Journal of Chromatography, 213 (1981) 99–103
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,854

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DIPHENYLINDENONYLTHIOHYDANTOIN DERIVATIVES OF AMINO ACIDS

I. N. MANCHEVA*
Institute of Chemical Technology, Department of Organic Chemistry, 1156 Sofia (Bulgaria)
R. N. NIKOLOV
Institute of Chemical Technology, Central Research Laboratory, 1156 Sofia (Bulgaria)
and
J. PFLETSCHINGER*
Max-Planck-Institute of Biochemistry, Department of Protein Chemistry, 8033 Martinsried, München (G.F.R.)
(First received February 11th, 1981; revised manuscript received April 6th, 1981)

SUMMARY

Conditions are proposed for high-performance liquid chromatographic elution of diphenylindenonylthiohydantoin amino acids in connection with the sequence determination of proteins with diphenylindenonyl isothiocyanate. A LiChrosorb RP-18 column was used for separations at ambient and at elevated temperature. The results obtained are compared with the published separations of the same derivatives.

INTRODUCTION

2-*p*-Isothiocyanophenyl-3-phenylindenone or diphenylindenonyl isothiocyanate (DIITC) was synthesized by Ivanov *et al.*[1] and proposed as a coloured reagent for sequencing proteins and peptides[2]. Using this reagent, an N-terminal amino acid was identified on a thin layer of silica gel G as a coloured (diphenyl)indenonylthiohydantoin (ITH) derivative[3] or as a fluorescent isobenzofuran derivative after additional treatment with sodium ethoxide[4].

The purpose of the present paper is to determine the conditions for separation and identification of these derivatives by high-performance liquid chromatography (HPLC), which could be used in routine sequence analysis of peptides and proteins with DIITC. During our investigations another communication[5] on HPLC separation of ITH-amino acids appeared, and these results will be compared with those reported here.

* Present address: Ciba-Geigy GmbH, D-7867 Wehr/Baden, G.F.R.

0021-9673/81/0000–0000/$02.50 © 1981 Elsevier Scientific Publishing Company

EXPERIMENTAL

Methods and reagents

A series 2/2 liquid chromatograph combined with an LC-55 UV–VIS variable-wavelength detector (both from Perkin-Elmer, Norwalk, CT, U.S.A.) and a Honeywell Model 194 1-mV recorder was employed. A pre-packed column (25 × 0.46 cm I.D.) with 10-μm LiChrosorb RP-18 (Knauer, Berlin, G.F.R.) was used for separations.

The solvents acetonitrile and ethyl acetate were UV grade (LiChrosolv; E. Merck, Darmstadt, G.F.R.). Buffer was prepared with reagent grade sodium acetate, adjusted to ±0.02 pH units and filtered through a 0.2-μm Millipore filter. Water was deionized and doubly distilled.

Preparation of ITH derivatives

ITH-amino acids were synthesized, purified and characterized as described earlier[6,7]. These compounds can be obtained in micromole amounts in the following way. To a solution of 3 μmole amino acid in 0.3 ml of 0.4 *M* dimethylallylamine buffer (pH 9.6) were added 0.3 ml of a pyridine solution of 3.39 mg (10 μmole) of DIITC. The reaction was carried out at 40°C under a nitrogen atmosphere. After 2 h the solution was evaporated to dryness in vacuum over phosphorus pentoxide and potassium hydroxide. The residue was dissolved in 0.2 ml of acetic acid–6 *M* hydrochloric acid (5:1 v/v) and the solution was warmed at 80°C for 10 min under nitrogen. The solvents were evaporated over potassium hydroxide.

HPLC

Standard solutions of 21 ITH-amino acids (50 pmole/μl) in methanol–ethyl acetate (1:1) were prepared. By mixing aliquots of these solutions, evaporation and dissolution in methanol, a standard mixture containing 5 pmole/μl of each ITH derivative was prepared. Samples (10 μl) were introduced through a Rheodyne injector into the column. The separation was performed at ambient temperature (24°C) and constant flow velocity (2 ml/min). The mobile phase was a mixture of 0.01 *M* aqueous sodium acetate (pH 4.80) and acetonitrile, in which the proportion of acetonitrile was linearly increased from 44 to 94% at a rate of 2% per min. Separated ITH-amino acids were detected at 267 nm, corresponding to their maximum absorbance in the UV-range.

