Journal of Chromatography, 270 (1983) 371–377
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,111

Note

Separation of phenylthiohydantoin–amino acids by high-performance liquid chromatography

PIETRO PUCCI*, GIOVANNI SANNIA and GENNARO MARINO
Istituto di Chimica Organica e Biologica, Università di Napoli, via Mezzocannone 16, I-80134 Naples (Italy)
(Received June 27th, 1983)

Since the pioneering work of Zimmerman *et al.*[1], reversed-phase high-performance liquid chromatography (HPLC) has been the most commonly applied method for the identification of phenylthiohydantoin (PTH) amino acids.

The following essential features are required for PTH–amino acids analysis: (1) high resolution; (2) short analysis time; (3) reproducibility. The last factor is, in our opinion, the most important one; limited reproducibility of peak retention time does not allow a confident assignment of the amino acid residue. Variation in retention time is essentially related to complex elution systems and/or to the detrimental effect of high-temperature procedures on the lifetime of the column.

Several simple HPLC procedures, using isocratic or binary gradient elution, have been reported[2–7] but the majority suffer either from long analysis times or the use of high temperatures during separation. Here we report a fast procedure (14 min) for the separation of PTH–amino acids by HPLC; the elution is performed with a binary system at a relatively low temperature (35°C). Hundreds of analyses have been performed with an extremely high reproducibility. Examples of PTH analysis from an automated sequencer run of pancreatic bovine ribonuclease (15 nmoles) are also given.

MATERIALS AND METHODS

HPLC analyses were performed on a Beckman Model 322 liquid chromatograph equipped with two high-pressure Model 110 pumps, a Model 210 sample injection valve (10-μl sample loop), a Model 153 UV detector set at 254 nm, a data processor recorder/integrator Chromatopac C-R1A and a Model 420 system controller programmer. The column was an Altex Ultrasphere ODS (5 μm, 4.6 × 250 mm), jacketed and held at constant temperature by means of a water-circulating bath.

The elution solvents comprised 10 m*M* sodium acetate buffer pH 4.85 (A) and acetonitrile (B). The sodium acetate buffer was adjusted to pH 4.85 (20°C) with dilute acetic acid and degassed under vacuum immediately before use. The flow-rate used was 1.5 ml/min and the temperature was 35°C. Elution was accomplished by the scheme shown in Table I.

Standard PTH–amino acids (Sigma, St. Louis, MO, U.S.A.) were dissolved in

0021-9673/83/$03.00

acetonitrile and stored at −20°C; solutions were found to be stable for months.

Sequence analysis was performed on a Beckman Model 890 C Sequencer modified with a cold-trap, using a double-coupling, single-cleavage, 1.0 *M* Quadrol program. The sequencer cell was first loaded with about 3 mg of Polybrene (Pierce). After six complete cycles, the protein sample was loaded (15 nmol of pancreatic bovine ribonuclease reduced and carboxymethylated) and submitted to a complete sequence cycle without heptafluoro-*n*-butyric acid.

Anilinothiazolinone amino acids recovered from the sequencer were dried in a Savant Speed-Vac concentrator and converted into PTH-amino acids in 1 *M* hydrochloric acid containing ethanethiol (2%) at 80°C for 10 min. The samples were evaporated to dryness, washed twice with 0.1 ml acetonitrile, dried and dissolved in various amounts of acetonitrile 10 m*M* sodium acetate buffer pH 4.85 (75:25, v/v). Aliquots containing about 0.5 nmoles of the PTH derivative were injected onto the column.

