Journal of Chromatography, 487 (1989) 161–166
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4510

Note

Isocratic separation of phenylthiohydantoin-amino acids by reversed-phase high-performance liquid chromatography

KOU HAYAKAWA* and JUN OIZUMI

Division of Metabolism, National Children's Medical Research Centre, 3-35-31 Taishido, Setagaya-ku, Tokyo 154 (Japan)

(First received July 5th, 1988; revised manuscript received September 27th, 1988)

A critical aspect of several protein sequencing techniques is the sensitivity of the determination and amino acid analyses after Edman degradation, i.e., the determination of phenylthiohydantoin (PTH)-amino acids [1]. Reversed-phase high-performance liquid chromatography (HPLC) has been used for this purpose previously [2–4]. However, as indicated by Tsunasawa et al. [5], HPLC methods are not satisfactory for the determination of picomole amounts owing to the limited reproducibility, sensitivity and resolution. Gradient elution methods [2, 4] are inconvenient and require relatively complex chromatographic systems compared with isocratic techniques. Reported isocratic elution systems [5] require precise sodium lauryl sulphate and acetonitrile concentrations, and the columns need occasional re-conditioning in order to achieve full separation of all PTH-amino acids.

We have developed a convenient isocratic system that permits the simultaneous separation of eighteen PTH-amino acids. Only the PTH-Met and PTH-Val peaks overlap, but these compounds can be separated either by modification of the solvent mixture or by increasing the column temperature.

EXPERIMENTAL

Chemicals and reagents

Trifluoroacetic acid (TFA, amino acid sequencing grade), methanol (liquid chromatography grade), acetonitrile (liquid chromatography grade) and standard PTH-amino acids were obtained from Wako (Osaka, Japan). PTH-Gln and PTH-Thr were dissolved in methanol at a concentration of 200 nmol/ml (stock

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solution) and stored at −80 °C; all other PTH-amino acids were stored at −20 °C. Immediately before use, the PTH-amino acids were mixed to give a solution containing 10 nmol/ml (working standard solutions). Usually samples of 3 μl (30 pmol) were injected.

Cysteine hydrochloride and 4-vinylpyridine were obtained from Nacalai Tesque (Kyoto, Japan), dithiothreitol and substance P from Boehringer (Mannheim, F.R.G.), 2-mercaptoethanol from Kanto Chemical (Tokyo, Japan) and hen egg-white avidin D from Vector Labs. (Burlingame, CA, U.S.A.). PTH-pyridylethylcysteine (PTH-PeC) was synthesized from cysteine hydrochloride and 4-vinylpyridine according to the method described by Friedman et al. [6].

Instruments

The HPLC apparatus consisted of a Hitachi (Tokyo, Japan) 655 pump with a Rheodyne 7125 sample injector (20-μl loop). For detection, a Hitachi Model 655A UV spectrophotometric detector was used at 269 nm; the data processor was a Hitachi Model 655-61.

The column was a Nucleosil 5-C_{18} (250 mm × 4.6 mm I.D.) (Macherey-Nagel, Düren, F.R.G.; Chemco, Osaka, Japan). The guard column (10 mm × 4.0 mm I.D.) was packed with Develosil ODS (Nomura Chemical, Seto, Aichi, Japan).

PTH-amino acid analysis

Eighteen amino acids were separated isocratically with acetonitrile–water (40:60) containing 0.1% TFA at a flow-rate of 1.0 ml/min. Degassing was not necessary. The column temperature was 23 °C and the column inlet pressure was approximately at 105 kg/cm^2. When necessary, a column oven (Hitachi 655A-52) was used.

Edman degradation of real samples

An amount of 1 nmol of hen egg-white avidin D (M_r = 68 000; biotin-binding glycoprotein composed of four subunits of M_r = 17 000) was processed for pyridylethylation by the method of Friedman et al. [6] and used as a real sample. The N-terminal amino acid sequence of avidin was known to be NH_2-Ala-Arg-Lys-Cys-Ser-. A 1-nmol amount of peptide substance P (M_r = 1348; N-terminal amino acid = Arg) was also used as a real sample. Manual Edman degradation was carried out essentially according to the method described by Tarr [7].

RESULTS AND DISCUSSION

We selected a commercially available fully end-capped Nucleosil 5-C_{18} column because of our previous findings [8]. This ODS gel matrix was found to work well at pH 2.0 and gave a good recovery and reproducibility in protein analyses of ovalbumin. We chose 0.1% TFA as the acidic modifier and acetonitrile was used because of its low viscosity.

