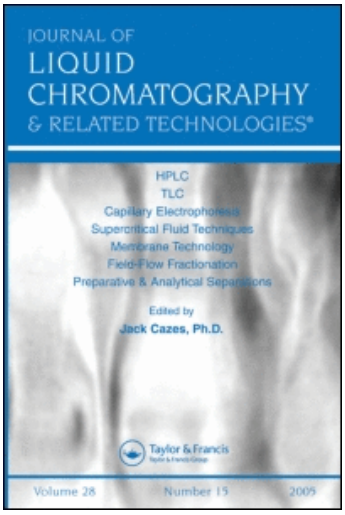

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HIGH PERFORMANCE CATION EXCHANGE CHROMATOGRAPHY OF BASIC PEPTIDES

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ABSTRACTS

S-Zephyr, a hydrophilic high performance cation exchanger, has been successfully used for the separation of a series of synthetic basic peptides. Due to the absence of any hydrophobic character of the support, a linear relationship was found between the retention time and the number of cationic charges. The ability to discriminate peptides according to their charge, even in case of one unit difference, are illustrated in the total separation of a series of seven peptides, which included mtr-arginine impurity derived from peptide synthesis. Preparative trials in overloading conditions were studied for each peptide to give in the best case 99.2% final purity with a dynamic sorption capacity of 18 mg per ml of sorbent.

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INTRODUCTION

The relationship between structure and biological function of proteins can usually be investigated through studies of peptides derived from enzymatic cleavages (1,2), or from automated solid-phase synthesis after sequence determination. In this way, preparative High Performance Reverse Phase liquid Chromatography is considered a promising technical tool in routine purification of peptides (3). Nevertheless, while this high performance technique can be successfully applied to hydrophobic compounds, reverse chromatography is often unappropriate for the separation of very hydrophilic peptides (4). To study hydrophilic peptidic sequences involved in antigenic sites, and more generally, water accessible sites of proteins (5) , gradient elution ion exchange chromatography (IEC) is more convenient. However, because of difficulties due to the low flow rates utilized with the classical soft gels generally employed, more rigid packing materials with smaller particles are better suited to high performance IEC. As described by Mant and Hodges (6), strong cation exchange chromatography is a useful mode for peptides separations. In this context a rigid sorbent constituted of mineral particles coated with dextran sulfate has been used for this purpose.

Most generally, electrostatic interaction strength between peptides and ion exchangers increases in proportion to the respective peptide charge density. Consequently peptide retention times vary as a function of the number of charges and the pH (7,8) . In practice,

however, the hydrophobic character of most HPLC ion exchange packings derived from cross-linked aliphatic bridges (9), or from polymeric hydrophobic matrices (10), leads to mixed-mode chromatography (6), rendering unpredictable the peptide behaviour unlike in reverse phase chromatography (11). The packing material used here has been designed in order to maximize hydrophilicity by directly coating mineral particles with dextran, forming a highly hydrated polymer layer. This approach aims to establish direct global charge versus retention time relationship for basic peptides by suppressing hydrophobic contribution effect.

With the use of such material it was our objective to try to demonstrate a linear relation between the retention time and the charge density of special basic peptides (up to 15 residues) synthesized according to Merrifield technique (12).

In this paper, we describe the use of S-ZEPHYR® to predict first the retention time of peptides according to their charge. The preparative potential of this cation exchanger is also described by loading up to 100 mg of peptide per milliliter of packing.

EXPERIMENTAL

Materials

0.22µm disposable MILLIPAK 200 GLCL filters were from Millipore Corporation (Bedford, MA, U.S.A.). HPLC-grade acetonitrile, Gen-apex quality tris(hydroxymethyl)-aminomethane, Gen-apex sodium chloride,

phosphate and acetate salts were obtained from Prolabo (Paris, France). All synthetic peptides were supplied by Neosystem (Strasbourg, France), except peptide +5 obtained from Rhône-Poulenc-Rorer (Vitry s/ Seine, France)

