Journal of Chromatography, 104 (1975) 359–365
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 7823

ANALYSIS OF Dns-AMINO ACIDS BY LIQUID CHROMATOGRAPHY

I. SELECTION OF OPTIMUM MOBILE PHASE COMPOSITION FOR SEPARATION OF Dns-AMINO ACIDS ON POLYVINYL ACETATE GEL

TAKEO YAMABE, NOBUHARU TAKAI and HIROSHI NAKAMURA
Institute of Industrial Science, University of Tokyo, Roppongi 7-chome, Minato-ku, Tokyo (Japan)
(First received May 13th, 1974; revised manuscript received July 9th, 1974)

SUMMARY

The separation of a mixture of ten Dns-amino acids (Dns-Gly, -Ala, -Val, -Leu, -Pro, -Hypro, -Met, -Ser, -Asn and -Gln) was carried out by liquid chromatography by using macroreticular polyvinyl acetate gel as a packing material. Different mobile phase systems were investigated, based mainly on mixtures of *n*-hexane with ethanol, methanol, chloroform, acetone, methyl ethyl ketone, ethyl acetate, dioxane and tetrahydrofuran. The solvent composition was fixed so as to elute all of the components of the sample mixture in a practical period of 2 h.

Satisfactory separation of the ten components was obtained with the *n*-hexane–ethanol (90:10) system. The presence of methanol as a modifier in the *n*-hexane was effective in reducing the elution time, but the separation was not as satisfactory. Chloroform or dioxane was useful only for the separation of Ser, Asn and Gln.

Acetone, methyl ethyl ketone, ethyl acetate and tetrahydrofuran were not suitable for practical separations of Dns-amino acids.

INTRODUCTION

The amino acid sequences of proteins and peptides are determined by the analysis of the amino end-group. The N-terminal amino acid can be modified with 1-fluoro-2,4-dinitrobenzene[1], phenylisothiocyanate[2] or 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl)[3]. The modified terminal amino acid is released by hydrolysis of the protein and can be identified by conventional methods. In many instances, however, proteins can be purified only in microgram amounts, and therefore a highly sensitive analytical method is necessary for the end-group determination. The Dns derivatives of amino acids have fluorescence spectra in the visible region with high quantum yields and can be detected by a fluorescence monitor. The merit of this method lies in its high sensitivity. A combination of the Dns derivatization with a stepwise degradation by the Edman procedure has been successfully applied to peptide sequence analysis[4,5]. Other workers applied the Dns derivatization

to the ultramicroanalysis of amino acids, which could not be detected by the ninhydrin method[6]. In these procedures, the Dns-amino acids are analyzed by thin-layer chromatography (TLC) on silica or polyamide gel, but the TLC method is troublesome and is not satisfactory for quantitative analysis. In view of the recent successful automation of the Edman technique[7], it has become desirable to improve the identification process for automation. High-performance liquid chromatography (LC) might be considered to be an appropriate technique for this purpose. However, suitable column packing materials have not been available. A column chromatographic separation of Dns-amino acids on a polyamide column has been reported by Deyl and Rosmus[8], but the procedure is not satisfactory for practical applications because of its long operation time.

In this paper, we describe the results of an investigation on packing materials and also an LC procedure for the analysis of Dns-amino acids.

EXPERIMENTAL

The column packing material was TSK-GEL-LS-140 (Toyo Soda Co., Tokyo, Japan), a macroreticular polyvinyl acetate gel (average diameter 10 μm), which was packed in a 50 cm $\times$ 3 mm I.D. glass column by the slurry technique. The slurry was prepared by dispersing the gel in acetone and was introduced into the slurry reservoir. Acetone was supplied by a KSD-88 piston-type pump (Kyowa Seimitsu, Tokyo, Japan), in order to force the slurry from the reservoir into the column.

The chromatographic system is shown in Fig. 1. The pump was a KSD-45 piston-type (Kyowa Seimitsu). The detector was an LDC Fluoro Monitor. A sample was injected on to the top of the column with a septum injector.