All reagents and solvents used in sequencer experiments were Sequanal grade from Beckman (Geneva). Acetonitrile was HPLC grade from Baker (Phillipsburg). Water was deionized and twice distilled from glass. All other reagents were Baker analyzed reagents.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of eighteen standard PTH-amino acids obtained

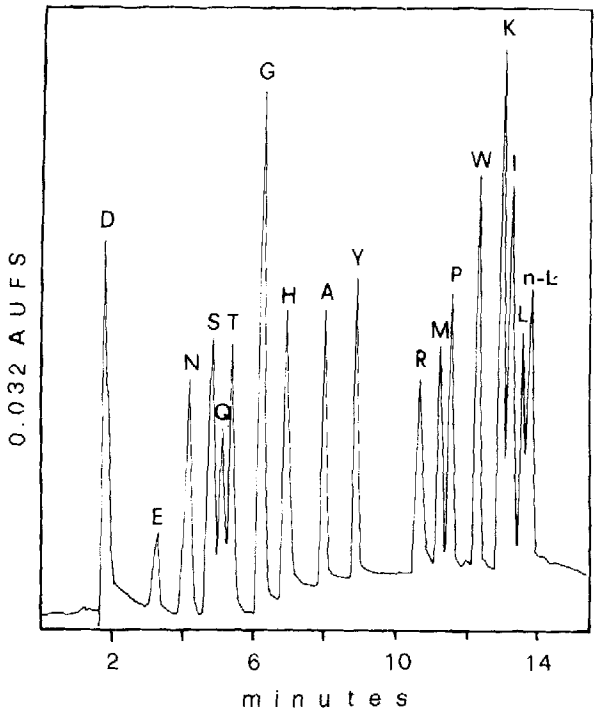

Fig. 1. Separation of standard PTH-amino acids by HPLC on a 25 × 0.46 cm Ultrasphere ODS (5-μm) column. Elution solvents: 10 m*M* sodium acetate buffer pH 4.85 (A) and acetonitrile (B). The gradient was as shown in Table I. Flow-rate: 1.5 ml/min. Temperature: 35°C. Sample size: 0.3 nmol of each PTH derivative. The peaks are labelled by one-letter abbreviations for the amino acids.

TABLE I

GRADIENT PROTOCOL FOR THE ELUTION OF PTH-AMINO ACIDS

Time (min)	*% B*	*Duration (min)*
0	26	1
1	32	0.5
1.5	54	7.5
14	90	2

under the conditions described in Materials and methods by using a gradient elution (Table I).

Attempts to obtain separation conditions as simple as possible led us to make the following choices. The octadecylsilane (ODS) type of column was chosen because such columns are available from many manufacturers and are easily standardizable. Acetonitrile was chosen because it is cheaper, gives a lower back pressure and has a lower heat of mixing than methanol and it does not need to be distilled, as does

TABLE II

RETENTION TIMES OF STANDARD PTH-AMINO ACIDS IN ORDER OF ELUTION

Values are the means from at least ten injections. Separation conditions as in Fig. 1.

Compound	*Mean retention time (min)*	*S.D. (min)*
PTH-Cys SO_3^-	1.44	0.02
PTH-Asp	1.75	0.01
PTH-Glu	3.22	0.03
PTH-Asn	4.10	0.03
PTH-Ser	4.74	0.03
PTH-Glu	5.02	0.03
PTH-Thr	5.26	0.02
PTH-Gly	6.09	0.03
PTH-Ser*	6.46	0.02
PTH-His	6.76	0.03
PTH-Ala	7.82	0.03
PTH-Tyr	8.71	0.03
PTH-Thr*	9.64	0.02
PTH-Arg	10.44	0.03
PTH-Met	11.01	0.02
PTH-Val	11.12	0.02
PTH-Pro	11.32	0.02
PTH-Ser*	11.40	0.02
PTH-Trp	12.08	0.03
PTH-Phe	12.65	0.02
PTH-Lys	12.71	0.03
PTH-Ile	12.95	0.03
PTH-Ser*	13.21	0.02
PTH-Leu	13.31	0.02
PTH-Nle	13.54	0.02

* By-product from the degradation of the PTH-amino acid; for discussion see text.

tetrahydrofuran. As hydrophilic eluent, a sodium acetate buffer was preferred because this salt is available in high purity and can easily be weighed.