Acetonitrile–water (40:60) containing 0.1% TFA was found to be the optimal element for the separation of eighteen PTH-amino acids in a single analysis. A

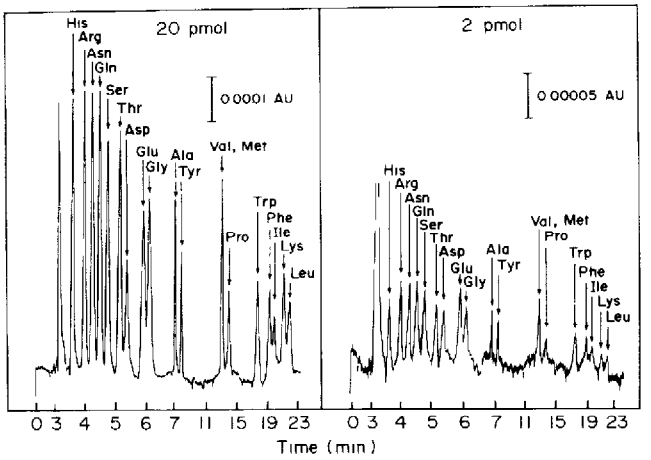

Fig. 1. Separation of eighteen PTH-amino acids. HPLC conditions as described under Experimental. Left, 20 pmol; right, 2 pmol. Eluent: acetonitrile–water (40 60) containing 0 1% TFA.

TABLE I

WITHIN-DAY REPRODUCIBILITY OF PTH-AMINO ACID ANALYSIS

Of each amino acid 30 pmol (3 μl) were injected PeC was determined separately according to Friedman et al. [6]. The yield of PeC synthesis was assumed to be 100%.

PTH-amino acid	Mean peak area	C.V. (%)	Mean peak height	C.V. (%)
His	23 019	6.9	2965	6.7
Arg	54 310	3.3	6213	8.0
Asn	36 552	3.1	4450	8.6
PeC	13 505	3.6	2080	2.6
Gln	25 458	6.7	4642	8.2
Ser	34 760	5.2	3008	9 1
Thr	19 984	22.0	1528	8.6
Asp	31 567	11.1	3890	6 7
Glu	19 152	4.9	2352	4.2
Gly	41 130	3.6	4782	5.7
Ala	44 970	4.0	3736	8.1
Tyr	55 360	5.3	3669	6.3
Pro	60 134	4.0	2368	6.1
Trp	73 902	4.6	2528	5.6
Phe	23 169	16.4	1275	7.5
Ile	29 295	8.2	1463	4.9
Lys	25 100	17.7	1266	7.0
Leu	38 370	8.3	1509	7.2

typical chromatogram showing the separation of these PTH-amino acids within 21 min is shown in Fig. 1.

PTH-cysteic acid was eluted at 3.13 min, which corresponded to the void volume. Cysteine had to be determined after derivatization of the SH residue [1]. We first tried to prepare S-carboxymethylcysteine using cysteine hydrochloride

TABLE II

CORRELATIONS FOR TWELVE AMINO ACIDS

Correlation between the amount of PTH-amino acid injected (pmol) and observed peak area (10^4 μV s), calculated from ten sample points.

PTH-amino acid	Linear regression line ($y=ax+b$)		Correlation coefficient (r)
	a	b	
Arg	0.11735	1.2144	0.9642
Asn	0.09330	0.4608	0.9898
Gln	0.00958	0.0139	0.9333
Ser	0 10238	0.2322	0.9992
Thr	0.08871	0.2414	0.9488
Asp	0 09901	0.3916	0.9962
Glu	0.05057	0.2146	0.9891
Gly	0.12653	0.0896	0.9980
Ala	0.13077	0.0256	0.9886
Tyr	0.17529	−0.2526	0.9959
Pro	0.16780	0.4310	0.9943
Trp	0.20554	0.5718	0.9896

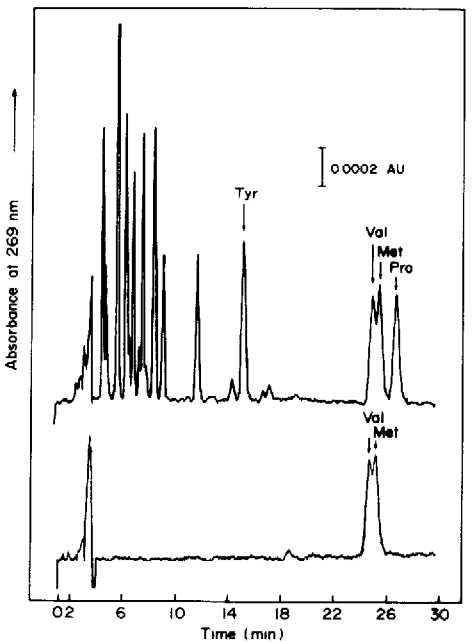

Fig. 2. Identification of methionine and valine. Conditions as described under Experimental. Flow-rate, 0.7 ml/min; eluent, acetonitrile–water (35:65) containing 0.1% TFA.

with dithiothreitol, iodoacetamide and iodoacetate. However, this procedure gave several PTH products. The synthesis of S-pyridylethylcysteine from cysteine hydrochloride and 4-vinylpyridine and dithiothreitol was successful [6]. PTH-PeC

