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Application of an anhydrotrypsin-immobilized precolumn for selective separation of peptides having arginine or lysine at their C-termini by column-switching high-performance liquid chromatography

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ABSTRACT

A column-switching high-performance liquid chromatographic (CS-HPLC) system which consisted of an anhydrotrypsin (AHT)-immobilized diol–silica precolumn and a reversed-phase analytical column was developed for the selective separation of peptides having Arg or Lys at their C-termini. Tuftsin (Thr-Lys-Pro-Arg) could be enriched almost quantitatively on the precolumn when loaded with water as a carrier solvent and the precolumn was washed with 10–30 m*M* acetate buffer (pH 5.0). An investigation of the affinity characteristics of 55 peptides to the AHT precolumn showed that among twelve peptides having Arg or $ArgNH_2$ at their C-termini and more than four amino acid residues, ten were retained almost quantitatively on the precolumn, and eight out of nine peptides having Lys at their C-termini were less retained. The peptide having D-Arg at its C-termini was not retained. However, twelve out of thirty peptides having no Arg or Lys at their C-termini were also retained, but the retention was greatly decreased, in contrast to the Arg peptides, when the precolumn was washed with 20 m*M* calcium chloride solution. The results indicate that the CS-HPLC system equipped with an AHT precolumn offers new selectivity in the HPLC separation of peptides.

INTRODUCTION

Column switching (CS) is a powerful technique in high-performance liquid chromatography (HPLC) for the separation of compounds of interest in multi-component samples. For the analysis of peptides that have a large number of isomers, the technique is an especially good candidate to give better resolution. Reversed-phase and immunoaffinity precolumns have so far been used for the CS-HPLC analysis of peptides [1–5]. The immunoaffinity method offers highly selective retention, but the preparation of antibody 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), a catalytically inert derivative of trypsin in which the active site Ser residue is converted into a dehydroalanine, is known to exhibit affinity

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toward peptides having Arg or Lys at their C-termini, and AHT-immobilized agarose has been used as a selective adsorbent for the isolation of the C-terminal peptides from tryptic or chymotryptic digests of proteins [6–10].

Recently, we have developed an AHT-immobilized diol–silica for the high-performance affinity chromatographic separation of peptides having Arg or Lys at their C-termini from the other [11]. The excellent characteristics of the AHT column with regard to separation speed and stability prompted us to investigate the possibility that it might be used as a precolumn in CS-HPLC.

In this study, we developed a CS-HPLC system in which peptides retained on an AHT-immobilized diol—silica precolumn were selectively transferred to and separated on a reversed-phase analytical column, and affinity characteristics of various peptides with respect to the AHT column were examined by using the system.

EXPERIMENTAL

Materials

Peptides were purchased from the Peptide Institute (Osaka, Japan) and Sigma (St. Louis, MO, U.S.A.). Diol–silica was prepared from LiChrospher Si 300 (10 μm) (E. Merck, Darmstadt, Germany) as described previously [11]. Other chemicals were of analytical-reagent grade. Deionized water (obtained with a Millipore RO-Q system) was used throughout.

Preparation of an AHT-immobilized precolumn

An AHT-immobilized precolumn (10 × 4.6 mm I.D.) was prepared as described previously [11]. In brief, diol–silica, prepared by silanization of LiChrospher Si 300 (10 μm) with 3-glycidoxypropyltrimethoxysilane under anhydrous conditions followed by hydrolysis of the epoxy groups with 0.01 *M* hydrochloric acid, was activated with 2,2,2-trifluoroethanesulphonyl chloride, and AHT was immobilized onto the diol–silica by shaking the activated gel in phosphate buffer (pH 8.0) containing AHT. The amount of AHT immobilized onto the diol–silica was 10 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 LC-6A pump (P1) (Shimadzu, Kyoto, 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

