Journal of Chromatography, 327 (1985) 327–332
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 538

COMPARISON OF REVERSED-PHASE AND CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR SEPARATING CLOSELY RELATED PEPTIDES: SEPARATION OF ASP^{76}-HUMAN PARATHYROID HORMONE (1–84) FROM ASN^{76}-HUMAN PARATHYROID HORMONE (1–84)

KUMIKO YOSHIZAWA KUMAGAYE, MICHIHIRO TAKAI, NAOYOSHI CHINO, TERUTOSHI KIMURA and SHUMPEI SAKAKIBARA*
Peptide Institute, Inc., Protein Research Foundation, Minoh-shi, Osaka 562 (Japan)

SUMMARY

Cation-exchange high-performance liquid chromatography (CE-HPLC) was compared with ordinary reversed-phase high-performance liquid chromatography (RP-HPLC) for separating closely related peptides. Some synthetic samples of bradykinin and angiotensins, which were homogeneous according to RP-HPLC, were found to be inhomogeneous when analyzed by CE-HPLC. On the other hand, diastereomeric peptides could be separated much more efficiently by RP-HPLC than by CE-HPLC. These results indicated that the purity of synthetic peptides should be checked not only by RP-HPLC but also by ion-exchange HPLC. In the case of human parathyroid hormone (hPTH), baseline separation of Asp^{76}-hPTH from Asn^{76}-hPTH by RP-HPLC was not possible, but was by CE-HPLC. Using this method we confirmed that the Asn residue in hPTH at position 76 could not be converted into the Asp residue under the conditions used to isolate and purify it from human organs.

INTRODUCTION

In 1978, Keutmann *et al.*[1] found the amino acid sequence of human parathyroid hormone (hPTH) to be a linear peptide with 84 amino acid residues when they applied Edman degradation reactions to an isolated hormone. The structure of the same peptide was deduced by Hendy *et al.*[2] from sequence analysis of the cDNA; both structures were identical except for the residue at position 76, which was thought to be Asp from the Edman degradation analysis and Asn from the DNA analysis. When the new structure was reported, the previous one with Asp at position 76 was suspected to be an artifact which might have been formed by spontaneous deamidation of the Asn residue during isolation or purification of the natural peptide. To verify this, we synthesized both Asn^{76}-hPTH and Asp^{76}-hPTH by the solution procedure[3,4], and examined their separation by ordinary reversed-phase high-performance liquid chromatography (RP-HPLC). Under isocratic conditions, the hormones

0021-9673/85/$03.30

were eluted very close together but with clearly different retention times. However, when injected into the same column as a mixture, the peptides were eluted together as a rather broad peak[4,5]. In the present study we have tried to establish conditions for separating such closely related peptides by HPLC.

Recently, ion-exchange type columns have been introduced for HPLC and their usefulness in separating peptides and proteins has been reported[6,7]. In order to elucidate the characteristic features of the new technique, we compared the resolving power of cation-exchange HPLC (CE-HPLC) with that of ordinary RP-HPLC by using some synthetic peptides as test samples. We also used the CE-HPLC technique to find the conditions for separation of the two hPTH analogues, and then applied them to determine whether or not Asp^{76}-hPTH is an artifact formed during isolation of hPTH from organs.

EXPERIMENTAL

Materials

Commercial samples of angiotensin I (AngI) and bradykinin (BK) were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.), UCB Bioproducts (Bruxelles, Belgium) and Bachem (Bubendorf, Switzerland). Other peptides were synthesized by solution procedures in our laboratory using previously reported methods[8]. The reagents and solvents for chromatography were of HPLC-reagent grade. The water used was distilled in a Toyo Aquarius Model GS-20N still (Toyo Kagakusangyo, Tokyo, Japan).

CE-HPLC

CE-HPLC was carried out on a Shimadzu liquid chromatograph Model LC-4A (Kyoto, Japan) equipped with a Rheodyne 7125 syringe-loading sample injector, a Shimadzu variable-wavelength UV detector Model SPD-2AS, a Shimadzu column oven Model CTO-2AS and a Shimadzu data processor Chromatopac C-R2AX. The column (250 × 4.6 mm I.D.) was packed with a cation-exchange resin, TSK gel CM-2SW (Toyo Soda, Tokyo, Japan). Two solvent systems were used: A, 10% acetonitrile in 20 m*M* sodium phosphate buffer (pH 6.0); B, 10% acetonitrile in 20 m*M* sodium phosphate buffer (pH 7.0) containing 0.5 *M* sodium chloride. Elution was performed at 42°C at a flow-rate of 0.8 ml/min; other conditions are as stated in the Figs.