The pH value chosen allows the best separation of the two pairs PTH-His/PTH-Gly and PTH-Arg/PTH-Met using the initial and final isocratic eluent compositions described below.

The initial isocratic step, 26% acetonitrile, was chosen to allow the separation of the triplet PTH-Gln, PTH-Ser and PTH-Thr; the final one, 54% acetonitrile, allows the separation of the pairs PTH-Lys/PTH-Ile and PTH-Leu/PTH-Nle. The gradient in between allows the separation of PTH-Gly, PTH-Ala, PTH-Tyr, PTH-Arg, PTH-Met and PTH-Pro.

The temperature of 35°C is the lowest which allows the separation of PTH-Arg/PTH-Met. However, the retention times of PTH-Met and PTH-Val as well as of PTH-Lys and PTH-Phe are too similar (see Table II) to enable their separation. Although eluent modification and/or temperature increase improves the separation, we prefer to leave the conditions as simple as possible and to make no further attempts to obtain a full separation of these two pairs. These derivatives can then be separated in 6 min using a single isocratic elution step, 50% acetonitrile–10 m*M* sodium acetate buffer pH 4.85, at the same temperature (Fig. 2).

Regarding the identification of side products arising from the conversion of serine- and threonine-thiazolinones into the corresponding phenylthiohydantoins[8], we carried out the following experiment. Samples of serine and threonine were sub-

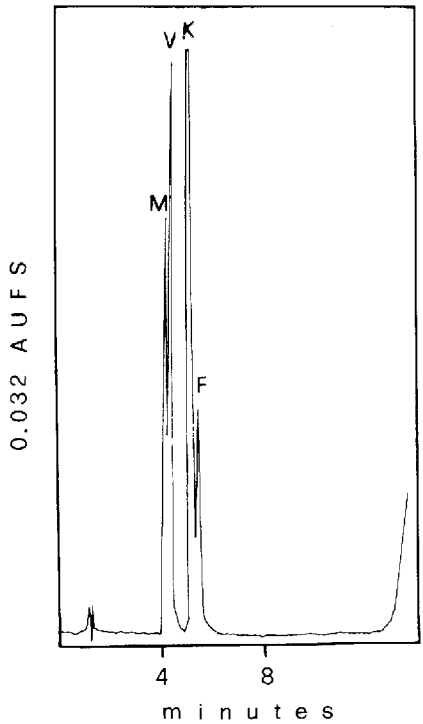

Fig. 2. Separation of PTH-Met, PTH-Val, PTH-Lys and PTH-Phe by HPLC under the conditions described in Fig. 1, except for isocratic elution performed with 50% solvent B.

mitted to a single sequencer cycle and to a conversion step; the dried products, after washing with acetonitrile, were dissolved in the starting elution buffer and analyzed. Each hydroxy-amino acid gave rise to a characteristic set of peaks together with the unmodified phenylthiohydantoins. These peaks appear fully separated from the other PTH-amino acids as shown in Table II and Fig. 3.