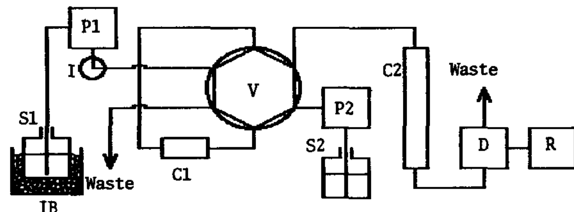

Fig. 1. Schematic diagram of the CS-HPLC system. P1, P2 = Pumps; C1 = precolumn; C2 = analytical column; I = injector; S1, S2 = eluents; IB = ice-bath; V = column-switching valve; D = detector; R = integrator.

injected onto the AHT precolumn (C1) through a Model NVI-3002 injector (Nihon Seimitsu Kagaku, Tokyo, Japan) equipped with a 1- or 5-ml loop. After removal of the sample from the loop, 1 ml of 10 m*M* acetate buffer (pH 5.0) was injected to eliminate non-specific adsorption of peptides, and the precolumn was further washed with the eluent for 5–10 min depending on the loop volume. The switching valve, a Model NHV-7000-6M (Nihon Seimitsu Kagaku), was then changed from "load" to "inject". A Model LC-5A pump (P2) (Shimadzu) was used for back-flushing the retained peptide from the precolumn onto an analytical column (C2) (Asahipak ODP-50; 250 × 4.6 mm I.D.) at a flow-rate of 0.7 ml/min with 0.1 *M* $NaClO_4$–[CH_3CN–0.14% H_3PO_4 (1–30:99–70, v/v)] (S2) as eluents. The column eluent was monitored at 214 nm with a Jasco Model 870-UV detector (Japan Spectroscopic, Tokyo, Japan) connected to a Chromatopac CR-1B integrator (Shimadzu).

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

RESULTS AND DISCUSSION

Optimization of the CS-HPLC system

A preliminary study using non-immobilized diol–silica in the precolumn showed that certain peptides were non-specifically retained. In order to eliminate such non-specific binding, washing of the precolumn with acetate buffer (pH 5.0) after loading a sample was examined. Fig. 2 shows the retention of a peptide, No. 14 (tuftsin), on the AHT and the diol–silica precolumns after washing with various concentrations of the acetate buffers. The non-specific binding of tuftsin to the diol–silica precolumn was eliminated completely by washing with 1 m*M* acetate buffer. On the other hand, almost quantitative retention was obtained at least up to 30 m*M* when the AHT precolumn was used, indicating that tuftsin was retained on the precolumn by its affinity to immobilized AHT.

Fig. 3 shows the effect of the flow-rate of S1 on the retention of tuftsin on the AHT precolumn. Almost quantitative retention was observed at flow-rates of 0.5–1.5 ml/min. As ligand–ligate interaction is generally decreased in affinity chromatogra-

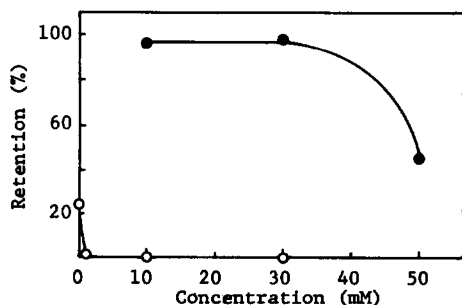

Fig. 2. Effect of concentration of acetate buffer (pH 5.0) on the retention of tuftsin on (○) diol–silica and (●) AHT precolumns. Tuftsin: 2 nmol in 400 μl.

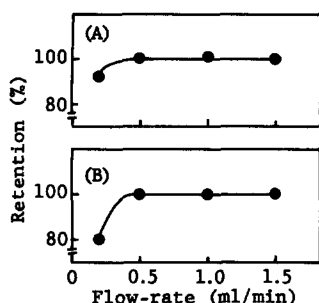

Fig. 3. Effect of flow-rate on retention of tuftsin on the AHT precolumn. Tuftsin: (A) 0.2 nmol in 400 μl; (B) 0.2 nmol in 1.6 ml.

phy with an increase in flow-rate, the lower retention at a flow-rate less than 0.5 ml/min seems strange. However, no further studies were carried out to clarify the phenomenon. In this study, a flow-rate of 0.5 ml/min was used.