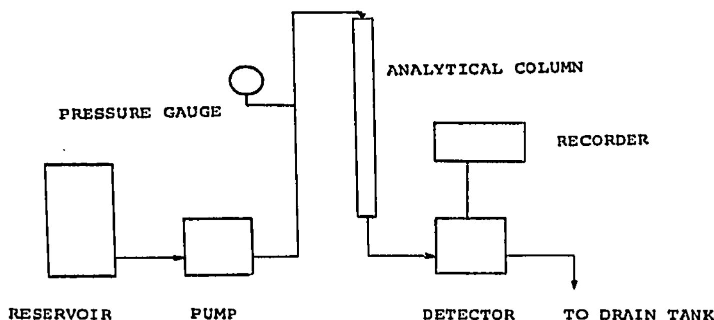


Fig. 1. Schematic diagram of chromatographic system.

All of the solvents used were of analytical-reagent grade, obtained from Wako, Osaka, Japan.

The sample solution of a mixture of Dns-amino acids was prepared by dissolving 5 mg each of Dns-Gly, -Ala, -Val, -Leu, -Pro, -Hypro, -Met, -Ser, -Asn and -Gln in 5 ml of methanol. Of these Dns-amino acids, Pro, Hypro, Ala, Met and Ser were piperidinium salts. The Dns-amino acids and solvents were used without further purification.

RESULTS AND DISCUSSION

We have previously reported the separation of amino acid derivatives by LC using polystyrene gel as the column packing material and methanol–water as the mobile phase[9]. The method gave a good separation of PTH-amino acids, but a poor separation of Dns-amino acids. Suitable packing materials must be selected for the practical separation of Dns-amino acids, and polyvinyl acetate gel may be considered to be an adequate packing material for this purpose. In this study, the separation of a mixture of ten Dns-amino acids was carried out by using polyvinyl acetate gel with various mobile phases in order to establish suitable mobile phase compositions. Ethanol, methanol, chloroform, acetone, methyl ethyl ketone, ethyl acetate, dioxane, tetrahydrofuran and *n*-hexane were employed as mobile phase components. Concentrations of 1% each of triethylamine and acetic acid were added to the mobile phases in order to eliminate peak tailing. It was known that peak tailing of carboxylic acids is eliminated by adding amines to the mobile phase and that of amines by adding acids.

With a single solvent (ethanol, methanol, chloroform, methyl ethyl ketone, acetone, ethyl acetate, dioxane or tetrahydrofuran), each of the ten components was eluted with the same elution time, whereas with *n*-hexane, none of the Dns-amino acids were eluted. Mixtures of *n*-hexane with the other solvents eluted the Dns-amino acids separatedly. The chromatographic behaviour was influenced by the components of the solvents and their concentrations. The solvent compositions were established so as to give adequate chromatograms within a practical period of time (2 h) by changing the composition in 10 vol.% steps. In Figs. 2–9, chromatograms of the sample solution obtained by various solvent systems are shown.

Fig. 2 shows the chromatogram obtained by using *n*-hexane–ethanol (90:10)

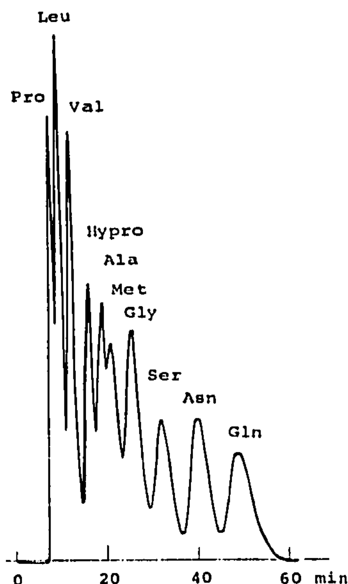
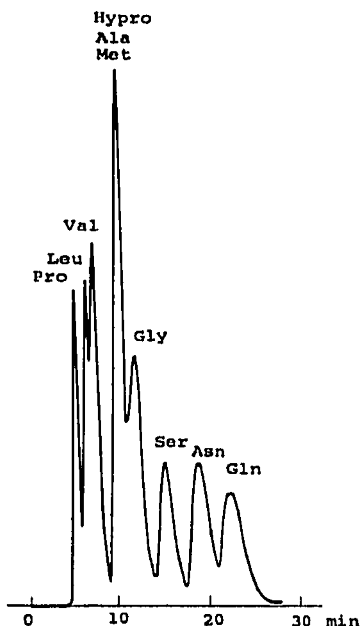


Fig. 2. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–ethanol–acetic acid–triethylamine (90:10:1:1). Flow-rate, 2.2 ml/min; pressure, 83 kg/cm^2.

as the mobile phase. In this case, Pro was eluted first, followed by Leu, Val, Hypro, Ala, Met, Gly, Ser, Asn and finally Gln. All ten components can be identified by their elution times.

