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Selective on-line enrichment and separation of peptides having aromatic amino acids at their C-termini by column-switching high-performance liquid chromatography using an anhydrochymotrypsin-immobilized precolumn

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ABSTRACT

A column switching high-performance liquid chromatographic system in which peptides retained on an anhydrochymotrypsin (AHC)-immobilized diol-silica precolumn were selectively transferred to and separated on a reversed-phase analytical column was developed. An investigation of the affinity characteristics of 40 peptides to the AHC precolumn showed that among eleven peptides having Tyr or Phe at their C-termini and more than five amino acid residues, ten were retained almost quantitatively on the precolumn, and two peptides having Trp at their C-termini were less retained. Two peptides having C-terminal $PheNH_2$ were also retained, but the peptide having C-terminal D-$PheNH_2$ was not retained. Among eighteen peptides having no aromatic amino acids at their C-termini, two were retained, one slightly and the other moderately. Calibration graphs for rat atrial natriuretic peptide constructed at various sample sizes were nearly identical, indicating that the peptide could be enriched by this system. The AHC precolumn showed no loss of analytical performance over 1 year, during which about 450 samples were analysed.

INTRODUCTION

High-performance liquid chromatography (HPLC) is now widely used for the determination of peptides. HPLC analysis of biological fluids for peptides, however, is restricted despite its high resolving power, and sample pretreatment is necessary in most instances to reduce the amount of interfering components and to enrich the analytes of interest. For this purpose, column switching (CS) is a powerful technique. Reversed-phase and immunoaffinity precolumns have so far been used [1–7]. The method using a reversed-phase precolumn is applicable to various peptides, but lacks selectivity. The immunoaffinity method offers high selectivity, but the preparation of antibody for each analyte is time consuming. Methods based on other bioaffinities such as enzyme–inhibitor or –substrate seem rational, but have not been used in the CS-HPLC analysis of peptides.

Anhydrotrypsin (AHT) and anhydrochymotrypsin (AHC) prepared by chemical modification of trypsin and chymotrypsin are known to be catalytically inert, but exhibit affinity toward peptides having Arg or Lys at their C-termini and those having aromatic amino acids at their C-termini, respectively [8–13]. Recently, we

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have developed AHT- or AHC-immobilized diol-silica columns for the high-performance affinity chromatographic separation of peptides [14,15]. The excellent characteristics of these columns with regard to separation speed and stability indicated the possibility that they might be used as precolumns in the CS-HPLC analysis of peptides. A CS-HPLC system equipped with the AHT precolumn was developed, and it was found that the AHT precolumn selectively retained peptides having Arg or Lys at their C-termini although there were exceptions, and showed no decrease in retention after exposure for about 800 cycles to acidic eluents containing acetonitrile during 1 year [16].

In this study, we developed a CS-HPLC system equipped with an AHC-immobilized diol-silica precolumn for the selective enrichment and separation of peptides having aromatic amino acids at their C-termini. The performance of the system was ascertained by examining the affinity characteristics of various peptides with respect to the AHC precolumn.

EXPERIMENTAL

Materials

Peptides were purchased from the Peptide Institute (Osaka, Japan), Sigma (St. Louis, MO, USA), Peninsula (Belmont, CA, USA), Aldrich (Milwaukee, WI, USA) and Tokyo Kasei Kogyo (Tokyo, Japan). Diol-silica was prepared from Matrex silica beads (30–50 μm, 50 nm)(Amicon, Lexington, MA, USA) as described previously [16]. Other chemicals were of analytical-reagent grade. Deionized water (obtained with a Millipore RO-Q system) was used throughout.

Preparation of an AHC-immobilized precolumn

An AHC-immobilized precolumn (10 × 4.6 mm I.D.) was prepared as described previously [15]. In brief, diol-silica, prepared by silanization of Matrex silica beads with 3-glycidoxypropyltrimethoxysilane under anhydrous conditions followed by hydrolysis of the epoxy groups with 0.01 *M* perchloric acid, was activated with 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride), and AHC was immobilized on the diol silica by shaking the activated gel in phosphate buffer (pH 8.0) containing AHC. The amount of AHC immobilized on the diol silica was 43 mg/g. The column was stored in water at 4°C when not in use.