Using the defined flow-rate and concentration of washing buffer, calibration graphs for tuftsin were constructed using various sample sizes. Linear relationships between the peak height and tuftsin concentration were observed at any sample size, but good reproducibility was obtained for sample sizes up to 1.6 ml (Table I). The detection limit (signal-to-noise ratio = 2) was 5 ng for a 1.6-ml injection (6.25 n*M*).

Affinity characteristics of various peptides

As tuftsin could be enriched and determined using the present CS-HPLC system, the retentions of various peptides on the AHT precolumn after washing with 10 or 30 m*M* acetate buffer (pH 5.0) were also examined. As shown in Table II, peptides that had Arg at their C-termini and more than four amino acid residues, except Nos. 14 and 16, were retained almost quantitatively on the AHT precolumn after washing with 10 m*M* acetate buffer. These peptides, except No. 7, were also retained in good yield after washing with 30 m*M* acetate buffer. No. 11, which had C-terminal D-Arg was not retained at all, whereas No. 5, which had C-terminal $ArgNH_2$, was retained almost quantitatively. The peptides that had Lys at their C-termini showed less affinity to the AHT precolumn, especially after washing with 30 m*M* acetate buffer.

TABLE I

CALIBRATION GRAPH FOR TUFTSIN

Sample volume (ml)	Regression equation[a]	Relative standard deviation (%)[b]
0.1	$y = 2.91x - 0.36$ ($r = 0.9999$)	1.6
0.4	$y = 2.53x + 0.12$ ($r = 0.9998$)	4.9
0.8	$y = 2.09x + 0.45$ ($r = 0.9997$)	2.4
1.6	$y = 2.28x - 0.02$ ($r = 0.9998$)	7.6
3.2	$y = 2.10x - 0.10$ ($r = 0.9998$)	14.2

[a] 0.1–20 nmol per injection. y = peak height (cm); x = injected amount (nmol).
[b] 0.2 nmol per injection ($n = 4$).

TABLE II

EFFECT OF WASHING WITH ACETATE BUFFER ON THE RETENTION OF PEPTIDES ON THE AHT AND DIOL–SILICA PRECOLUMNS

No.	Peptides[a]	Retention (%)			
		AHT precolumn		Diol–silica precolumn	
		10 mM[b]	30 mM[b]	10 mM[b]	30 mM[b]
1	Tyr-Arg	4	0	0	–[c]
2	Bz-Gly-Arg	0	–	0	–
3	Gly-Gly-Arg	0	–	–	–
4	Thr-Lys-Pro-Arg	97	101	0	–
5	Tyr-Ile-Gly-Ser-ArgNH_2	90	92	0	–
6	His-Leu-Gly-Leu-Ala-Arg	97	92	0	–
7	Tyr-Gly-Gly-Phe-Leu-Arg	105	21	0	–
8	Tyr-Gly-Gly-Phe-Leu-Arg-Arg	99	96	1	1
9	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg	91	97	7	4
10	Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg	80	89	0	–
11	Dnp-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg	0	–	0	–
12	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	97	100	3	2
13	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg	93	99	10	5
14	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg	17	7	0	–
15	Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	89	88	3	2
16	Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg	0	–	0	–
17	Bz-Gly-Lys	0	–	0	–
18	Lys-Trp-Lys	96	105	2	2
19	Thr-Pro-Arg-Lys	87	103	0	–
20	Pro-Phe-Gly-Lys	44	0	0	–
21	Tyr-Gly-Gly-Phe-Met-Lys	68	4	1	–
22	Tyr-Gly-Gly-Phe-Leu-Lys	55	4	1	–
23	Ser-Ile-Gly-Ser-Leu-Ala-Lys	20	0	0	–
24	Val-His-Leu-Thr-Pro-Val-Glu-Lys	89	84	0	–
25	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys	52	61	7	4
26	Gly-Gly-Gly	0	–	–	–
27	Tyr-Pro-Phe	0	–	0	–
28	Tyr-Tyr-Phe	0	–	0	–
29	Glu-Val-Phe	0	–	0	–
30	Met-Leu-Phe	0	–	0	–
31	Val-Ala-Ala-Phe	0	–	0	–
32	Ala-Ala-Ala-Ala	0	–	–	–
33	Arg-Gly-Asp-Ser	0	–	–	–
34	Gly-Arg-Gly-Asp	0	–	0	–
35	Arg-Pro-Lys-Pro	87	87	0	–
36	Phe-Gly-Gly-Phe	0	–	0	–
37	Phe-Leu-Glu-Glu-Val	0	–	0	–
38	Tyr-Pro-Phe-Pro-Gly	0	–	0	–
39	Tyr-Gly-Gly-Phe-Leu	0	–	0	–
40	Tyr-Gly-Gly-Phe-Met	0	–	0	–
41	Arg-Ser-Arg-His-Phe	52	62	19	6
42	Arg-Lys-Asp-Val-Tyr	109	20	0	–
43	Lys-Val-Ile-Leu-Phe	78	32	0	–
44	Arg-Val-Tyr-Ile-His-Pro-Phe	86	86	2	1