RP-HPLC

RP-HPLC was performed on a Hitachi liquid chromatograph Model 638-30 equipped with a multi-wavelength UV monitor Model 635M (Tokyo, Japan); the column (150 × 4.0 mm I.D.) was packed with Nucleosil 5 C_{18} (Macherey-Nagel, Düren, F.R.G.). Chromatography was carried out at a flow-rate of 1.0 ml/min at ambient temperature unless stated otherwise.

Test of conversion of Asn^{76}-hPTH into Asp^{76}-hPTH

Asn^{76}-hPTH (15 μg) was dissolved in 75 μl each of water, 0.1 *M* ammonium acetate buffer (pH 2,5, 5.0 or 7.5), 0.1 *M* ammonium hydrogen carbonate buffer (pH 8.7) or 4% trichloroacetic acid (TCA). After the solution had been kept for 1 day or

5 days at room temperature, 35 μl from each solution were lyophilized twice; the residue was dissolved in 20 m*M* sodium phosphate buffer (pH 6.0) and subjected to HPLC. With the TCA solution, the whole mixture was lyophilized after it had been kept for 1 day at room temperature, and the residue was subjected to HPLC after it had been dissolved in a 20 m*M* sodium phosphate buffer (pH 6.0).

RESULTS AND DISCUSSION

As reported previously[9], various angiotensin II (AngII) analogues, such as β-Asp[1]-, D-Asp[1]-, D-Tyr[4]-, Val[5]-, Leu[5]-, des-Ile[5]-, D-His[6]- and D-Phe[8]-AngII, were clearly separated by RP-HPLC, except for β-Asp[1]-AngII, which is always eluted together with Asp[1]-AngII in our RP-HPLC system. The separation could be slightly improved by increasing the pH of the buffer system, but it was far from a baseline separation. CE-HPLC enabled a clear separation of the two AngII analogues as shown in Fig. 1. These results indicate that the purity of synthetic peptides containing Asp residue(s) should be checked not only by RP-HPLC but also by CE-HPLC. Particularly important is the detection of β-Asp-containing peptide since some Asp residue(s) in peptides have a great tendency to be converted into β-Asp residue(s) during various steps of their synthesis. We applied these techniques to test the homogeneity of commercially available samples of AngI. As expected in RP-HPLC, all samples looked homogeneous, but in CE-HPLC they were found to be contaminated by β-Asp-AngI, in the range of 2.3–17%, and by various other minor contaminants; a typical elution pattern is shown in Fig. 2.

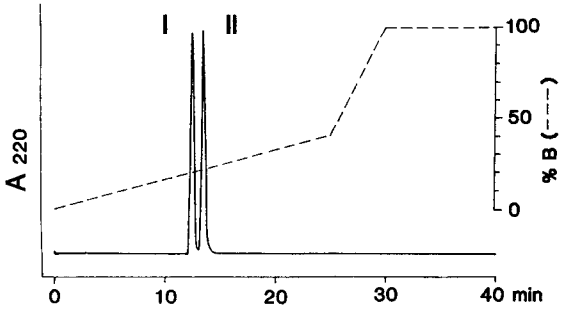

Fig. 1. Separation of β-Asp[1]- and Asp[1]-angiotensin II by CE-HPLC. Column: TSK gel CM-2SW (250 × 4.6 mm I.D.). The column was equilibrated with solvent A, and then the peptide was eluted with solvent B using a gradient as shown in the figure. See text for other details. Peaks: I = β-Asp[1]-AngII; II = Asp[1]-AngII.

Next, we tested the homogeneity of commercial bradykinin (BK) samples since this peptide is also widely available. Only one commercial sample gave a single peak in RP-HPLC, but the shape was somewhat broader than that of our standard sample. Even the best sample gave a relatively large extra peak in CE-HPLC as shown in Fig. 3; the peak area was about 13% of the total. The structure of the component in the side peak was examined by chymotryptic mapping in HPLC and by amino acid analysis; it was confirmed to contain a normal bradykinin sequence (1–8), identical with that isolated from an authentic sample, together with one Orn residue instead of one Arg residue. Thus, the main component of the side peak was Orn[9]-BK.

