatization with 5-dimethylaminonaphthalenesulphonyl (Dns or dansyl) chloride (Dns-Cl) is known to enable a sensitive HPLC analysis of amino acids, as do derivatization with *o*-phthalaldehyde, phenylisothiocyanate, fluorescamine or 2,4-dinitrofluorobenzene. Dansyl derivatization was originally been developed by Gray and co-workers[1–3] and applied to the sequential analysis of peptides and proteins. Dns-amino acids have usually been separated in the reversed-phase mode, followed by fluorometric or UV spectrophotometric detection. They have mostly been separated by gradient elution[4–11], but isocratic separations have also been reported[12].

The increase in mass sensitivity is especially favourable to the analysis of biomedical samples. Since concentration of solutes in the column effluent increases with decreasing column dimensions, it is possible to increase the mass sensitivity by using a small-bore column and an appropriate detection system. Koroleva *et al.*[13]

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have examined the micro HPLC analysis of Dns-amino acids using fluoroplastic or glass micro packed columns (0.5 mm I.D.). In their study, fluorometric detection was used resulting in a detection limit of *ca.* 10 fmol owing to the low dispersion in micro HPLC as well as the improved detection system. However, the total analysis time was longer (70 min).

This paper describes the analysis of amino acids by micro HPLC with dansyl derivatization and UV spectrophotometric detection. Both isocratic and gradient separations of Dns-amino acids are demonstrated.

EXPERIMENTAL

Reagents

Dns derivatives of the following amino acids were obtained from Pierce (Rockford, IL, U.S.A.): alanine (Ala), asparagine (Asn), aspartic acic (Asp), cysteine (Cys) (both mono- and di-Dns derivatives), glutamic acid (Glu), glycine (Gly), hydroxyproline (Hyp), isoleucine (Ile), leucine (Leu), methionine (Met), norleucine (Nle), norvaline (Nval), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp) and valine (Val). These Dns-amino acids were obtained as piperidinum salts, except Dns-Glu, Dns-Nval and Dns-Val. Dns-Glu and Dns-Val were obtained as the acid forms and Dns-Nval as a cyclohexylamine salt. Dansyl amide (Dns-NH_2) and Dns-Cl were also purchased from Pierce Chemical. The above amino acids were examined since other Dns-amino acids were difficult to obtain. Ammonium acetate, distilled water, acetonitrile and glycine were obtained from Wako (Osaka, Japan) and employed without any purification. Other reagents were also obtained from Wako, unless otherwise noted.

Apparatus

The liquid chromatograph was constructed from a Famille-300S (JASCO: Japan Spectroscopic, Tokyo, Japan) or a Microfeeder (Azumadenkikogyo, Tokyo, Japan) equipped with a gas-tight syringe MS-GAN 050 (Terumo, Tokyo, Japan) as a pump, a microvalve injector ML-422 (0.02 μl, JASCO), gradient equipment, a micro packed fused-silica column, a UV spectrophotometer UVIDEC-100II (JASCO) and a recorder. The Famille-300S pump withstood a pressure of 500 kg/cm^2, while the Microfeeder pump withstood a pressure of 70 kg/cm^2. The former pump was operated in the constant-pressure mode and the latter in the constant-flow mode. Preparation procedures for the separation columns were the same as in previous work[14,15]. LiChrosorb RP-18 (5 μm; Merck, Darmstadt, F.R.G.) and ODS-Hypersil (3 μm; Shandon, Cheshire, U.K.) were employed as packing materials. The gradient equipment comprised a magnetic stirrer and a hand-made mixing vessel (volume 410 μl), enabling exponential elution profiles to be obtained, as described previously[16].

Dns derivatization

Dns derivatives of amino acids in soy sauce or in sake were prepared under the following conditions. Soy sauce was diluted 7.5 times in 0.17 *M* sodium bicarbonate and 0.1 *M* sodium hydroxide. The pH of the sample solution was adjusted to 9.7 and 2 ml of the solution were placed in a vial. Dns-Cl (0.14 ml of 10% (w/v) in acetone) was added to the prepared solution followed by 2.06-ml acetonitrile to

homogenize the sample solution. The vial was stoppered and incubated at 38°C for 110 min in an oven. The sample solution was finally centrifuged at 1210 *g* for 2 min. In the case of sake, the pH was adjusted to 9.7 with 0.17 *M* sodium bicarbonate and 0.2 *M* sodium hydroxide. One ml of the sample solution and 0.13 ml of 1% Dns-Cl in the mixture of acetone and acetonitrile were placed in a vial and incubated at 38°C for 90 min in the oven. The sake sample could be subjected to analysis without centrifugation.