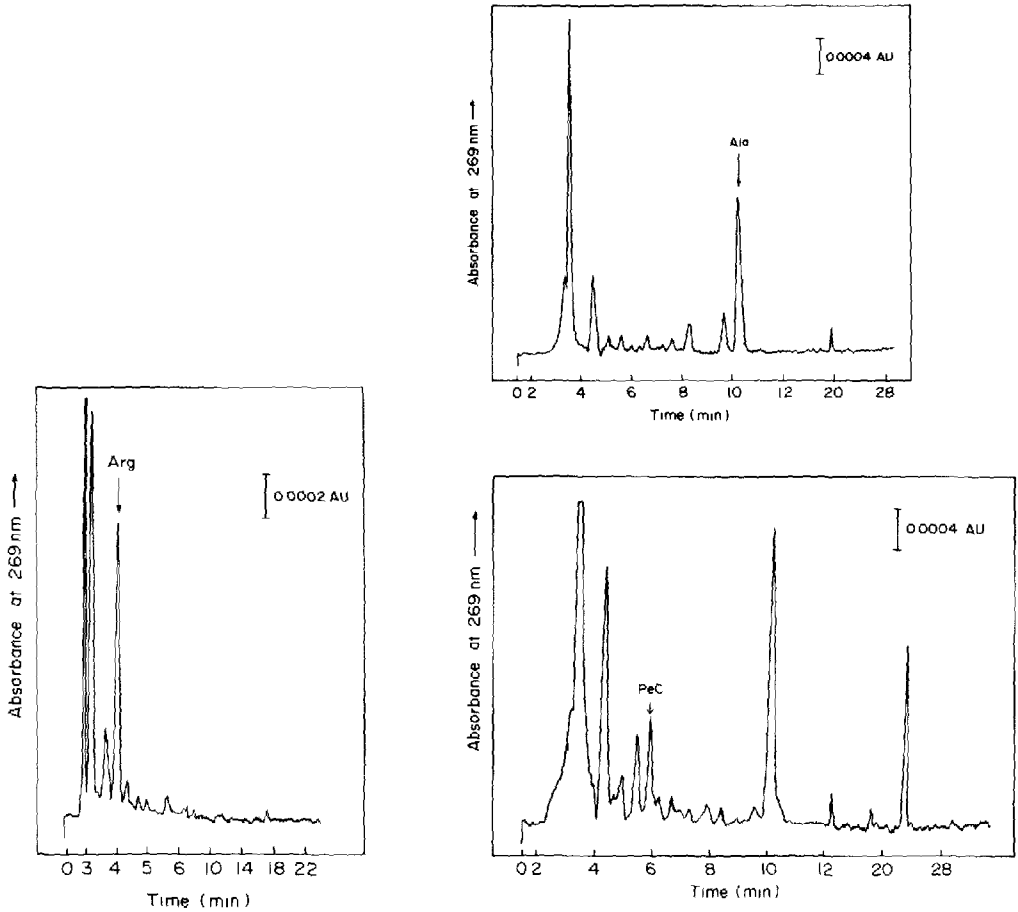

Fig. 3. Application of the method to substance P. N-Terminal Arg is shown. Conditions as described under Experimental.

Fig. 4. Application of the method to the analysis of glycoprotein avidin. Top, N-terminal Ala; bottom, 4th amino acid from the N-terminal end, Cys, detected as PeC. A flow-rate of 0.7 ml/min was used in this instance to improve the separation. Other conditions as described under Experimental.

eluted as a single peak between PTH-Asn and PTH-Gln.

The method is reproducible, as shown in Table I. The within-day coefficients of variation (C.V.) for most of the PTH-amino acids were within 3.1–22%. The large C.V. values for PTH-Thr, PTH-Asp, PTH-Phe and PTH-Lys are due to incomplete separation of these peaks. The resolution factors for Thr-Asp, Phe-Ile and Lys-Leu were 0.88, 0.82 and 0.93, respectively. Peak-height measurements are preferable to peak-area calculations in these instances (Table I). The detection limits of these compounds were approximately 1 pmol. Graphs of the amount of PTH-amino acids injected versus peak areas were linear for all the PTH-amino acids tested except PTH-Arg in the range 0–50 pmol. Typical results for twelve amino acids are given in Table II.

PTH-Val and PTH-Met were separated at room temperature by using aceto-

nitrile–water (35:65) containing 0.1% TFA (Fig. 2). PTH-Val and PTH-Met were eluted at 24.8 and 25.3 min, respectively. In this instance, the detection limit was 5 pmol and the resolution factor was 0.90. Elevation of column temperature to 40°C also allowed the separation of PTH-Val and PTH-Met using acetonitrile–water (40:60) (PTH-Val, 11.2 min; PTH-Met, 11.5 min; detection limit, 3 pmol; resolution factor, 0.86).

Examples of the application of the method to the analysis of commercial peptide (substance P) and glycoprotein (hen egg-white avidin) are shown in Figs. 3 and 4, respectively. N-Terminal Arg of substance P was identified (Fig. 3). N-Terminal Ala and 4th amino acid from the N-terminal end Cys (PeC) were identified from derivatized (pyridylethylated) avidin (Fig. 4). Hence the proposed separation system is useful for Edman degradation analysis.

ACKNOWLEDGEMENT

This work was supported by a grant from the Japanese Ministry of Health and Welfare.

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