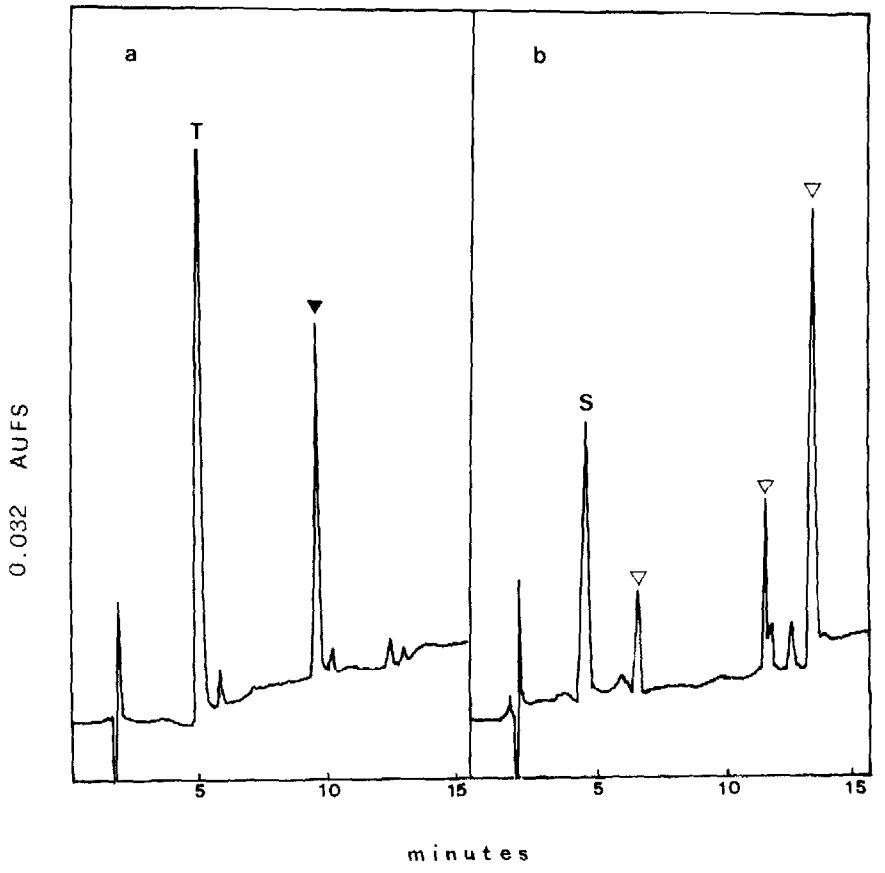

Fig. 3. Separation of degradation products present when samples of threonine (a, ▼) and serine (b, ▽) were submitted to an automatic Edman degradation cycle followed by a conversion step, and then directly injected onto the column. Conditions as in Fig. 1. The relative intensities of the peaks are dependent on the length of time before conversion.

The average elution times of the PTH-amino acid standards and the serine and threonine by-products are reported in Table II. The values are the means from at least ten injections made at two- or three-week intervals.

To illustrate the application of the procedure, a sequence analysis was performed on 15 nmoles of reduced and carboxymethylated bovine ribonuclease. The HPLC analyses of four cycles (1, 7, 19 and 20) are illustrated in Fig. 4, 1%–10% of each cycle was injected onto the column. Although several background peaks are present, the amino acid residues are easily identified on the basis of their accurately reproducible retention times.