CS-HPLC

The set-up of the CS-HPLC system used is shown schematically in Fig. 1. A Model KHD-26 pump (P1) (Kyowa Seimitsu, Tokyo, Japan) was used for sample loading at a flow-rate of 0.5 ml/min with ice-cold water (S1) as the eluent. Peptide solution was injected on to the AHC precolumn (C1) through a Model 7125 injector (Rheodyne, Cotati, CA, USA) equipped with a 0.5-ml loop. After the injection, the precolumn was washed with S1 for 5 min, and then 0.5 ml of 20 m*M* acetate buffer (pH 5.0) was injected twice at an interval of 2 min to eliminate non-specific adsorption of peptides. The precolumn was further washed with S1 for 5 min. The switching valve, a Model E1E 002 (Senshu Kagaku, Tokyo, Japan), was then changed from "load" to "inject". A Model LC-6A pump (P2) (Shimadzu, Kyoto, Japan) was used for flushing the retained peptide from C1 on to the analytical column (C2) (Capcell Pak C_{18}; 150 × 4.6 mm I.D.) at a flow-rate of 1 ml/min with acetonitrile–10 m*M* H_3PO_4 (2–30:98–70, v/v) containing 0.1 *M* $NaClO_4$ and 0.5 *M* NaCl (S2) as eluents. This is the so-called straight-flushing mode [17]. The CS-HPLC system in which the retained peptide was flushed from C1 by interchanging the outlets of the switching valve connected to the injector and to waste and reversing the flow direction was also examined in certain cases (back-flushing mode [17]). The column

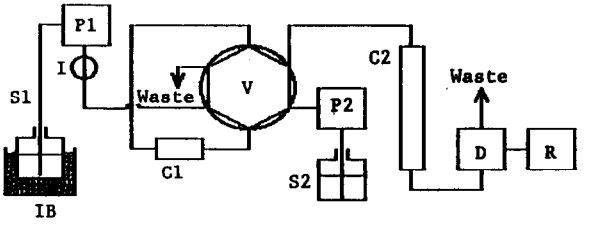

Fig. 1. Schematic diagram of the CS-HPLC system. P1 and P2 = pumps; C1 = precolumn; C2 = analytical column; I = injector; S1 and S2 = eluents; IB = ice-bath; V = switching valve; D = detector; R = integrator.

eluent was monitored at 214 nm with an SPD-6A detector (Shimadzu) connected to a Chromatopac C-R6A integrator (Shimadzu).

The percentage retention of each peptide on the AHC precolumn was calculated by dividing the peak area obtained with the precolumn by that obtained by injecting 50 μl of solution containing the corresponding amount of the peptide directly on to the analytical column.

RESULTS AND DISCUSSION

CS-HPLC conditions

The same HPLC conditions as those in the previous study for the AHT precolumn [16] were initially employed to examine the affinity characteristics of peptides with respect to the AHC precolumn. However, a preliminary study using several peptides showed that No. 15 gave a broad peak. No. 16 gave a sharper peak although eluted at a similar position. The peak broadening of No. 15 can therefore probably be attributed to the higher affinity of the peptide for the AHC precolumn. The eluents that enabled No. 15 to be eluted sharply were investigated, and addition of sodium chloride was found to be effective. Although the addition of sodium chloride was not necessarily required for all other peptides, eluents containing 0.5 *M* sodium chloride were used throughout this study for convenience.

Fig. 2 shows the effect of the flushing mode. A higher peak height was obtained by straight-flushing for No. 15, but by back-flushing for No. 22. However, no significant difference in peak area was observed between these flushing modes. Although the preferred flushing mode was expected to differ depending on the kinds of peptides involved, straight-flushing was employed exclusively in this study.

The CS-HPLC conditions other than those described above, such as the flow-rate of S1 and the kinds of washing solvents, were almost the same as those in the previous study with the AHT precolumn [16].