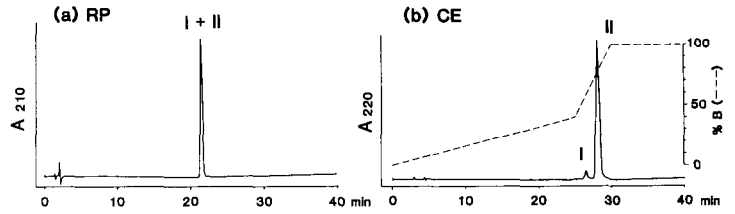

Fig. 2. RP- and CE-HPLC profiles of a commercial AngI. (a) RP-HPLC. Column: Nucleosil 5C18 (150 × 4 mm I.D.). Gradient: 10–60% acetonitrile in 10 m*M* H_3PO_4–K_2HPO_4 (pH 2.6) containing 50 m*M* Na_2SO_4. (b) CE-HPLC. Column and elution conditions as in Fig. 1. Peaks: I = β-Asp[1]-AngI; II = Asp[1]-AngI.

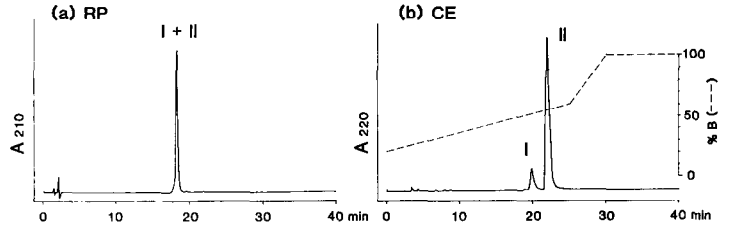

Fig. 3. RP- and CE-HPLC profiles of a commercial bradykinin. (a) RP-HPLC. Column and elution conditions as in Fig. 2. (b) CE-HPLC. Column as described in Fig. 1. It was equilibrated with 20% B in A before applying the peptide; then the peptide was eluted with solvent B using a gradient as shown. Peaks: I = Orn[9]-BK; II = BK.

Today, many peptides are synthesized by a solid-phase procedure and purified simply by a RP-HPLC system; the present results clearly indicate that the purification of synthetic peptides by RP-HPLC is not sufficient to obtain homogeneous products.

With regard to the separation of peptide diastereomers, the usefulness of RP-HPLC has been recognized by many groups, and several examples of diastereomer separation have been reported by Dizdaroglu's group[10,11], who used a weak anion-exchange column. We also tried to separate D-Ala[73],Asp[76]-hPTH (39–84) from the L-peptide by our CE-HPLC, but we were unsuccessful despite the good separation of the same compounds in our RP-HPLC system[5]. A similar result was obtained with a pair of shorter peptides, D-Glu[22]-hPTH (18–28) and its L-isomer. However, in the case of human growth hormone-releasing factor (hGRF), D-Leu[22]-hGRF(1–44)-NH_2 was clearly separated from its original L-peptide not only by RP-HPLC[12] but also by CE-HPLC as shown in Fig. 4. From these observations, we concluded that some diastereomeric isomers can be separated by IE-HPLC but the system may not be suitable in general for detecting racemization during peptide synthesis.

Finally, we examined the separation of Asp[76]-hPTH (1–84) from Asn[76]-hPTH by the CE-HPLC system. Clear separation was achieved even when a 1:1 mixture was applied, as shown in Fig. 5. This CE-HPLC technique was then used to detect possible conversion of the Asn residue into Asp under the various conditions used to isolate and purify natural hPTH; that is, the peptides were kept in water, 0.1 *M* ammonium acetate solution (pH 2.5, 5.0 or 7.5), 0.1 *M* ammonium hydrogen carbonate solution (pH 8.7) or 4% TCA solution for a maximum of 5 days at room

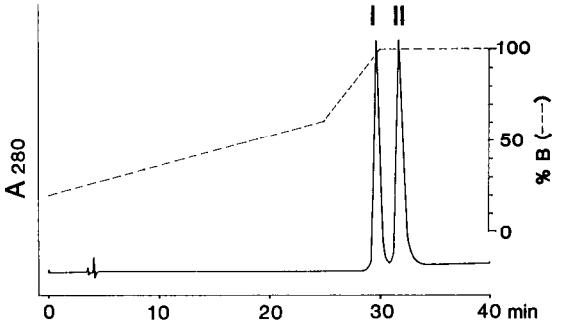

Fig. 4. Separation of D-Leu^{22}-hGRF(1–44)–NH_2 and the all-L-peptide by CE-HPLC. Column and elution conditions as in Fig. 3b. Peaks: I = D-Leu^{22}-hGRF(1–44)-NH_2; II = hGRF(1–44)-NH_2.

