Journal of Chromatography, 346 (1985) 413–416
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 068

Note

Separation of phenylthiocarbamyl amino acids by high-performance liquid chromatography on Spherisorb octadecylsilane columns

CHAO-YUH YANG* and FELIX I. SEPULVEDA
Baylor College of Medicine and Department of Medicine, The Methodist Hospital, 6565 Fannin Street, Houston, TX 77030 (U.S.A.)
(Received July 10th, 1985)

The role of the reagent phenylisothiocyanate (PITC) for amino terminal degradation, discovered by Edman in 1956, has become increasingly important. All current liquid, solid, and gas phase protein sequencers are designed on the principal of Edman degradation chemistry[1]. Many investigators have improved the analysis of phenylthiohydantoin (PTH) amino acids through the use of reversed-phase high-performance liuid chromatography (RP-HPLC) as a means for identification and quantification[2–5].

The application of PITC for precolumn derivatization of amino acids and the quantification of phenylthiocarbamyl (PTC) derivatives by RP-HPLC have been published[6]. A commercial system, PICO-TAG, is available from Waters Assoc. Most precolumn modification methods for amino acid analysis are very sensitive because of fluorescent amino acid derivatives[7–10] or color chromophores of the reagent[11]; however, these applications have been limited by either a lack of proline reaction from the *o*-phthaladehyde method or an excess of reagent that interferes with the separation processes utilizing dansyl chloride[10] and dimethylaminoazobenzene-4-sulfonylchloride[11], respectively. The application of PITC for amino acid analysis is rapid, sensitive, and simple to quantify, overcoming these disadvantages.

Our improvements of this method facilitate the separation of PTC-amino acid derivatives for analysis of smaller amounts of material in shorter periods of time. In this report we describe the separation of 22 PTC-amino acids by HPLC on Spherisorb ODS column in low picomole concentration.

EXPERIMENTAL

Chemicals

PITC, triethylamine (TEA), a standard mixture of amino acids, and a kit of individual amino acid standards were purchased from Pierce. Acetonitrile was an HPLC-grade product from Burdick and Jackson Labs. Water was deionized, then purified with the Milli-Q water system. Two peptides used for amino acid analysis were oxidized insulin A-chain and glucagon. Both peptides were obtained from Sigma.

0021-9673/85/$03.30 © 1985 Elsevier Science Publishers B.V.

Coupling of amino acids with PITC

PITC-amino acid derivatives were prepared according to the Waters PICO-TAG procedure with some improvement. The procedure involved two steps: (1) hydrolysis of the sample and (2) derivatization. Before sealing the samples in a vacuum for hydrolysis at 110°C for 24 h, the dry samples in small tubes (6 × 50 mm) were placed in the reaction vial with 200 μl of 6 *M* HCl. The hydrolyzed samples were dried and redried by adding 20 μl of ethanolic solution (ethanol–water–TEA, 1:1:1) to ensure that a trace amount of ammonia was left. For derivatization, the samples were coupled with 20 μl of PITC solution (ethanol–water–TEA–PITC, 7:1:2:1) for 10 min, dried again in a Speedvac (Savant Instruments), and reconstituted in sample diluent for analysis. The sample diluent was a 0.5 *M* sodium phosphate buffer, pH 7.4, and 5% acetonitrile.

The 22 PTC-amino acid standard was prepared by adding 1 nmol of each of the individual amino acids (carboxymethylcysteine, Trp, Gln, S-sulfocysteine (Cys-SO_3H), hydroxyproline, and Asn) to 1 nmol of the Pierce amino acid mixture (Asp, Glu, Ser, Gly, His, Thr, Ala, Arg, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe, Lys) which was hydrolyzed and derivatized simultaneously with samples using the PICO-TAG procedure described above.