Affinity characteristics of various peptides

Table I shows the retentions of various peptides on the AHC precolumn after washing with

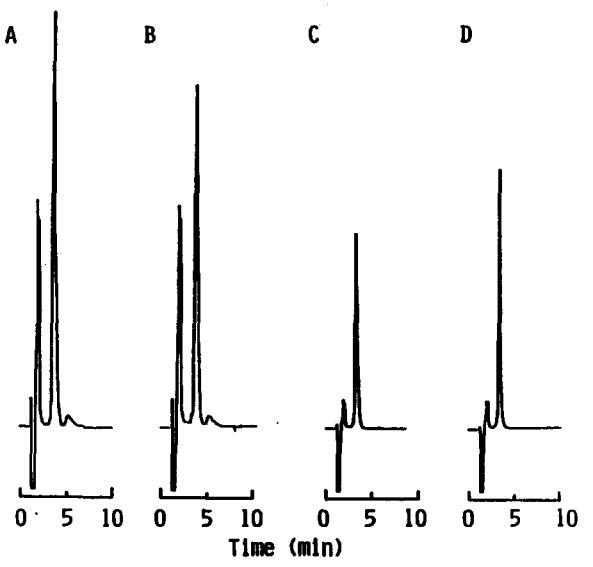

Fig. 2. Effect of flushing mode on peak shapes of (A, B) No. 15 and (C, D) No. 22. A and C, straight-flushing; B and D, back-flushing. Eluent S2, CH_3CN–10 m*M* H_3PO_4 (30:70, v/v) containing 0.1 *M* $NaClO_4$ and 0.1 *M* NaCl.

20 or 60 m*M* acetate buffer (pH 5.0). The peptides that had Tyr at their C-termini and more than five amino acid residues, except No. 9, were retained almost quantitatively on the precolumn after washing with 20 m*M* acetate buffer. These peptides, except Nos. 11 and 21, were also retained in good yield after washing with 60 m*M* acetate buffer. The peptides that had Phe at their C-termini and more than five amino acid residues were retained almost quantitatively after washing with 20 m*M* acetate buffer, and also in good yield, except Nos. 13 and 15, after washing with 60 m*M* acetate buffer. Nos. 7 and 17, having C-terminal $PheNH_2$, were retained, whereas No. 8, that had the same sequence as No. 7 but C-terminal D-$PheNH_2$ was not retained at all. The peptides having Trp at their C-termini, except No. 1, showed less affinity for the AHC precolumn. Among eighteen peptides that had no aromatic amino acids at their C-termini, three were retained slightly (Nos. 38 and 40) or moderately (No. 34).

On the other hand, almost all the peptides examined showed no or only a slight retention to the diol-silica precolumn, indicating that the retained peptides were recognized with the im-