Peptides

All arginine residues were protected during the synthesis by the fluorhydric acid labile tosyl (p-toluen-sulfonyl) group except for peptide +5. In this last peptide, arginine residues were protected by pmc [2,2,5,7,8-pentamethylchromo-6-sulfonyl] group (13). Structure of peptide +4b was the same as the peptide +5 except that arg-4 was protected with mtr [4-methoxy-2,3,6-trimethyl-benzenesulfonyl] blocking group (14). All peptides were firstly desalted on Sephadex G10 immediately after synthesis, except peptides +4b and +5 that were directly purified by cation exchange. All studied peptides were classified according to their overall charge at neutral pH, as follows:

+1 [Leu-Arg-Pro-Leu-Tyr-Ile-Asp-Phe-Lys-Arg-Asp-Leu]

+2 [Ac-Leu-Lys-Leu-Lys-NH2]

+3 [Ile-Thr-Arg-Gln-Arg-Tyr-NH2]

+4a [Lys-Ser-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile]

+4b [Leu-Lys-Gln-Arg(mtr)-Arg-Arg-Ala-Gly-Gln-Leu-Val-Asn-Tyr-Lys-Gly]

+5 [Leu-Lys-Gln-Arg-Arg-Arg-Ala-Gly-Gln-Leu-Val-Asn-Tyr-Lys-Gly]

+7 [Ala-Asn-Arg-Phe-Arg-Arg-Pro-Ala-Ser-Tyr-Val-Ser-Lys-Gly-Tyr-Lys-Thr-Arg-Arg-Leu-Ala]

Instrumentation

Chromatographic separations were performed on Merck-Clevenot (Nogent s/ Marne, Fance) Lichrograph L-6200, coupled to a L-4000 variable wavelength UV detector (5 mm and 2 mm optical pathlength for analytical and preparative flow cell, respectively) and D-2000+ integrator.

Low pressure Minipuls 3 peristaltic pump from Gilson (Villiers le Bel, France) was employed for peptide desalting on Sephadex G/10. PH-ion detector from IBF-Biotechnics (Villeneuve la Garenne,France) was used for low pressure and high pressure chromatography. Chargpro/ PC-Gene software (Genofit, Grand-Lancy, Switzerland) used at Rhône-Poulenc-Rorer (Vitry s/ Seine, France) was utilized to determine theoritical charge of peptides according to their sequence.

Columns

HPLC column S-Zephyr/10, 90 x 10 mm I.D., 20 µm particle size, 1000 A pore size for HPCEC (High Performance Cation Exchange Chromatography), was obtained from Sepracor-IBF Bioprocessing division (Villeneuve la Garenne, France). Low pressure Sephadex G-10 packed in glass column (177 ml bed length 880 x 16 mm I.D.), was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden).

Methods

Analytical cation exchange

0.1 mg of peptides in 200 µl solution were injected into a column of Zephyr/10, previously equilibrated in 50 mM sodium acetate (pH 3.5 or 4.5), sodium phosphate (pH 5.5, 6.5 or 7.5) or tris,HCl

at pH 8.6. Flow rate was 3.8 cm.min^{-1}. Elution was performed using a linear salt gradient from 0 to 1.5M started immediately after the injection of the sample. Gradient time was 20 minutes for individual peptides or 45 minutes with peptides mixtures. Detection was effected at 280 nm for peptides +1,+3,+5 and +7 and at 220 nm for peptides +2 and +4. A continuous detection of conductivity was measured at the outlet of the column to verify gradient slopes and validate the performance of the pump-gradient former. Retention times (RT) were calculated after integration of the dead time necessary for salt elution at the column outlet (4.4 min. equivalent to 13 ml).

Preparative cation exchange

• Dynamic sorption capacity measurement (dSC)

Sorption capacity of the packing material was determined for each peptide by means of breakthrough curves. A solution of 100 mg/ml was prepared in 50 mM phosphate adsorbtion buffer pH 7.5 and injected into the column at 1 ml.min^{-1} until peptide appeared in the flow through of the saturated column. Dynamic sorption capacity was then calculated in dividing peptide amount injected up to the saturation per the column volume.