RESULTS AND DISCUSSION

According to literature data, the best separation of PTH (3-phenyl-2-thiohydantoin)-amino acids was achieved by Zimmerman *et al.*[8] on a Zorbax-ODS column at 62°C, using acetonitrile and 0.01 *M* sodium acetate (pH 4.5) as solvents. Under the conditions described above, all twenty PTHs are separated in about 20 min.

First we tried to separate ITH-amino acids at ambient temperature, using acetonitrile and sodium acetate buffer at different molarities (0.005–0.02 *M*) and different pHs (4.0–6.0) as eluents. The best results were achieved on the LiChrosorb RP-18 column with 0.01 *M* sodium acetate (pH 4.80) and gradient elution from 44 to

94% acetonitrile at 2% per min, the flow-rate being 2 ml/min. The RP-18 (10 μm) column packing was preferred because of its availability, relatively acceptable price and greater lifetime than Zorbax-ODS packing.

As shown in Fig. 1, a standard mixture containing 21 ITH-amino acids gives 19 peaks, the analysis being completed in 28 min. ITH-Arg is the last to be eluted with 89% acetonitrile, because of its high affinity to the column packing. Two pairs of ITHs, Met/Trp and Leu/Ile, are not separated under these conditions.

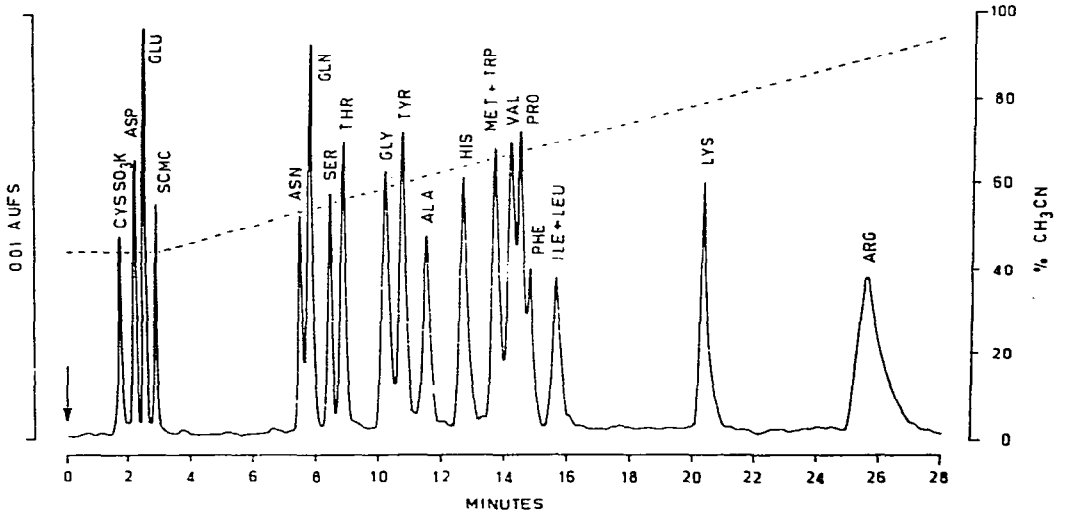

Fig. 1. Gradient elution of ITH-amino acids on a Knauer LiChrosorb RP-18 column (25 × 0.46 cm). Solvents: A = acetonitrile; B = 0.01 *N* sodium acetate (pH 4.80). Gradient of A from 44 to 94% at 2% per min, flow-rate 2.0 ml/min. Temperature: 24°C. Sample size: 10 μl, containing 50 pmole of each ITH. Detection: UV absorbance at 267 nm. SCMC = S-carboxymethylcysteine.

An additional separation of ITH-Met and ITH-Trp can be achieved by isocratic elution with acetonitrile, containing 22% ethyl acetate. The concentration of the combined organic solvent is 44% with respect to the mixture of organic solvent and sodium acetate buffer (pH 4.80) (Fig. 2). It is seen that at ambient temperature the two pairs of ITHs Met/Trp and Leu/Ile were separated simultaneously without prolonging the analysis time. The retention times of these derivatives are comparable for both separations discussed. The addition of ethyl acetate to the organic solvent is the important factor. Because of the lower polarity of the ethyl acetate, its presence in the eluent provides a more significant decrease of the hydrophobic interactions between the ITH derivatives and the alkyl ligands of the reversed phase. A necessary precondition for a successful separation of ITH-Met and ITH-Trp is the absence of the amino acids proline and valine. These give overlapping peaks between those of ITH-Met and ITH-Trp. Because of the considerable width of all these peaks, they were coeluted as a single peak.