TABLE I

AFFINITY CHARACTERISTICS OF PEPTIDES FOR THE AHC PRECOLUMN

No.	Peptide[a]	Retention (%)[b]			
		AHC precolumn		Diol-silica precolumn	
		20 m*M*[c]	60 m*M*[c]	20 m*M*[c]	60 m*M*[c]
1	Gly-Trp	0	–[d]	0	–[d]
2	Gly-D-PheNH$_2$	0	–	0	–
3	Gly-Leu-Tyr	0	–	0	–
4	Met-Leu-Phe	0	–	0	–
5	Phe-Gly-Gly-Phe	0	–	0	–
6	Asp-Arg-Val-Tyr	0	–	0	–
7	Phe-Met-Arg-PheNH$_2$	103	102	4	3
8	Phe-Met-Arg-D-PheNH$_2$	0	–	0	–
9	Arg-Lys-Glu-Val-Tyr	60	39	0	–
10	Arg-Ser-Arg-His-Phe	88	90	5	1
11	Arg-Lys-Asp-Val-Tyr	84	57	1	0
12	Ser-Met-Glu-Val-Arg-Gly-Trp (δ-melanocyte-stimulating hormone)	68	68	0	–
13	Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin III)	91	62	6	0
14	Tyr-Gly-Gly-Phe-Met-Arg-Phe	82	73	1	–
15	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (angiotensin II)	93	47	2	1
16	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	96	82	4	1
17	Asp-Tyr-Met-Gly-Trp-Met-Asp-PheNH$_2$ (cholecystokinin octapeptide 26–33)]	52	34	9	8
18	Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (α-mating factor)	94	95	7	5
19	Trp-His-Trp-Leu-Ser-Phe-Ser-Lys-Gly-Glu-Pro-Met-Tyr (α-SK2 mating factor)	104	100	4	7
20	Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp (endothelin 1)	43	15	0	–
21	Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr [atrial natriuretic peptide (human)]	89	67	16	18
22	Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr (atrial natriuretic peptide (rat)]	96	96	15	12
23	Ala-Ala-Ala-Ala	0	–	0	–
24	Pro-Phe-Gly-Lys	0	–	0	–
25	Arg-Gly-Asp-Ser	0	–	0	–
26	Gly-Arg-Gly-Asp	0	–	0	–
27	Arg-Pro-Lys-Pro	6	2	4	3
28	Tyr-Gly-Gly-Phe-Met (Met-enkephalin)	0	–	0	–
29	Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin)	0	–	0	–
30	Tyr-Pro-Phe-Pro-Gly (β-casomorphin 5)	0	–	0	–
31	Phe-Leu-Glu-Glu-Val	0	–	0	–
32	Tyr-Gly-Gly-Phe-Leu-Arg	4	4	4	5
33	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	0	–	2	–
34	Arg-Val-Tyr-Ile-His-Pro-Ile	61	5	10	8
35	Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr (peptide T)	0	–	0	–
36	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu	2	1	1	1
37	Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu	0	–	0	–
38	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin)	18	15	5	2
39	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro (β-neoendorphin)	0	–	2	0
40	Ser-Gly-Ser-Ala-Lys-Val-Ala-Phe-Ser-Ala-Ile-Arg-Ser-Thr-Asn-His	16	13	16	14

[a] 1 nmol per 50-μl injection.
[b] Calculated on the basis of peak area.
[c] Concentration of acetate buffer (pH 5.0).
[d] Not determined.

mobilized AHC. The results with the diol-silica precolumn seem to indicate that the non-selective retention of No. 40 observed with the AHC precolumn is attributable to the support.

Ishii and co-workers [11–13] investigated the chromatographic behaviour of seventeen peptides (six C-terminal Tyr peptides, three C-terminal Phe peptides, four C-terminal Trp peptides and four other peptides) on an AHC-agarose column, and reported that peptides having aromatic amino acids at their C-termini, except two C-terminal Tyr dipeptides, were retained on the column. These characteristics generally coincide with our results, but there are several discrepancies. No. 6, which showed an affinity for the AHC-agarose column, was not retained on the precolumn in this study. This, however, is probably attributable to the difference in the evaluation method used for the affinity. In this study, the affinity was expressed as the percentage of peptides retained on the precolumn after elution with a definite volume of S1 and the washing buffer, whereas that for the AHC-agarose column as the pH values of eluents that enabled peptides to be eluted from the column. Therefore, it is possible that peptides showing weak affinity are washed out from the precolumn. In fact, the collection and analysis of the eluate from the precolumn showed that 99% of No. 6 was recovered in the eluate within 5 min after injection.

Another problem is the retention of Nos. 34 and 38, which have no aromatic amino acids at their C-termini. Although these two were not examined, it was reported previously that peptides having no aromatic amino acids at their C-termini showed no affinity for the AHC-agarose column [11,13]. We recently encountered a similar discrepancy: twelve out of thirty peptides having no Arg or Lys at their C-termini were retained on the AHT precolumn [16]. It is interesting that the non-selective adsorption of No. 34 was observed with both precolumns. As these precolumns were prepared by using diol-silica as a support and tresyl chloride as an activating reagent for immobilization of ligands, these common factors may be responsible for the non-selective adsorption.