• Preparative purification of peptides

Separation was done by peptide adsorption at 1 ml.min^{-1}, followed by a salt gradient at 3 ml.min^{-1}. Concentration of salt was between 0 to 1.5M, separation time was 20 minutes and UV detection

was performed at OD 2.5 AUFS. Quantity of injected sample per run was up to the sorption capacity of the column. Yield and peptide purity were determined by analytical HPLC and compared with calibration curves obtained using pure lyophilized peptides.

The separated pure peptides collected in the presence of salts were desalted prior lyophilization on a Sephadex G-10 column in the presence of 10% acetic acid in water.

RESULTS AND DISCUSSION

* **General peptides behaviour**

In figure 1 (a & b), retention times for the series of investigated peptides are shown to vary very slightly with pH and tend to decrease when pH increases. Assuming that the sulfonate groups of the sorbent were completly ionized at pH above 2 (pKa is about 1), the difference in peptide retention times obviously comes from the charge variation according to the pH. The only peptide that did not follow this behaviour was peptide +1 which showed a stronger pH dependence at low pH. This was due to the presence of two aspartic residues that contribute to decrease the ionic charge at pH 3.5.

To correlate the whole charge of the peptides with their retention times, we used a specific software program (CHARGPRO) able to define with accuracy the total charge of any peptide according to the pH buffer (figure 2). This theoretical charge, calculated as a function of each residue charge, is considered very accurate,

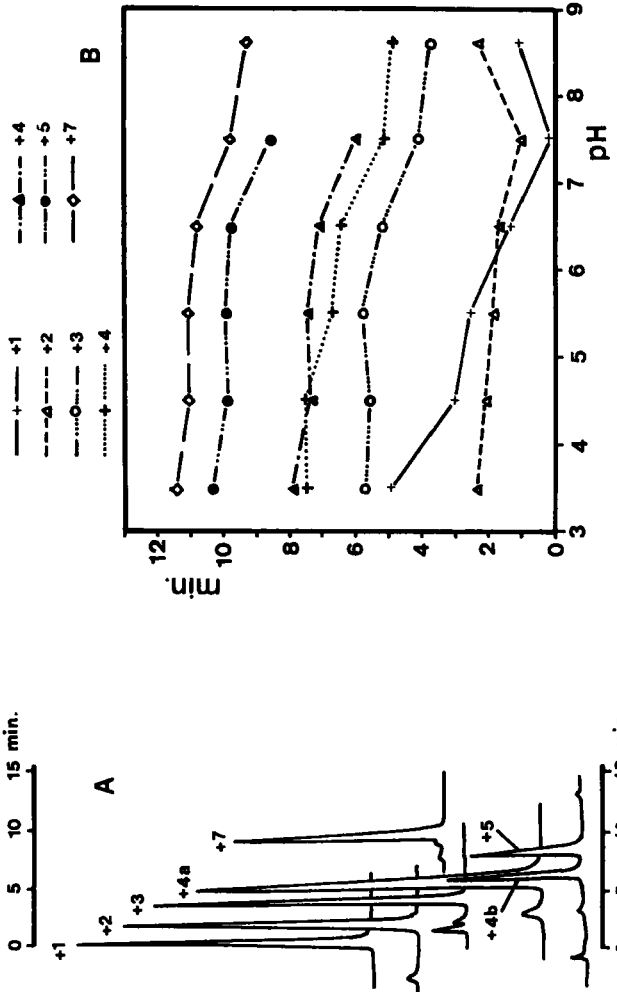

FIGURE 1 Analytical cation exchange chromatography of seven peptides on S-Zephyr /10

Peptide: overall charge (+1 to +7, pH7.5); load (0.1 mg each in 200µl loop)
Column : 10 mm I.D x 90 mm ; **Flow rate** : 3 ml/min.
Elution linear gradient : sodium chloride from zero to 1.5 M in 20 min.
a: Seven chromatographies of seven peptides in phosphate buffer 50 mM, pH7.5
b: Influence of pH on peptide retention times in 50 mM buffer
[acetate (pH 3.5 to 6.5), phosphate (pH 7.5) and tris,HCl (pH 8.6)]