In Fig. 3, the chromatogram obtained with *n*-hexane–methanol–chloroform (85:5:10) is shown. The Dns-amino acids were eluted in the same order as in Fig. 2, except for Hypro, Ala and Met, which appeared with the same elution time. However, the elution time was reduced without decreasing the resolution of the other components.

With *n*-hexane–chloroform (80:20), the Dns-amino acids containing polar groups, such as Hypro, Ser, Asn and Gln, were separated satisfactorily, but the separation of the other less polar Dns-amino acids was less satisfactory, as shown by Leu and Val and Ala and Met in Fig. 4. The peak of Hypro shifted after Gly and the elution order was partly different from that with *n*-hexane–ethanol (90:10) (Fig. 2).



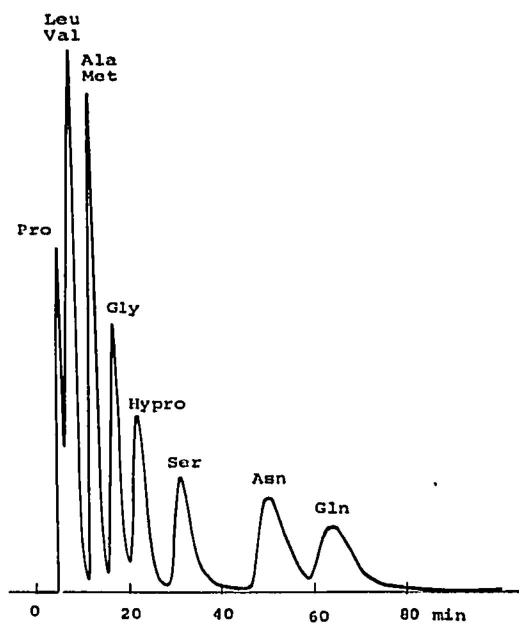
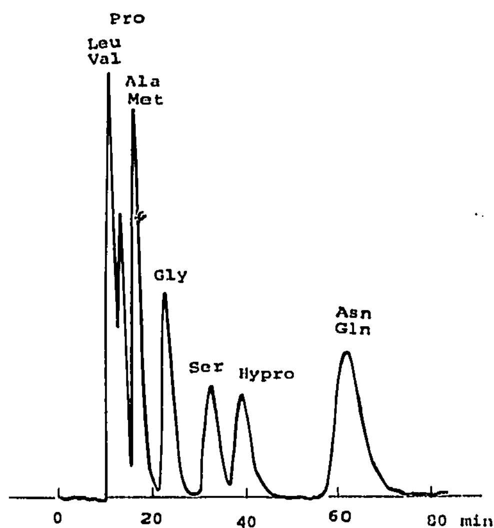


Fig. 3. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–methanol–chloroform–acetic acid–triethylamine (85:5:10:1:1). Flow-rate, 1.7 ml/min; pressure, 73 kg/cm^2.

Fig. 4. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–chloroform–acetic acid–triethylamine (80:20:1:1). Flow-rate, 1.4 ml/min; pressure, 68 kg/cm^2.

Fig. 5 shows the chromatogram obtained with *n*-hexane–acetone (80:20). Pro was eluted after Val and Hypro after Ser. The separation was not satisfactory, because Leu and Val, Ala and Met and Asn and Gln were not resolved.

The replacement of acetone with methyl ethyl ketone in the mobile phase gave a similar chromatographic behaviour (Fig. 6). Although the separation of Ser and Hypro was adequate, Asn and Gln could not be separated.



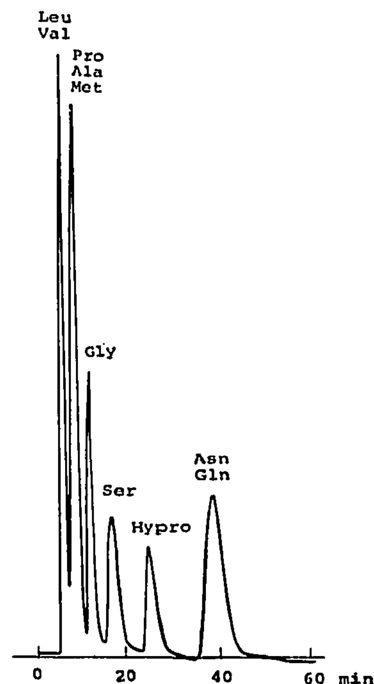


Fig. 5. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–acetone–acetic acid–triethylamine (80:20:1:1). Flow-rate, 1.1 ml/min; pressure 88 kg/cm².