In a previous study using the AHT precolumn [16], the use of 20 m*M* calcium chloride instead of acetate buffer as the washing solvent increased the selectivity of the precolumn, although decreasing concurrently the retention of some of the C-terminal Arg peptides and most of the C-terminal Lys peptides. The effect of the addition of calcium chloride to 20 m*M* acetate buffer (pH 5.0) was therefore examined in this study. As shown in Fig. 3, the retention of No. 34, a non-selectively adsorbed peptide, was depressed with increasing concentration of calcium chloride, and disappeared almost completely on addition of 5 m*M* calcium chloride. However, the retention of No. 13, a C-terminal Phe peptide, was also decreased. On the other hand, the retention of No. 22, a C-terminal Tyr peptide, was hardly affected by the addition of calcium chloride at least up to 5 m*M*. The retention of these peptides, Nos. 34, 13 and 22, were 23, 57 and 91%, respectively, when 15 m*M* sodium chloride, which had the same ionic strength as 5 m*M* calcium chloride, was added to the buffer. The retentions of other peptides having aromatic amino acids at their C-termini, Nos. 14, 16, 18, 19, 20 and 21, were 13, 19, 106, 95, 4 and 90%, respectively, when 20 m*M* acetate buffer containing 5 m*M* calcium chloride was used as the washing solvent. These results indicate that the addition of calcium chloride enhances the selectivity of the AHC precolumn, although decreasing concurrently the retention of some of the

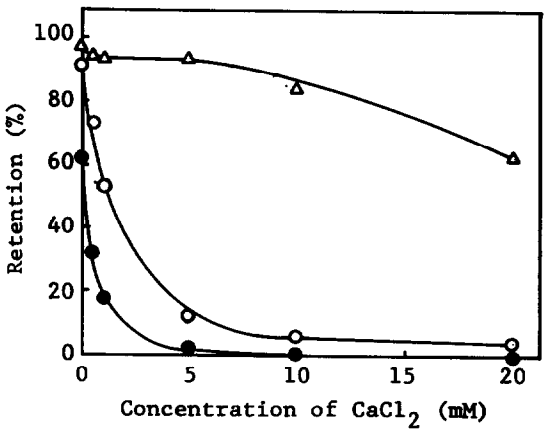

Fig. 3. Effect of addition of calcium chloride on the retention of peptides. ○, No. 13; △, No. 22; ●, No. 34.

peptides having aromatic amino acids at their C-termini. The reason why calcium chloride effectively suppresses the non-selective adsorption of peptides on AHC and AHT precolumns is obscure, but is under investigation in connection with the common factors for the preparation of both precolumns as described above.

Separation of a model peptide mixture

Fig. 4 shows the separation of a model peptide mixture by CS-HPLC. Peptides having aromatic amino acids at their C-termini were retained almost quantitatively, and other peptides were completely removed. Therefore, the CS-HPLC system equipped with theAHC precolumn is useful for the selective separation of peptides having aromatic amino acids at their C-termini.

Enrichment

As No. 22 showed a fairly high affinity for the AHC precolumn, as demonstrated in Fig. 3, an enrichment experiment was carried out using this peptide. Prior to the enrichment experiment, the optimum retention conditions for No. 22 were investigated. Back-flushing was employed because it gave a higher peak height for No. 22, as already shown in Fig. 2.

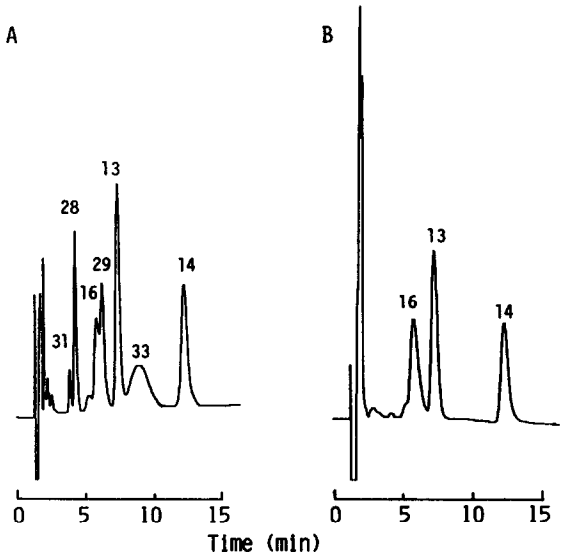

Fig. 4. HPLC separation of a model peptide mixture. A, without precolumn; B, with the AHC precolumn. Eluent S2, CH_3CN–10 m*M* H_3PO_4 (26:74, v/v) containing 0.1 *M* $NaClO_4$ and 0.5 *M* NaCl.