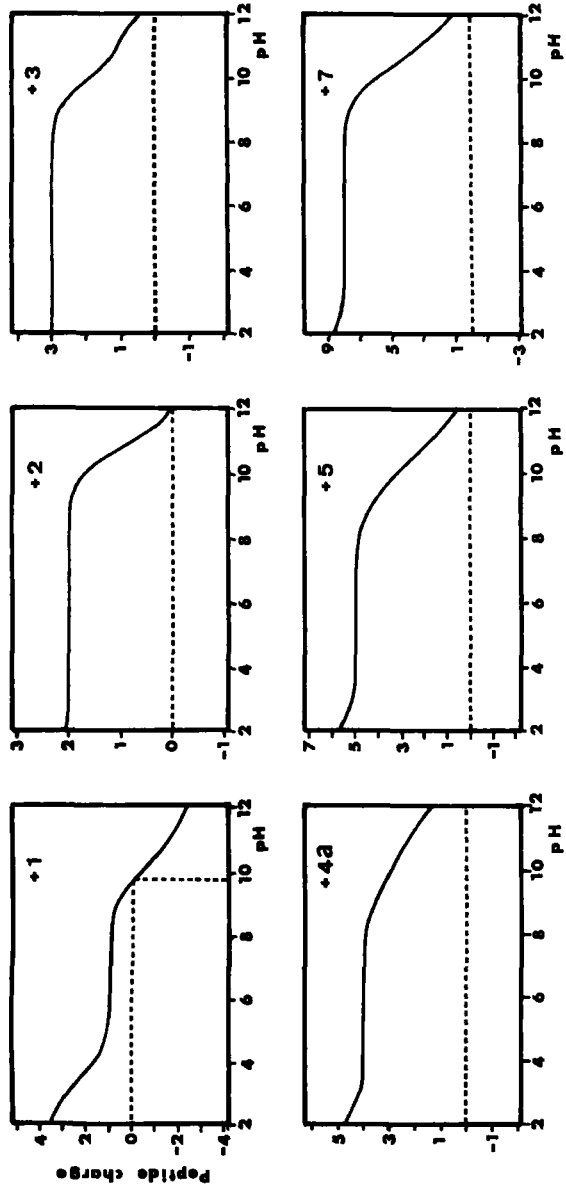

FIGURE 2 Theoritical calculation of overall positive charge for peptides +1 to +7 (see sequences in experimental)
Y axis: peptide charge
X axis: pH ranging from 3 to 12

particularly with all charged amino-acids of any peptide with less than 15 residues in aqueous solution.

Figure 3a shows a linear relationship between the peptide positive charge and retention time for seven peptides studied at pH 7.5 and 8.6. The linear correlation between the positive charge of the molecules and the salt concentration necessary for elution indicates a pure ionic interaction between the peptides and the cation exchanger. At pH 3.5, 4.5, 5.5 and 6.5, peptide retention times were significantly higher (figure 3b); the salt molarity to elute the peptide was also higher. Only peptide +2 showed a retention time to that observed at pH 7.5 and 8.6. This peptide alone did not show a linear relation between its charge and its retention time.

To demonstrate that no hydrophobic interaction occured between the sorbent and the peptides (6), the influence of acetonitrile in adsorption and elution buffers has been studied. Figure 4a summarizes all results on the influence of acetonitrile at different concentrations for the studied peptides and shows no real significant modification in the retention times (RT). About 10% RT decrease was found when acetonitrile was at 10% concentration. When plotting the RT versus calculated hydrophobicity Rekker's constants (11) (figure 4b), no relation was apparent. For Rekker's constants ranging from 1.5 and 3.0, the modification of RT was only +/- 10%.