RESULTS AND DISCUSSION

Isocratic separation

A mixture of acetonitrile and 0.13 *M* ammonium acetate (pH = 6.8) was employed as the mobile phase. Fig. 1 illustrates the relationship between the logarithm of the retention time and the mobile phase composition on the RP-18 column. The elution order of most of the Dns-amino acids is independent of the mobile phase

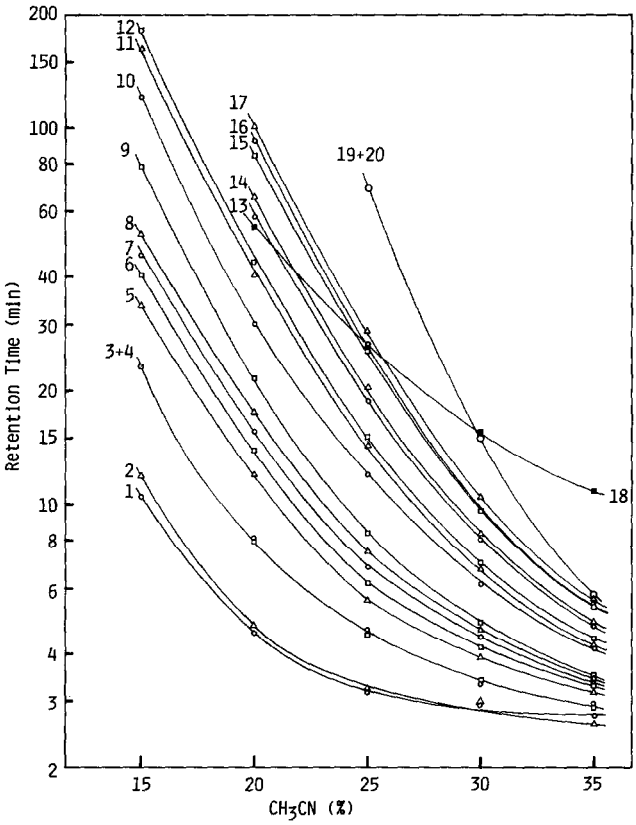

Fig. 1. Relationship between the retention time of Dns-amino acids and the mobile phase composition. Column: LiChrosorb RP-18, 153 × 0.26 mm I.D. Mobile phase: acetonitrile–0.13 *M* ammonium acetate (pH = 6.8). Flow-rate: 2.1 μl/min. Dns-amino acids: 1 = Asp; 2 = Glu; 3 = Hyp; 4 = Asn; 5 = Ser; 6 = Thr; 7 = Gly; 8 = Ala; 9 = Pro; 10 = Val; 11 = Nval; 12 = Met; 13 = Ile; 14 = Leu; 15 = Nleu; 16 = Trp; 17 = Phe; 18 = NH_2; 19 = di-Cys; 20 = Cys.

composition, while the retention behaviour of Dns-NH_2 is different from that of Dns-amino acids. The resolutions of Dns-Hyp from Dns-Asn and di-Dns-Cys from Dns-Cys are poor. Similar retention behaviour was observed for the ODS-Hypersil column.

A typical isocratic separation of Dns-amino acids is shown in Fig. 2; it was carried out within 25 min. The reproducibility of retention time obtained with the isocratic separation is expected to be better than that with the gradient separation. Although the pump was operated at 220 kg/cm^2 in the constant-pressure mode, the reproducibility of the retention time was satisfactory (less than 1% for several analyses). However, a pump which can precisely feed the mobile phase at low flow-rates under high pressure should be employed to improve the reproducibility of the retention time. The column should sometimes be washed with an acetonitrile-rich solution in order to remove retarded solutes, otherwise the retention times of Dns-amino acids were observed to decrease after several analyses. About 20 pmol of each Dns-amino acid were detected sensitively and the detection limits were 70–310 fmol at a signal-to-noise ratio of 2.