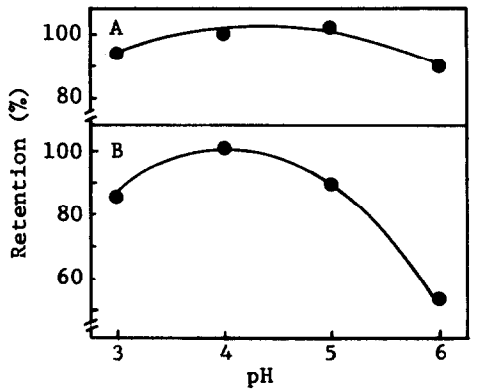

Fig. 5. Effect of pH of 60 m*M* acetate buffer on retention of No. 22. The retention was calculated on the basis of (A) peak area and (B) peak height.

Fig. 5 shows the effect of the pH of the washing buffer. The highest and quantitative retention was obtained at pH 4.0 when evaluated on the basis of peak height. The marked decrease in retention at pH 6.0 can probably be attributed to broadening of the sample zone in the precolumn, because the retention was not decreased so much when evaluated on the basis of peak area. Fig. 6 shows the effect of the concentration of washing buffers. The retention was not influenced with acetate buffer at least up to 0.3 *M*, but decreased gradually with increasing

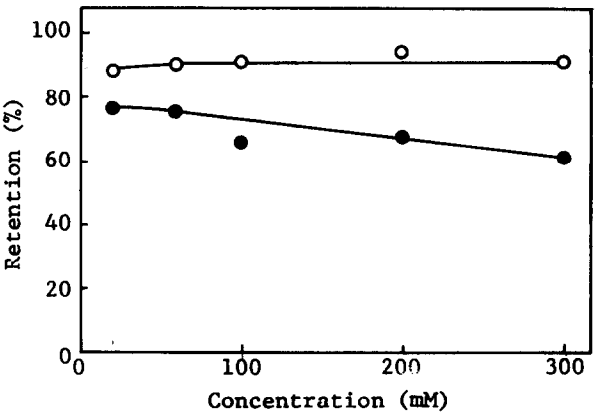

Fig. 6. Effect of concentration of washing buffers on retention of No. 22. The retention was calculated on the basis of peak height. ○ = Acetate buffer (pH 4.0); ● = phosphate buffer (pH 4.0).

concentration of phosphate buffer. Acetate buffer (pH 4.0, 60 m*M*) was therefore employed as the washing solvent.

The efficiency of enrichment was evaluated on the basis of the linearity and slope of calibration graphs constructed at three sample sizes (0.0625–1 nmol per 50-, 250- and 500-μl injection). The linear relationships ($r = 0.9975$–0.9991) between the peak height and the injected amount and the good reproducibility [relative standard deviations ($n = 4$) for 0.25 nmol = 2.5–5.4%] were observed at any sample size. The slope of the graph decreased with increase in sample size, but the slope obtained with 500-μl injections was still about 73% of that with 50-μl injections, indicating that the peptide could be enriched by this system.

Stability of the AHC precolumn

The stability of the AHC precolumn was evaluated by measuring the retentions of several peptides having aromatic amino acids at their C-termini after various periods of operation. The precolumn showed no decrease in retention after exposure for about 450 cycles to acidic eluents containing up to 30% acetonitrile during 1 year; the retentions of Nos. 13, 18 and 22 1 year after the preparation of the precolumn were 92, 100 and 94%, respectively.

CONCLUSIONS

The results demonstrate that in the CS-HPLC separation of peptides the AHC precolumn offers improved selectivity different from that obtained with the reversed-phase and immunoaffinity precolumns used previously. The AHC precolumn may be useful for on-line pretreatment in the HPLC determination of peptides having aromatic amino acids at their C-termini in biological fluids because of its high stability. In addition, this CS-HPLC system would be applicable for the rapid isolation and on-line identification of C-terminal peptide fragments from tryptic digests of proteins having aromatic amino acids at their C-termini. It would similarly be applicable for C-terminal peptide fragments from chymotryptic digests of proteins having no aromatic amino acids at their C-termini if the system were to be changed so that the non-retained fraction from the AHC precolumn is introduced into the analytical column.

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