The ability of S-Zephyr to separate peptides strictly according to their charge even in case of one unit difference is illustrated in the total separation of the series of six peptides

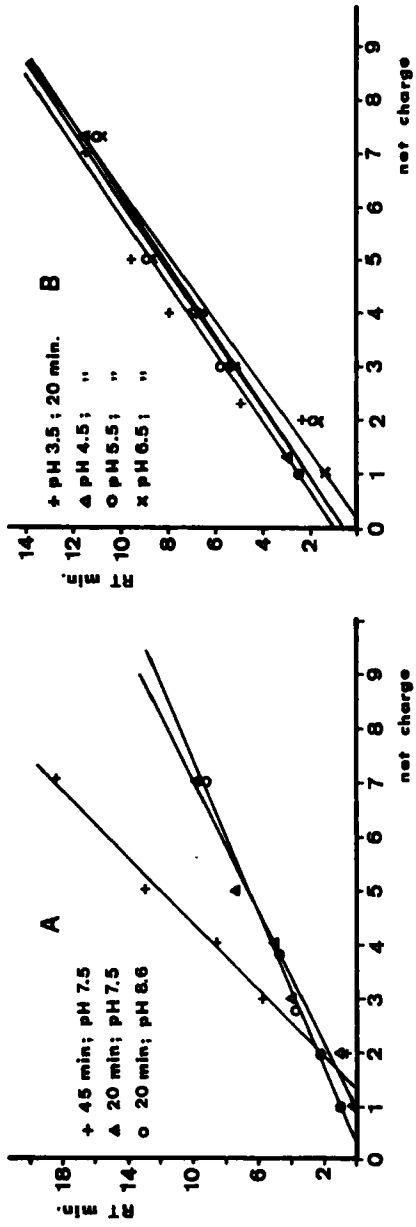

FIGURE 3 Influence of overall charge on retention time for seven peptides chromatographied on S-Zephyr/10 at basic pH (3a) and acidic pH (3b).

Peptide: load (0.1 mg each in 200µl loop) ; Column : 10 mm I.D x 90 mm ;
Flow rate: 3 ml/min.; Adsorption buffer : 50 mM acetate (pH 3.5 to 6.5), phosphate (pH 7.5) and tris (pH 8.6);
Elution linear gradient : sodium chloride from zero to 1.5M in 20 or 45 min.

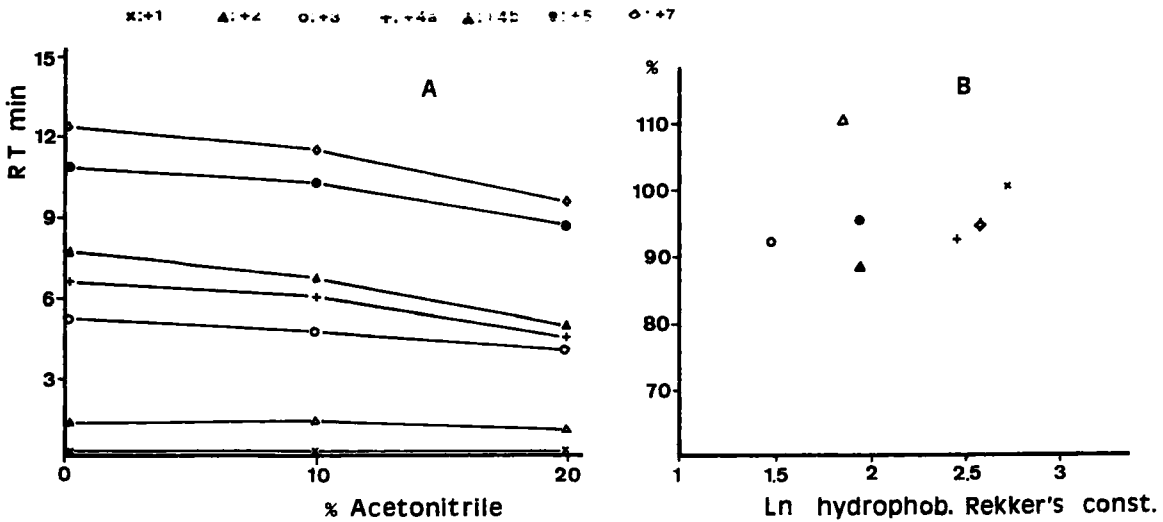

FIGURE 4 Influence of acetonitrile amount in mobile phase **(4a)** and peptide hydrophobicity **(4b)** on retention time for seven peptides chromatographied on S-Zephyr/10 at acidic pH.