RP-HPLC separation of PITC-amino acids

A Hewlett-Packard HP 1090 liquid chromatograph and a Spherisorb ODS II (3 μm, 150 × 4.6 mm I.D.; Custom LC, Houston, TX, U.S.A.) were used for the separation. The PITC derivatives were identified using an HP 1040A HPLC detection system at 254 nm. The chromatographic conditions were: column temperature 47°C; flow-rate 0.8 ml/min; buffer A: 0.03 *M* sodium acetate, 0.05% TEA, and 6% acetonitrile (pH 6.4); buffer B: 40% water in acetonitrile; mobile phase isocratic at 0% B for 1.5 min; first linear gradient from 0 to 12% B in 10 min; second linear gradient from 12% to 48% in 10 min, and final change to 95% B in 0.1 min; maintenance at 95% B isocratic flow for 5 min; return of the system to 0% B in 5 min.

RESULTS AND DISCUSSION

The separation of 50 pmol of the 22 PTC-amino acids on Spherisorb ODS (150 × 4.6 mm) is shown in Fig. 1. All amino acid derivatives, including ammonia and an unknown peak, were well separated under those conditions in 22 min. To achieve a better resolution between PTC-Cys-SO_3H and PTC-Asp, an isocratic elution of buffer A was used for 1.5 min.

The characteristics of PTC-amino acids separated on an ODS column with sodium acetate buffer system are similar to those of dimethylaminoazobenzenethiohydantoin (DABTH) amino acids and PTH-amino acids. This means that (1) the retention times of acidic and basic amino acid derivatives can be influenced by altering the pH of the sodium acetate buffer, (2) the position of the His and Arg derivatives can be altered by changing the salt concentration[12–16], and (3) the retention times of His and Arg derivatives gradually change during the lifetime of the column[5,12,17–19].

An elevated column temperature improves the resolution of the PTC-amino acids and reduces the pressure drop over the column. The retention time of PTC-Arg can be decreased by increasing the salt concentration of buffer A. Hence, the position

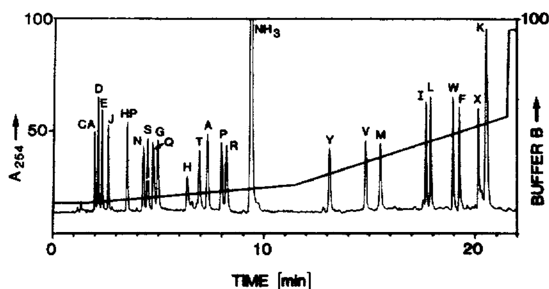

Fig. 1. HPLC chromatogram of a standard mixture of 22 PTC-amino acids (50 pmol each) on a column packed with Spherisorb ODS, 3 μm column. Chromatographic conditions are described in the Experimental section. The solvent gradient is indicated in the figure. CA = Cysteic acid; D = aspartic acid; E = glutamic acid; J = carboxymethcysteine; HP = hydroxyproline; N = asparagine; S = serine; Q = glutamine; G = glycine; H = histidine; T = threonine; A = alanine; P = proline; NH_3 = ammonia; Y = tyrosine; V = valine; M = methionine; I = isoleucine; L = leucine; W = tryptophan; F = phenylalanine; X = unknown peak; K = lysine.

of the PTC-Arg on the chromatogram can be changed as desired in order to achieve a better separation. Using the Spherisorb ODS column and the conditions described in the Experimental section, the PTC-Arg can be changed from its position behind PTC-Pro to a position between PTC-His and PTC-Thr by changing the sodium ace-

TABLE I

AMINO ACID ANALYSIS OF GLUCAGON AND INSULIN A-CHAIN OXIDIZED FORM BY RP-HPLC OF PTC-AMINO ACIDS

A = Number of residues found; B = number of residues expected based upon the known sequences.

Amino acid	*Glucagon*		*Insulin A-chain*	
	A	*B*	*A*	*B*
Cys-SO_3H	–	–	4.12	4
Asp	4.10	4	1.88	2
Glu	3.00	3	4.22	4
Ser	3.82	4	1.99	2
Gly	1.08	1	1.09	1
His	0.82	1	–	–
Thr	2.92	3	–	–
Ala	1.18	1	1.00	1
Pro	–	–	–	–
Arg	2.10	2	–	–
Tyr	1.92	2	1.94	2
Val	1.10	1	1.74	2
Met	1.10	1	–	–
Ile	–	–	0.76	1
Leu	1.86	2	2.26	2
Phe	1.89	2	–	–
Lys	1.11	1	–	–
Trp	n.d.*	1	–	–
Total	28.00	29	21.00	21

* n.d. = Not determined.

tate concentration from 0.03 to 0.153 *M*, while the order of the other PTC-amino acids remains the same.