The yield of the dansylation reaction was estimated by using glycine as a test solute. Glycine was dissolved in 0.2 *M* sodium bicarbonate (pH = 10.0) and mixed with a Dns-Cl solution. Incubation was carried out at 38°C for 30–60 min. The results

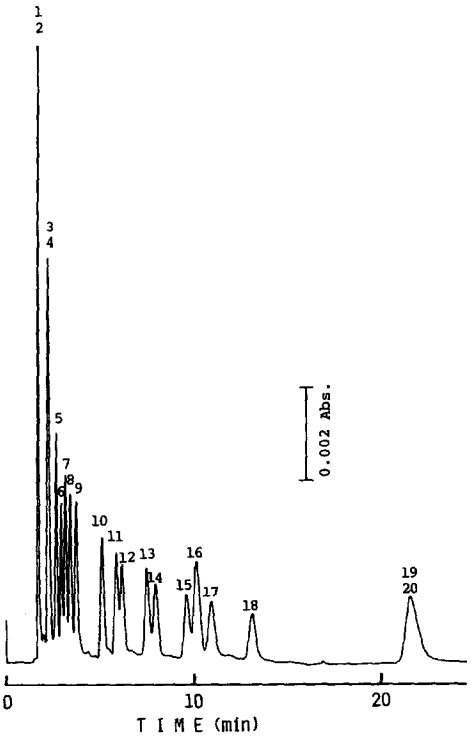

Fig. 2. Isocratic separation of Dns-amino acids. Column: ODS-Hypersil, 250 × 0.34 mm I.D. Mobile phase: acetonitrile–0.13 *M* ammonium acetate (pH 6.8) (27:73). Inlet pressure: 220 kg/cm^2. Flow-rate: 8.0 μl/min. Wavelength of UV detection: 222 nm. Samples: as in Fig. 1, each 20 pmol.

TABLE I

THE YIELD (%) OF THE DANSYLATION REACTIONS

Incubation temperature: 38°C.

Reaction time (min)	*Mole ratio (Dns-Cl/Gly)*			
	1.14	*2.30*	*5.41*	*10.82*
30	43	65	88	76
60	43	65	–	–

are shown in Table I. The yield of the dansylation reaction depends on the mole ratio of Dns-Cl to glycine. The yield was calculated by comparing the chromatographic peak heights of the standard Dns-Gly and of prepared samples. The reaction does not proceed after 30 min.

Fig. 3 demonstrates the separation of amino acids in soy sauce. About 0.2 nmol of amino acids in 1.3 nl of soy sauce were detected sensitively.

Gradient separation

Solvent-gradient elution improves selectivity and reduces the analysis time[4–11]. Fig. 4 demonstrates the gradient separation of Dns-amino acids on a 10-cm column. The content of acetonitrile in the mobile phase was changed by the home-made gradient system[16]. Dns-Asp and Dns-Glu could not be separated by isocratic elution, while they were satisfactorily separated with gradient elution. Dns-Hyp is not resolved from Dns-Asp nor di-Dns-Cys from Dns-Cys. In the isocratic elution, the peak heights tended to decrease with increasing retention volume, while the variance of the peak heights with the retention volume was relatively small in the gradient

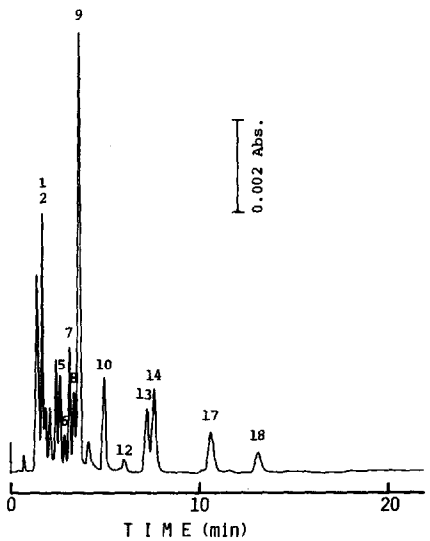

Fig. 3. Isocratic separation of amino acids in soy sauce (1.3 nl). Operating conditions as in Fig. 2, except the sample.