Peptide: load (0.1 mg each in 200µl loop) ; **Column** : 10 mm I.D x 90 mm ;
Flow rate: 3 ml/min.;
Adsorption buffer : 50 mM acetate pH 5.5 with 10% acetonitrile **(4b)** and with 0, 10 and 20% acetonitrile **(4a)**.
Elution linear gradient : sodium chloride from zero to 1.5 M in 20 min.

(4b)
Y axis: % RT variation in adding 10% acetonitrile in mobile phases
X axis: Logarithm of Rekker's hydrophobicity constant of peptides (13)
Ln [1+1.1 Sum(k x n)]
where k is the Rekker's Cte of each amino-acide and n its number

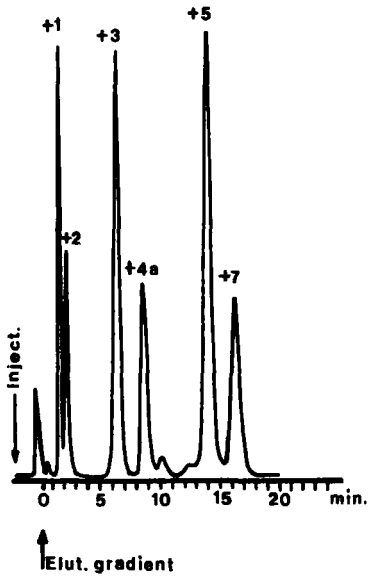

FIGURE 5

Analytical cation exchange chromatography of six peptides on S-Zephyr/10.

Peptide: overall charge (+1 to +7, pH7.5); load (0.1 mg each in 200µl loop)
Column : 10 mm I.D x 90 mm ; **Flow rate** : 3 ml/min.
Adsorption buffer : sodium phosphate buffer 50 mM, pH 7.5
Elution linear gradient : sodium chloride from zero to 1.5 M in 45 min.

(figure 5), where the salt gradient time was 45 minutes. Interestingly, peptides +4b and +5, having exactly the same structure and differing only by the modification of an arginine residue (arginine-4 free or mtr protected), were perfectly resolved.

These observations indicate that with hydrophilic peptides possessing cationic charges, it was possible to calculate and predict retention time values when separated on a hydrophilic column like S-Zephyr. Associating the separation efficiency of this HPLC sorbent and its charge-dependent selectivity, S-Zephyr demonstrated a particularly high discriminating power in separating complex mixtures of peptides.

• **Preparative separation of peptide +5**

It was observed that a mixture containing 100 µg peptides +4b and +5 (55% and 45% p/p respectively), could be separated in under 20 minutes using a salt gradient from zero to 1.5M (gradient slope: 7.5 $mM.min^{-1}$) To optimize the separation before increasing the loading to pass from an analytical to a preparative separation, the gradient slope was modified. It was found that by decreasing the gradient slope from 75 to 30 $mM.min^{-1}$, the resolution increased from 3.0 to 4.2. In this more favourable situation, the amount of injected sample was progressively increased until the conditions of "touching band" state (15,16) were reached (see figure 6). This situation was achieved when 14.5 mg of peptide mixture was injected into the column; which corresponded to 2.1 mg of peptide per ml of sorbent. The quantity of peptides separated in these conditions was 145 times higher than in previously studied analytical conditions.

Fractions from four "touching band" preparative chromatography representing 63.5 mg were analyzed in high performance cation exchange and pooled so as to have three entities: 99.2% pure +4b, 99.2% pure +5 a mixture of +4b and +5 peptides. After desalting chromatography and lyophilization in the presence of 10% acetic acid, the yield of the operation was close to 75% for both peptides +4b and +5 and their puritis were 98.8% and 97.7%, respectively.

These results demonstrated the ability of S-Zephyr to separate preparative quantities of peptides in good conditions after a limited number of optimization experiments, essentially restricted to the gradient slope.