(Continued on p. 64)

TABLE II *(continued)*

No.	Peptides[a]	Retention (%)			
		AHT precolumn		Diol–silica precolumn	
		10 m*M*[b]	30 m*M*[b]	10 m*M*[b]	30 m*M*[b]
45	Arg-Val-Tyr-Ile-His-Pro-Ile	92	91	0	–
46	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	1	0	0	–
47	Tyr-Gly-Gly-Phe-Met-Arg-Phe	73	71	0	–
48	Ser-Met-Glu-Val-Arg-Gly-Trp	0	–	0	–
49	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	60	58	1	–
50	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	78	77	1	1
51	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu	97	97	0	–
52	Ala-Ser-Thr-Thr-Thr-Asp-Tyr-Thr	0	–	0	–
53	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro	71	69	3	2
54	Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu	0	–	0	–
55	Arg-Arg-Leu-Ile-Glu-Asn-Ala-Glu-Tyr-Ala-Ala-Arg-Gly	100	100	0	–

[a] 1 nmol per 50-μl injection (5 nmol for Nos. 3, 26 and 32).
[b] Concentration of acetate buffer (pH 5.0).
[c] Not determined.

Among thirty peptides that had no Arg or Lys at their C-termini, twelve were retained considerably on the AHT precolumn, as opposed to our expectations. On the other hand, almost all the peptides examined showed no affinity to the diol–silica precolumn, indicating that the retained peptides were recognized with the immobilized AHT.

Ishii and co-workers [6–10] investigated the chromatographic behaviour of twenty peptides (twelve C-terminal Arg peptides, six C-terminal Lys peptides and two other peptides) on an AHT–agarose column, and reported that Arg or Lys peptides having more than three amino acid residues, except those having D-Arg or $ArgNH_2$, were retained on the column, and that the Lys peptides showed less affinity to the column. These characteristics generally coincided with our results, but there are several discrepancies. Peptides Nos. 2 and 16, which showed a strong affinity to the AHT–agarose column, were not retained on the AHT precolumn in this study. In order to assess the possibility that these were retained but not eluted from the precolumn, the eluates from the precolumn were collected and analysed by HPLC. The eluates contained 90% (No. 2) and 94% (No. 16) of the loaded peptides, indicating that these were not retained on the AHT precolumn. Because the eluent used in this study (water and 10–30 m*M* acetate buffer, pH 5.0) was different from that in previous studies (20 m*M* calcium chloride–50 m*M* acetate buffer, pH 5.0), the retention behaviour of these peptides on the AHT precolumn pre-equilibrated with 20 m*M* calcium chloride or 20 m*M* calcium chloride–10–50 m*M* acetate buffer (pH 5.0) was also investigated. However, none of them were retained on the precolumn at all. Therefore, the discrepancy in retention between our study and previous studies is likely to be attributable to differences in the chromatographic supports and the method of immobilization of AHT.

The retention of $ArgNH_2$ peptide No. 5 also seems to conflict with the results of the previous investigation in which Bz-$ArgNH_2$, in contrast to Bz-Arg, is not retained on the AHT–agarose column. However, the number of $ArgNH_2$ peptides examined in both studies seems too small to draw conclusions.