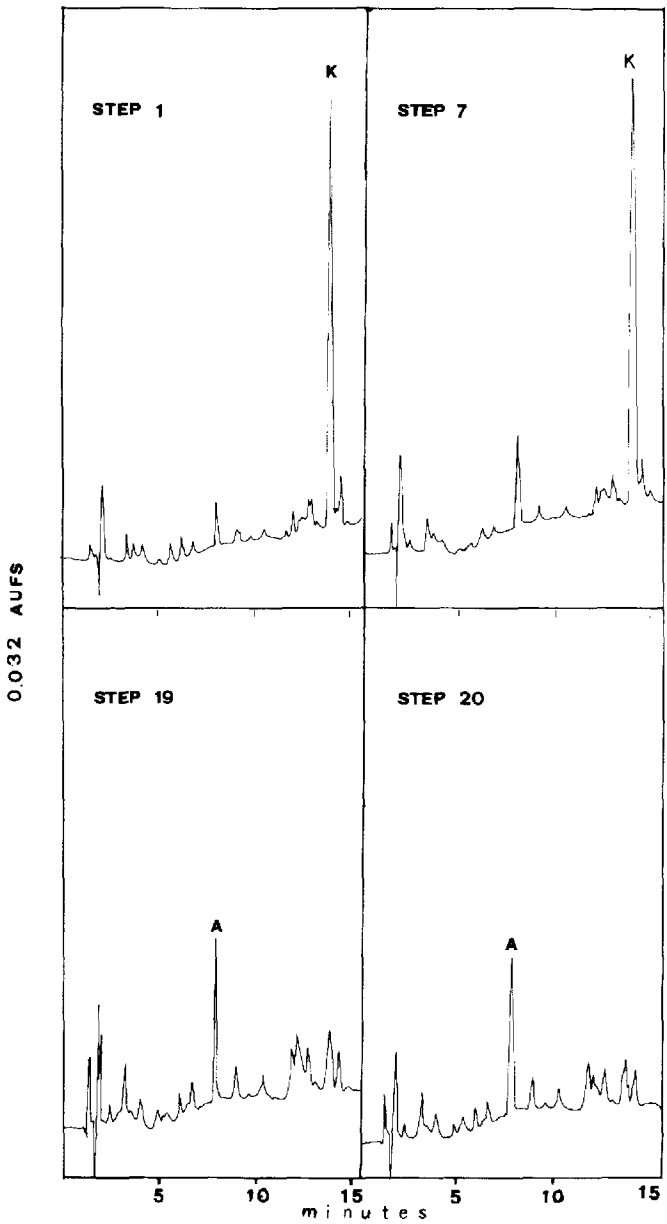

Fig. 4. Selected steps from the automated Edman degradation of RNAase A. After the conversion step, the residue was taken up in 1 ml of acetonitrile 10 m*M* sodium acetate buffer pH 4.85 (75:25, v/v). An amount from 1% to 10% was injected onto the column. HPLC conditions as in Fig. 1.

CONCLUSIONS

A very simple procedure for the separation of PTH–amino acids by HPLC is illustrated. The procedure is relatively fast (14 min) and uses a relatively low tem-

perature, thus allowing longer column lifetimes. The results are highly reproducible, the observed variation in retention time being at most ± 0.03 min. Peaks arising at the PTH-Met/PTH-Val and/or PTH-Lys/PTH-Phe positions may be simply resolved as necessary by a further 6-min procedure.

The practical application of the procedure is shown by the sequence analysis of bovine pancreatic ribonuclease. Peaks of PTH-amino acids are well separated from background peaks and a high signal-to-noise ratio is achieved in the chromatogram of 10% of the sample at the twentieth cycle of a 15-nmol analysis.

ACKNOWLEDGEMENTS

We are indebted to Mr. Fulvio Gargiulo for technical assistance. This work is part of a project on the levels of Structural Organization in Proteins, financially supported by the Italian Ministry of Education (MPI). The sequencer and HPLC equipment belong to the Protein Structure Service of Centro di Endocrinologia Sperimentale, CNR (Naples, Italy).

REFERENCES

1 C. L. Zimmerman, E. Apella and J. J. Pisano, *Anal. Biochem.*, 77 (1977) 569–573.
2 A. S. Bhown, J. E. Mole, A. Weissinger and J. C. Bennet, *J. Chromatogr.*, 148 (1978) 532–535.
3 M. Abrahamsson, K. Gröningsson and S. Castensson, *J. Chromatogr.*, 154 (1978) 313–317.
4 P. W. Moser and E. E. Rickli, *J. Chromatogr.*, 176 (1979) 451–455.
5 N. D. Johnson, M. W. Hunkapiller and L. E. Hood, *Anal. Biochem.*, 100 (1979) 335–338.
6 G. E. Tarr, *Anal. Biochem.*, 111 (1981) 27–32.
7 D. Hawke, Y. Pau-Miau and J. E. Shively, *Anal. Biochem.*, 120 (1982) 302–311.
8 M. Fraenkel-Contrat, J. Morris and A. L. Levy, in D. Glick (Editor), *Methods in Biochemical Analysis*, Vol. 2, Interscience, New York, 1955, pp. 359–425.