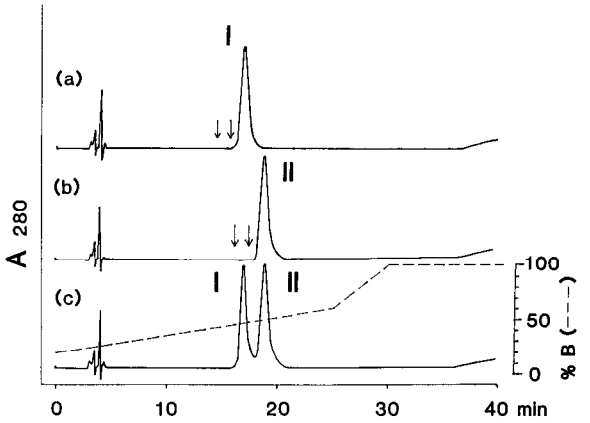

Fig. 5. Separation of Asp^{76}- and Asn^{76}-hPTH(1–84) by CE-HPLC. (a) Application of compound I only; (b) application of compound II only; (c) application of a mixture of I and II. Column and elution conditions as in Fig. 3b. Peaks: I = Asp^{76}-hPTH(1–84); II = Asn^{76}-hPTH(1–84). Arrows indicate the peak positions of Met(O)-containing peptides.

temperature[13]. No clear evidence of conversion of the Asn residue into Asp was obtained. Recently, Gleed *et al.*[14] confirmed, by using radioimmunoassay, that our synthetic Asn^{76}-hPTH had better cross-reactivity with an antibody, raised against an isolated natural hPTH, than our Asp^{76}-hPTH. Furthermore, the cross-reaction curve of Asn^{76}-hPTH was completely parallel to that of the natural hormone. From this evidence, together with the results of the above conversion tests, we concluded that the Asp^{76} structure claimed for hPTH might have been due to misreading the results of the original Edman degradation reactions.

REFERENCES

1 H. T. Keutmann, M. M. Sauer, G. N. Hendy, J. L. H. O'Riordan and J. T. Potts, Jr., *Biochemistry*, 17 (1978) 5723.
2 G. N. Hendy, H. M. Kronenberg, J. T. Potts, Jr. and A. Rich, *Proc. Nat. Acad. Sci., U.S.A.*, 78 (1981) 7365.
3 T. Kimura, T. Morikawa, M. Takai and S. Sakakibara, *J. Chem. Soc., Chem. Commun.*, (1982) 340.

4 T. Kimura, M. Takai, K. Yoshizawa and S. Sakakibara, *Biochem. Biophys. Res. Commun.*, 114 (1983) 493.
5 T. Kimura, K. Yoshizawa, M. Takai and S. Sakakibara, in T. Y. Liu, S. Sakakibara, A. N. Schechter, K. Yagi, H. Yajima and K. T. Yasunobu (Editors), *Frontiers in Biochemical and Biophysical Studies of Proteins and Membranes*, Elsevier, New York, 1983, pp. 81–88.
6 F. E. Regnier, Anal. Biochem., 126 (1982) 1.
7 M. Dizdaroglu, in W. S. Hancock (Editor), *Handbook of the Use of HPLC for the Separation of Amino acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, pp. 23–43.
8 T. Kimura, M. Takai, Y. Masui, T. Morikawa and S. Sakakibara, *Biopolymers*, 20 (1981) 1823.
9 S. Sakakibara, H. Sokabe, T. Nakajima, M. C. Khosla, S. Kumagaye and T. X. Watanabe, in B. Lofts (Editor), *Proceedings of the Ninth International Symposium on Comparative Endocrinology*, Hong Kong University Press, Hong Kong, in press.
10 M. Dizdaroglu and M. G. Simic, *J. Chromatogr.*, 195 (1980) 119.
11 M. Dizdaroglu, M. G. Simic, R. Rioux and S. St-Pierre, *J. Chromatogr.*, 245 (1982) 158.
12 N. Chino, S. Kumagaye, Y. Masui, T. Kimura and S. Sakakibara, in E. Munekata (Editor), *Peptide Chemistry 1983*, Protein Research Foundation, Osaka, 1984, pp. 285–290.
13 H. T. Keutmann, P. M. Barling, G. N. Hendy, G. V. Segre, H. D. Niall, G. D. Aurbach, J. T. Potts, Jr. and J. L. H. O'Riordan, *Biochemistry*, 13 (1974) 1646.
14 J. H. Gleed, G. N. Hendy, T. Kimura, S. Sakakibara and J. L. H. O'Riordan, *J. Endocrinol*, in press.