Another serious problem is the retention of some of the peptides that have no Arg or Lys at their C-termini on the AHT precolumn. However, it should be noted that only two such peptides were examined previously, and that the recovery of C-terminal peptides having no Arg or Lys at their C-termini from the AHT–agarose column loaded with tryptic digests of proteins was reported sometimes to be low [8]. Therefore, the retention of these peptides observed in our study seems not to conflict necessarily with the previous observations. In order to clarify whether these peptides are recognized at the same site of immobilized AHT as the peptides having Arg or Lys at their C-termini, the retentions of No. 50 on the AHT precolumn and that presaturated with tuftsin were compared. No difference in retention between the two columns was observed, as shown in Fig. 4, indicating the presence of multiple sites on the immobilized AHT for affinity of peptides. However, it still remains uncertain what factor governs the retention of some of the peptides having no Arg or Lys at their C-termini.

Reinvestigation of the washing solvents

The above results indicated that the selectivity of the AHT precolumn was not necessarily satisfactory because of the retention of some of the peptides having no Arg or Lys at their C-termini. Therefore, other washing solvents were investigated. As trypsin had a calcium binding site and was stabilized with the ion [12], washing with 20 m*M* calcium chloride was compared with that with 60 m*M* sodium chloride, which had the same ionic strength. The effect of these washings on retention of the selected peptides is shown in Fig. 5. The retention of some of the Arg peptides, Nos. 7 and 10, and most of the Lys peptides, Nos. 20–24, was decreased greatly by using 60 m*M* sodium chloride, which had a higher ionic strength than 10 and 30 m*M* acetate

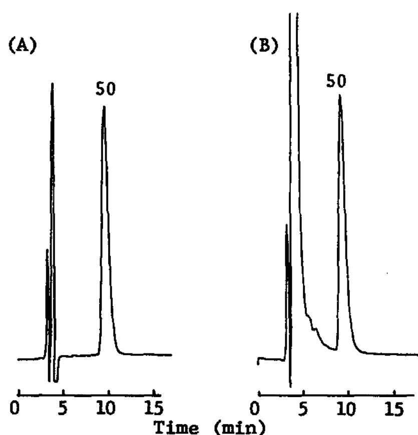

Fig. 4. Effect of presaturation of the AHT precolumn with tuftsin on the retention of peptide No. 50. (A) AHT precolumn; (B) AHT-precolumn presaturated with tuftsin (0.5 μmol). No. 50: 1 nmol in 50 μl. Mobile phase: 0.1 *M* $NaClO_4$–[CH_3CN–0.14% H_3PO_4 (30:70, v/v)].

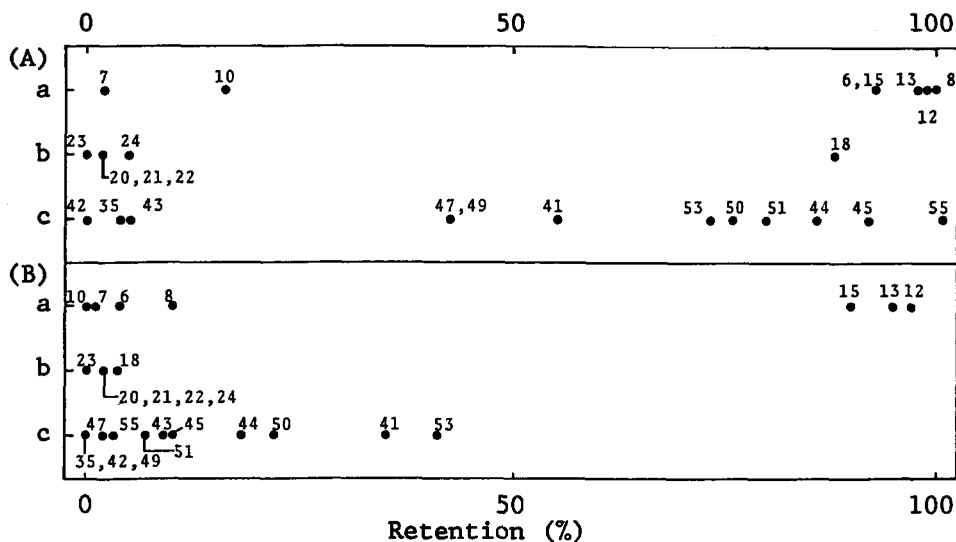

Fig. 5. Effect of washing solvents on retention of peptides on the AHT precolumn. (A) 60 mM NaCl; (B) 20 mM $CaCl_2$. (a) Peptides having Arg at their C-termini; (b) peptides having Lys at their C-termini; (c) peptides having no Arg or Lys at their C-termini. Peptide numbers as in Table II.