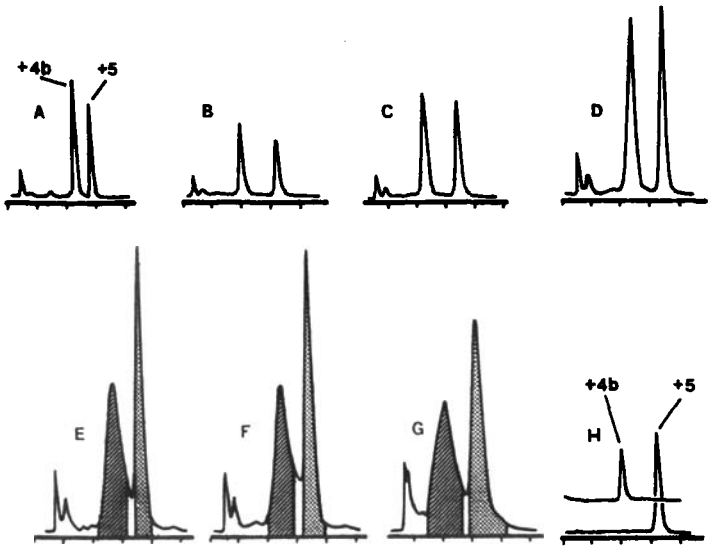

FIGURE 6

Scale up approach in HPCEC of peptides mixture +4b and +5 on S-Zephyr/10.

Column : 10 mm I.D x 90 mm ; **Flow rate** : 3 ml/min.
Adsorption buffer : sodium phosphate buffer 50 mM, pH 7.5

a) Peptide load: 0.1 mg
Elution linear gradient : sodium chloride from zero to 1.5 M in 20 min.
b) Peptide load: 0.1 mg
Elution linear gradient : sodium chloride from 0.225 M to 0.825 M in 20 min.
c) Peptide load: 1 mg
Elution linear gradient : sodium chloride from 0.225 M to 0.825 M in 20 min.
d) Peptide load: 4 mg
Elution linear gradient : sodium chloride from 0.225 M to 0.825 M in 20 min.
e) Peptide load: 14.5 mg
Elution linear gradient : sodium chloride from 0.225 M to 0.825 M in 20 min.
f) Peptide load: 20 mg
Elution linear gradient : sodium chloride from 0.225 M to 0.825 M in 20 min.
g) Peptide load: 14.5 mg
Elution linear gradient : sodium chloride from 0.375 M to 0.75 M in 20 min.
h) Analytical control of lyophilized pure peptides +4b and +5, derived from four preparative chromatographies (type g) and desalting on gel filtration.
Elution linear gradient : sodium chloride from 0.225 M to .825 M in 20 min.

• Overloading preparative chromatography of peptides

A peptide purification effect in overloading mode as described elsewhere (16,17,18), is expected essentially from a displacement effect of impurities by the main peak component. Using this approach, large peptide quantities, nearing the maximal sorption capacity of the sorbent, have been purified (19,20,21).

To find the overloading separation conditions, increasing quantities of peptide +1, +2, +3, +4a and 7 solutions (100 $mg.ml^{-1}$ of in tris buffer pH 7.5) were injected into the column of S-Zephyr. When overloading capacity was reached, it was considered that they could be utilized as a limit condition for preparative purposes; at this point and after elution under salt gradient, the peptides of interest were recovered and analyzed.

Yields and purity compared to the crude material were calculated on the final desalted and lyophilized peptide. Table 1 summarizes the results for the peptides +1, +2, +3, +4 and +7.

Maximal dynamic sorption capacity of S-Zephyr for most studied peptides was rather high, ranging from 6.3 to 18.3 mg per ml of sorbent under the described conditions. Only the sorption capacity for peptide +2 was quite low (1.4 mg per ml of sorbent), due to the synergistic effect of a low positive charge (+2, pH 7.5) and a very low hydrophobic character (figure 4b, peptide +2 is the least hydrophobic). It was in any case demonstrated that adsorption and elution in saturation condition did not disturb the mechanism of peptide purification (44 mg and 128 mg of +1 and +3 respectively adsorbed), where the less retained impurities of +3 were displaced

TABLE N°1

PREPARATIVE CATION EXCHANGER

peptide	anal RT (min.)	purity (%)	solubility (mg/ml)	MDCP (mg/ml)	purity (%)	yield (%)
+1	0.05	99.3	> 100	6.3	99.5	73
+2 imp	1.00 less retained	86.0	> 100	1.4	97	/
+3 imp	4.10 less retained	88.0	> 100	18.3	99.2	89
+4 imp	5.10 less retained	84.0	ar. 1	>14.3	86	95
+7 imp	9.70 less retained	96.0	> 100	>10	98	95

+1 to +7 (main peaks)
"imp" (impurity of each peptide)

by the main component (figure 7). Peptides +2 and +7 were also separated to a very high degree of purity (>97% pure).