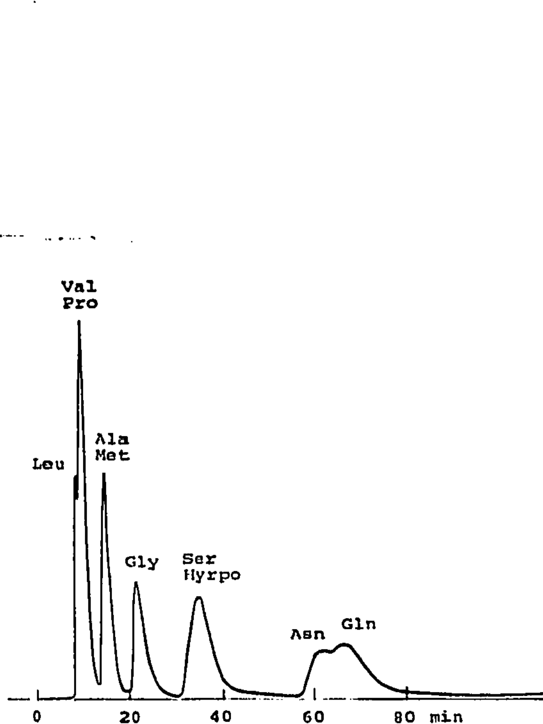
Fig. 6. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–methyl ethyl ketone–acetic acid–triethylamine (70:30:1:1). Flow-rate, 1.7 ml/min; pressure, 65 kg/cm².

With *n*-hexane–ethyl acetate (70:30), the peaks for almost all of the components overlapped (Fig. 7). By increasing the proportion of *n*-hexane in the mobile phase, the separation could be improved, but this caused an increase in elution time and was impractical.

Fig. 8 shows the chromatogram obtained with *n*-hexane–dioxane (70:30). The relatively less polar Dns-amino acids could not be separated from each other, but the polar compounds were separated satisfactorily. The chromatographic behaviour is similar to that with *n*-hexane–chloroform (80:20) (Fig. 4).

With *n*-hexane–tetrahydrofuran (70:30), the separation of Asn and Gln was not successful, even by prolonged elution with a change in the solvent composition, as shown in Fig. 9. This solvent system caused a large pressure drop in the column and a decrease in the flow-rate. Therefore, this solvent system is unsuitable for the separation of Dns-amino acids.

From the results described above, it can be concluded that polyvinyl acetate gel is a useful packing material for the separation of Dns-amino acids. The separation behaviour was dependent on the composition of the mobile phase. Of the solvent systems investigated, *n*-hexane–ethanol gave the best result. Methanol was found to be effective in reducing the elution time, as shown in Fig. 3, for example. Chloroform or dioxane was useful only for the separation of Ser, Asn and Gln. Acetone, methyl



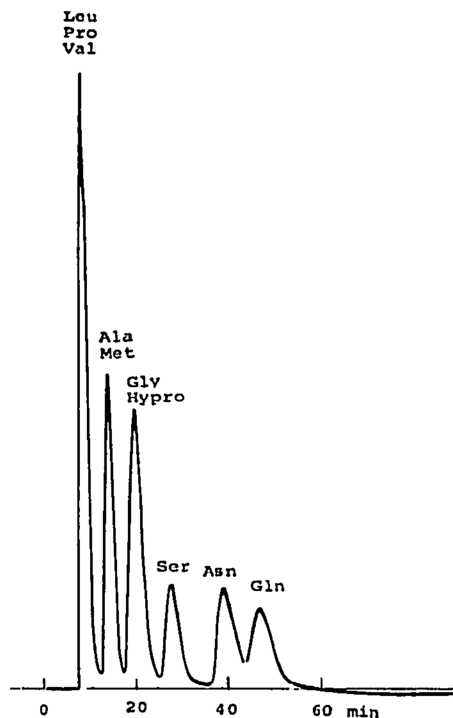


Fig. 7. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–ethyl acetate–acetic acid–triethylamine (70:30:1:1). Flow-rate, 1.6 ml/min; pressure, 65 kg/cm^2.