buffers. The retention of many of the peptides having no Arg or Lys at the C-termini, however, was not changed or changed only slightly. On the other hand, the retention of all of these peptides with no Arg or Lys was decreased greatly by using 20 mM calcium chloride, while some Arg peptides, Nos. 12, 13 and 15, remained unchanged. These results indicated that the selectivity of the AHT precolumn was enhanced by the use of 20 mM calcium chloride although some of the Arg peptides became unretained. The difference in retention between the Arg peptides and peptides with no Arg or Lys supports the notion that multiple sites on the immobilized AHT are responsible for peptide affinity.

Selective separation of peptides by CS-HPLC

The above results indicate that the selectivity of the AHT precolumn can be changed by changing the washing solvent. Fig. 6 shows the HPLC of a model peptide mixture after washing with various solvents. No. 28, which had Phe at the C-termini, was removed by washing with 10 mM acetate buffer (B) and, in addition, No. 22, which had Lys at the C-termini, was removed by washing with 30 mM acetate buffer (C). When the precolumn was washed with 20 mM calcium chloride, all of the peptides that had no Arg at C-termini were removed (D). Therefore, changing the concentration and/or type of washing solvent is useful for controlling the selectivity of the precolumn.

Stability of the AHT precolumn

We determined the stability of the AHT precolumn by measuring the retention of tuftsin after various periods of operation. The AHT precolumn showed no decrease in retention after exposure for about 800 cycles to acidic eluents containing

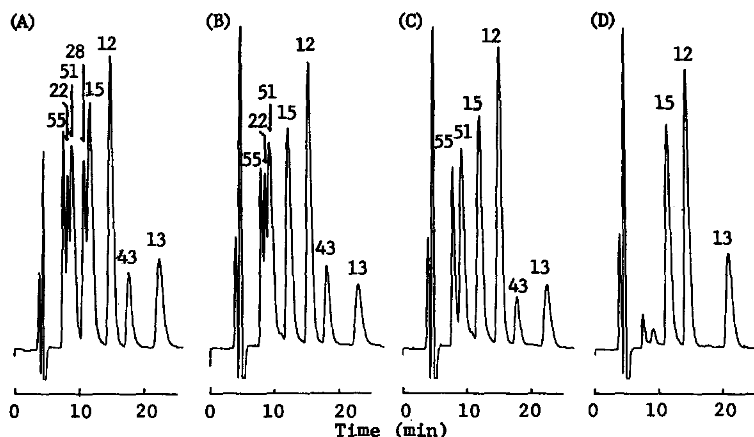

Fig. 6. HPLC of a model peptide mixture after washing the AHT precolumn with various solvents. (A) The mixture (50 μl) containing 1 nmol each of peptides was chromatographed without the AHT precolumn (B)–(D) the mixture (400 μl) containing 1 nmol each of peptides was chromatographed with the AHT precolumn [washing solvent: B, 10 m*M* acetate buffer (pH 5.0); C, 50 m*M* acetate buffer (pH 5.0); D, 20 m*M* $CaCl_2$]. Mobile phase: 0.1 *M* $NaClO_4$–[CH_3CN–0.14% H_3PO_4 (30:70, v/v)].

acetonitrile during 1 year: the retention (2 nmol per 400-μl injection) at 2, 5, 8 and 12 months after the preparation of the column were 96, 104, 105 and 93%, respectively.

CONCLUSIONS

Reversed-phase and immunoaffinity precolumns have been used for the CS-HPLC separation of peptides [1–5]. The use of the AHT precolumn offers new selectivity in the CS-HPLC separation of peptides. The AHT precolumn may be useful for on-line sample pretreatment in the HPLC determination of peptides having Arg or Lys at their C-termini in biological fluids because of its high stability.

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