An acceptable separation of ITH derivatives can be obtained also at elevated temperature with isocratic elution. Under these conditions the greater solubility differences between the ITHs in the eluent lead to a better resolution of ITHs (Asn/Gln) and (Tyr/Ala/His) as shown in Fig. 3. This separation was performed with a Hewlett-

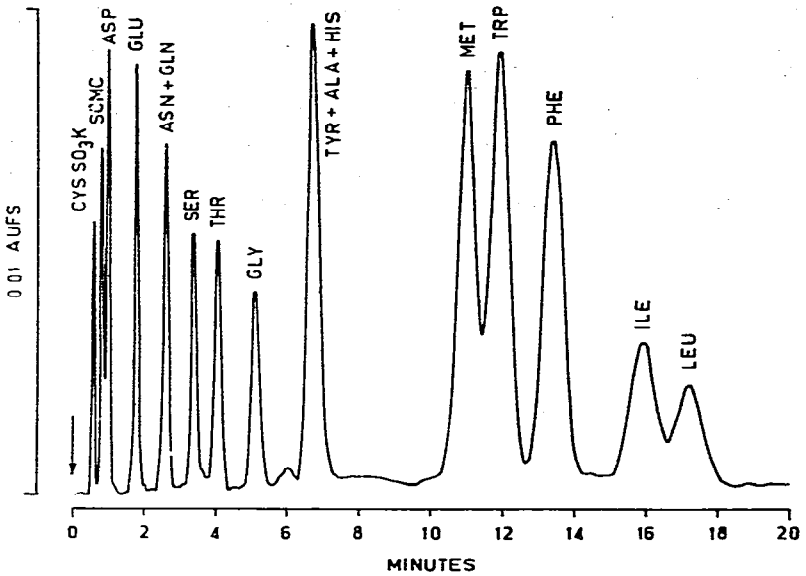

Fig. 2. Isocratic elution of ITH-amino acids. Solvents: A = acetonitrile–ethyl acetate (78:22); B = 0.01 *N* sodium acetate (pH 4.80). Elution with A:B (44:56), flow-rate 4 ml/min. Other conditions as in Fig. 1.

Packard Model 1084 B liquid chromatograph at increased temperature (62°C). The eluent was acetonitrile–ethyl acetate (85:15) + 0.01 *N* sodium acetate buffer (48:52).

Under the conditions for HPLC separation of twenty ITH-amino acids (without ITH-Cys-SO_3H) reported by Nasimov *et al.*[5], three pairs of ITH derivatives, Tyr/His, Arg/Met and Leu/Ile, remain unresolved. Moreover, we consider as incorrect the position of ITH-Arg, in Fig. 1 of that paper. It is an artifact of the same derivative. As established by us, ITH-Arg appears last in a chromatogram with gradient elution using 89% acetonitrile. On the other hand, Nasimov *et al.*[5] affirmed, that after conversion under conditions described for PTH[9], it is convenient to identify ITH-His and ITH-Arg separately by HPLC analysis of the water layer after extraction of the other ITH-amino acids with ethyl acetate. It was found in previous work[2,6,7] that, for complete cyclization of more hydrophobic (diphenyl)-indenonylthiocarbamoyl (ITC)-amino acids to ITH derivatives, acetic acid–6 *M* HCl (5:1) is more suitable than 1 *M* HCl usually used for PTHs[9]. After cyclization under these conditions, all the ITH-amino acids, including ITH-Arg, -His and -Cys-SO_3H, are extractable with ethyl acetate and do not remain in the water layer. Moreover, ITC-Arg, -His, ITH-Arg and -His have a fairly low solubility in 1 *M* HCl even at 80°C. They are more soluble in organic solvents and have to be detected in the organic layer together with the other ITH derivatives. This is the reason for the importance of the separation of the ITH pairs.

In the present work the conditions for HPLC elution of the ITH-amino acids allow the separation of nearly all derivatives. Only the separation of ITH-Met and ITH-Trp was limited.