Fig. 8. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–dioxane–acetic acid–triethylamine (70:30:1:1). Flow-rate, 1.4 ml/min; pressure, 78 kg/cm^2.

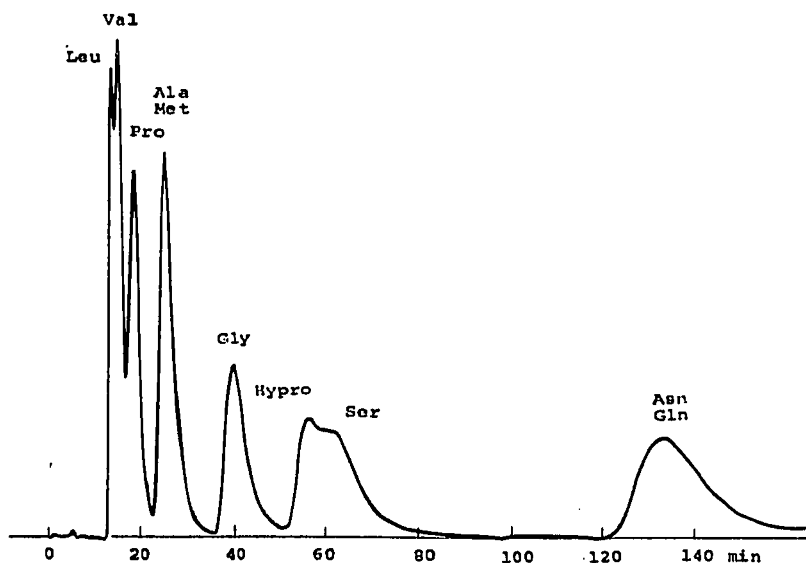


Fig. 9. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–tetrahydrofuran–acetic àcid–triethylamine (70:30:1:1). Flow-rate, 1.0 ml/min; pressure, 98 kg/cm^2.

ethyl ketone, ethyl acetate and tetrahydrofuran were not suitable for practical separation of Dns-amino acids.

The elution order of the Dns-amino acids, except Pro and Hypro, was not changed on changing the modifiers in *n*-hexane. The elution time of a series of Dns-amino acids, α-alkylamino acids, such as Leu, Val, Ala and Gly, is dependent on the bulkiness of the alkyl groups, and Leu was eluted first and Gly last. The relatively polar Dns-amino acids that contain –OH or $-CONH_2$ groups, such as Ser or Asn, required relatively long elution times. Such chromatographic behaviour resembles that of normal phase chromatography. Pro and Hypro showed unusual elution behaviour and the elution times were markedly influenced on changing the modifiers in *n*-hexane. With a polar modifier, such as methanol or ethanol, Pro was eluted first and Hypro after Val (Figs. 2, 3, 4 and 8), but with a less polar modifier, Pro was eluted after Val and Hypro after Gly or Ser (Figs. 5–7 and 9). This is considered to be due to a difference of interaction of pyrrolidine ring in Pro and Hypro with the solvent.

Incidentally, Pro, Hypro, Ala, Met and Ser were used as piperidinium salts. Of these, Pro and Hypro showed unusual chromatographic behaviour. This may imply that the unusual behaviour is not due to the difference in the form of the sample and that all ten Dns-amino acids exist in the form of triethylamine salts in the solvent system used.

ACKNOWLEDGEMENT

Several helpful discussions with Dr. S. Shiraishi of the Institute of Industrial Science, University of Tokyo, are gratefully acknowledged.

REFERENCES

1 F. Sanger, *Biochem. J.*, 39 (1945) 507.
2 P. Edman, *Arch. Biochem.*, 22 (1949) 475.
3 W. R. Gray, *Methods Enzymol.*, (1967) 139.
4 W. R. Gray and J. F. Smith, *Anal. Biochem.*, 33 (1970) 36.
5 A. M. Waner, P. Platt and K. Weber, *J. Biol. Chem.*, 247 (1972) 3242.
6 V. A. Spivak, V. M. Orlov, V. V. Shcherbukhin and Ya. M. Varshavsky, *Anal. Biochem.*, 35 (1970) 227.
7 P. Edman and G. Begg, *Eur. J. Biochem.*, 1 (1967) 80.
8 Z. Deyl and J. Rosmus, *J. Chromatogr.*, 69 (1972) 129.
9 T. Yamabe, H. Nakamura and N. Takai, *Seisan-Kenkyu*, 26 (1974) 158.