Amino acid analysis of an oxidized insulin A-chain and glucagon, obtained with the system as described in the Experimental section, is listed in Table I. Those data appear to be reliable at the 30-pmol level. According to the stable baseline shown in Fig. 1 (using 50 pmol of each amino acid), quantification of samples with less than 10 pmol of material is feasible.

The advantages of the present system for amino acid analysis are the following: (1) high resolution to separate all 22 PTC-amino acids; (2) possibility to analyze the samples containing carboxymethylcysteine and cysteic acid; (3) ability to shorten the analysis time as compared with the original method[6]; (4) possibility to analyze sample at the low picomole level; (5) ability to substantially save solvent consumption due to the slower flow rate (0.8 ml/min).

Over the past few years, RP-HPLC techniques have been improved in instrumental design, detection sensitivity, packing material for columns, and solvents. In order to increase detection sensitivity and to save solvent consumption and time, the use of microbore column has increased. Commercial columns are available from a number of different companies. Since the mobile phase flow-rate is proportional to the product of the linear velocity and the column cross-sectional area, solvent consumption can be reduced proportionally to the square of the column radius[20]. Thus the microbore column reduces solvent consumption and increases mass sensitivity. The application of microbore columns for PTC-amino acid analysis would enable the analysis of amino acids at the femtomole level.

ACKNOWLEDGEMENTS

We thank Tseming Yang and Billy Touchstone for excellent technical assistance, Mrs. Susan Kelly for the artwork, and Mrs. Marjorie Sampel for preparation of this manuscript. This work was supported by a Specialized Center of Research on Atherosclerosis (HL27341).

REFERENCES

1 P. Edman, *Acta Chem. Scand.*, 10 (1956) 761.
2 L. E. Henderson, T. D. Copeland and S. Ososzlan, *Anal. Biochem.*, 102 (1980) 1–7.
3 G. E. Tarr, *Anal. Biochem.*, 111 (1981) 27–32.
4 J. Simmons and D. H. Schlesinger, *Anal. Biochem.*, 104 (1980) 254–258.
5 N. D. Johnson, M. W. Hunkapiller and L. E. Hood, *Anal. Biochem.*, 100 (1979) 335–338.
6 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65–74.
7 W. Voelter and K. Zech, *J. Chromatogr.*, 112 (1975) 643–649.
8 K. V. J. Swedas, J. L. Galaev, I. L. Borislow and I. V. Berezin, *Anal. Biochem.*, 101 (1980) 188.
9 J. C. Hodgin, *J. Liq. Chromatogr.*, 2 (1979) 1047.
10 T. Yamabe, N. Takai and H. Nakamura, *J. Chromatogr.*, 104 (1975) 359–365.
11 J. Y. Chang, R. Knecht and D. G. Braun, *Biochem. J.*, 199 (1981) 547–555.
12 C. Y. Yang and S. J. Wakil, *Anal. Biochem.*, 137 (1984) 54–57.
13 J. T. Chang, *Biochem. J.*, 199 (1981) 557–564.
14 K. Sugden, G. B. Cox and C. R. Loscombe, *J. Chromatogr.*, 149 (1978) 377–390.
15 E. Lundanes and T. Greibrokk, *J. Chromatogr.*, 149 (1978) 241–254.
16 W. D. Annan, *J. Chromatogr.*, 173 (1979) 194–197.
17 L. Scottrup-Jensin, T. E. Petersen and S. Magnusson, *Anal. Biochem.*, 107 (1980) 456–460.
18 S. J. Dimari, J. P. Robinson and J. H. Hash, *J. Chromatogr.*, 213 (1981) 91–97.
19 F. Lottspeich, *Hoppe-Seyler's Z. Physiol. Chem.*, 361 (1980) 1829–1834.
20 R. P. W. Scott (Editor), *Samll Bore Liquid Chromatography Columns*, Wiley, New York, 1984.