ITH-amino acids were detected under UV light at 267 nm. For most ITH derivatives the molar extinction coefficient at 265–268 nm is *ca.* 50,000 or approximately three times higher than for PTH derivatives ($\approx$17,000)[2]. Under identical

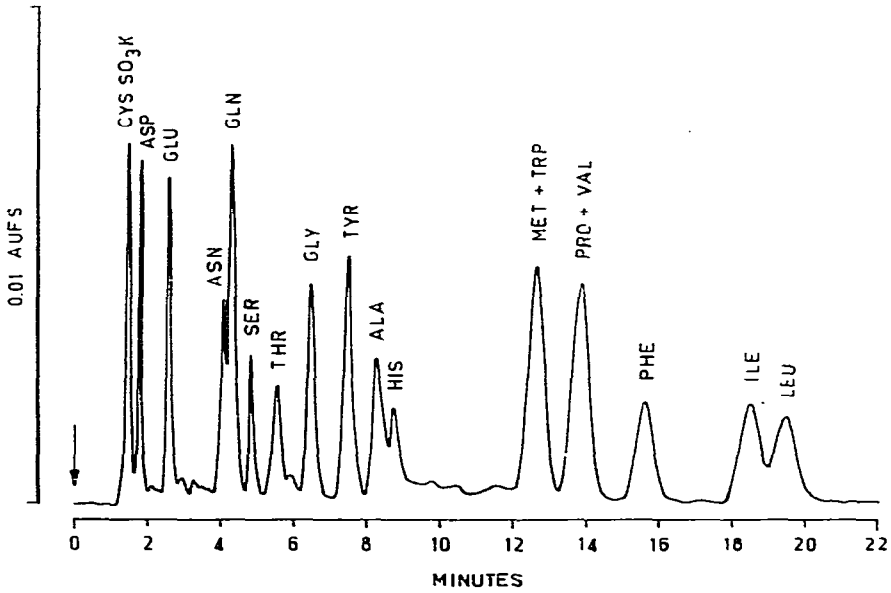

Fig. 3. Isocratic elution of ITH-amino acids. Solvents: A = acetonitrile–ethyl acetate (85:15); B = 0.01 *N* sodium acetate (pH 4.80). Elution with A:B (48:52), flow-rate 2 ml/min. Temperature: 62°C. Other conditions as in Fig. 1.

conditions, this difference permits a higher sensitivity for detection of ITH derivatives by HPLC. 2–5 pmole amounts of ITH-amino acids can be reliably detected. This indicates that the sensitivity of UV detection of the ITH derivatives is 5–8 times higher than fluorescent detection (10–40 pmole) of the same derivatives after separation by thin-layer chromatography on silica gel G[4].

ACKNOWLEDGEMENTS

The authors are indebted to Professor Dr. G. Braunitzer, Max-Planck-Institute of Biochemistry, Martinsried, Munich, G.F.R., for providing the Knauer columns. The helpful discussion with Professor Dr. Ch. P. Ivanov, Institute of Chemical Technology, Sofia, Bulgaria, is gratefully acknowledged.

REFERENCES

1 Ch. P. Ivanov, I. N. Mancheva and S. Gaitandjiev, *C.R. Acad. Bulg. Sci.*, 20 (1967) 799.
2 Ch. P. Ivanov and I. N. Mancheva, *Anal. Biochem.*, 53 (1973) 420.
3 Ch. P. Ivanov and I. N. Mancheva, *J. Chromatogr.*, 75 (1973) 129.
4 Ch. P. Ivanov and I. N. Mancheva, *C.R. Acad. Bulg. Sci.*, 28 (1975) 1399.
5 I. V. Nasimov, N. B. Levina, V. V. Schemyakin, B. V. Rosinov, I. A. Bogdanova and V. G. Merimson, in Chr. Birr (Editor), *Methods in Peptide and Protein Sequence Analysis*, Elsevier North-Holland Biomedical Press, Amsterdam, 1980, p. 475.
6 Ch. P. Ivanov and I. N. Mancheva, *C.R. Acad. Bulg. Sci.*, 21 (1968) 785.
7 Ch. P. Ivanov and I. N. Mancheva, *C.R. Acad. Bulg. Sci.*, 25 (1972) 349.
8 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569.
9 P. Edman and G. Begg, *Eur. J. Biochem.*, 1 (1967) 80.