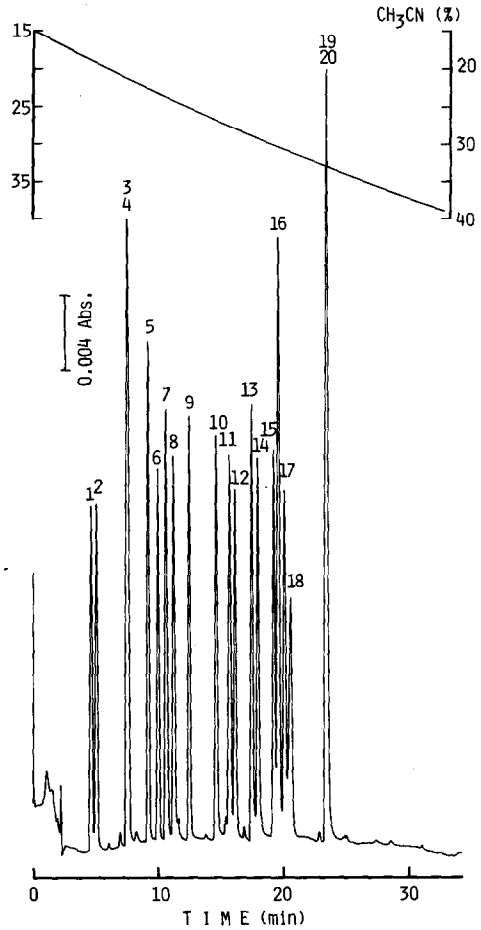

Fig. 4. Gradient separation of Dns-amino acids. Column: ODS-Hypersil, 100 × 0.34 mm I.D. Mobile phase: acetonitrile–0.13 *M* ammonium acetate, gradient profile as indicated. Flow-rate: 4.2 μl/min. Wavelength of UV detection: 222 nm. Samples: as in Fig. 1, each *ca.* 40 pmol.

elution. Thus, the detection limit of solutes eluted later is improved with the gradient elution. Most separations of amino acids involve protein hydrolysate containing as many as 25 compounds. Only eighteen amino acids were examined here, which is not sufficient for most applications. However, several peaks which may be due to other impure Dns-amino acids also appeared on the chromatogram.

Fig. 5 shows the gradient separation of amino acids in soy sauce. The advantages of gradient elution are apparent from Figs. 3 and 5. The resolution of weakly retained solutes is improved compared with the isocratic separation.

Fig. 6 shows the gradient separation of amino acids in sake. Dansylic acid (Dns-OH) (also seen in Fig. 5) may originate from the hydrolysate containing excess of Dns-Cl. About 60 pmol of amino acids in 11 nl of sake are detected. Several unidentified peaks appeared on the chromatograms (Figs. 5 and 6). Other amino acids that have not been considered here should be examined for various applications.

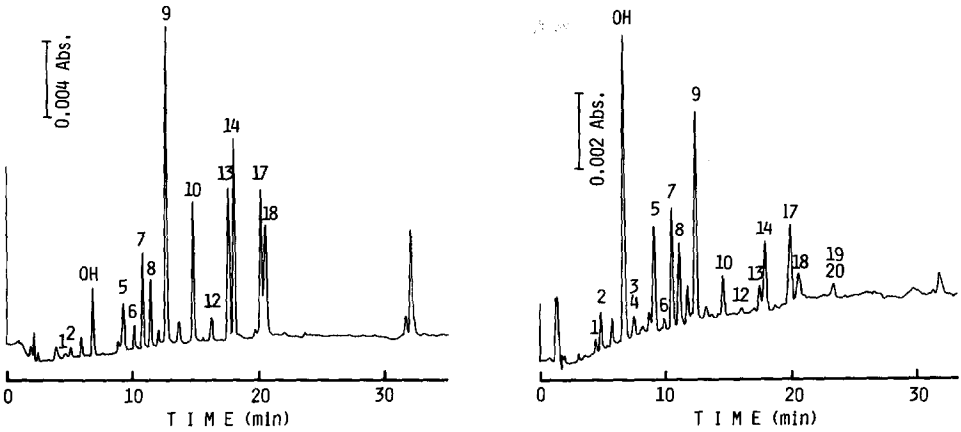

Fig. 5. Gradient separation of amino acids in soy sauce (1.3 nl). Operating conditions as in Fig. 4, except the sample. OH = Dansylic acid.

Fig. 6. Gradient separation of amino acids in sake (11 nl). Operating conditions as in Fig. 4, except the sample. OH = Dansylic acid.

CONCLUSION

Dns-amino acids were satisfactorily separated within 30 min by micro HPLC in the reversed-phase mode. Both gradient and isocratic separations of Dns-amino acids were successful. The former mode improved solute resolution, while the latter mode will be of use in routine work. The detection limit of 0.07–0.3 pmol was obtained with a UV detector, and will be further improved with fluorometric detection. This system was applied to the analysis of amino acids in soy sauce and in sake.

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