Separation yields were always high (73 to 89%); highest values were found when saturation level was not reached and when no loss in the flow through occured (peptides +4 and +7).

Desalting operations on G-10 column decreased the yield. The best yields (76-79%) were obtained when large quantities were injected into the columns (e.g. peptides +3, 128 mg; +4, 100 mg and +7b, 70 mg). To avoid losses in recovery, when the amount of sample was small (like peptide +1: 44 mg), we used a smaller G-10 column.

Theoretically, desalting steps should not affect peptide purity. Nevertheless, purity decreased a little with peptides +1 and

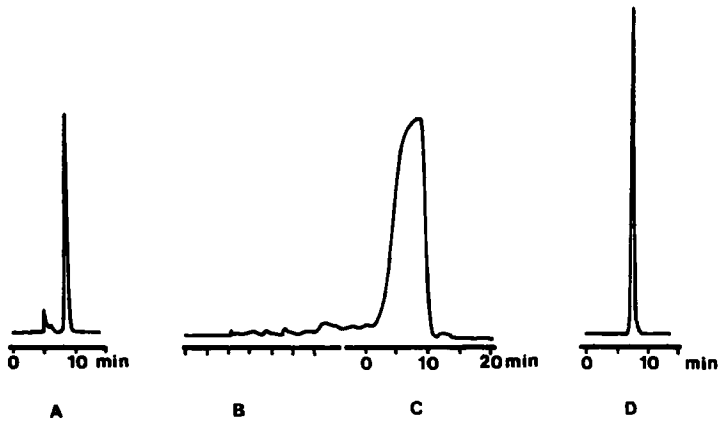

FIGURE 7

Preparative cation exchange chromatography of peptide +3 on S-Zephyr/10.

Column : 10 mm I.D x 90 mm ; Adsorption buffer : 50 mM sodium acetate pH 7.5
Elution linear gradient : sodium chloride from zero to 1.5 M in 20 min.
a) analytical control of crude peptide +3 on S-Zephyr/10
Peptide: load (0.1 mg each in 200µl loop); Flow rate : 3 ml/min. ;
b) adsorption step; 128 mg peptide +3 (6 x 20 + 8 mg ; loop injection)
Flow rate : 1 ml/min.
c) elution step
Flow rate : 3 ml/min.
d) Analytical control of lyophilized pure peptide +3, derived from preparative chromatographies (b and c) and desalting on gel filtration.

+7. This was explained by the storage of peptide in high sodium chloride solution (especially for +7) even at -20°C. Several experiments demonstrated the absolute necessity to desalt immediately the purified peptide after cation exchange. A possible alternative to desalting by gel filtration is the use of ammonium acetate, a lyophilizable salt (22) for elution. However, according to our experience, a single lyophilization run does not eliminate all salts and instead, several lyophilization runs were necessary for complete desalting.

CONCLUSION

In this paper it was demonstrated that using S-Zephyr, the retention time at pH 7.5 or 8.6 for seven different peptides was a linear function of their whole charges (1 to +7). This specific behaviour of small peptides can be advantageously considered to predict first the retention time of an unknown peptide, and more interestingly to predict if the impurities can be easily separated. This modelling approach of cation exchange chromatography of peptides, less developped than reverse phase chromatography, can help the user in the appropriate choice of a more effective separation method based on the charged amino-acid composition. Unlike mixed mode chromatography, pure hydrophilic ion exchange on S-Zephyr® eliminates unpredictable retention behaviour due to hydrophobicity.

High performance hydrophilic cation exchange chromatography with S-Zephyr seems a very powerful and reliable tool for routine peptide purification, even at preparative scale. This should certainly facilitate investigation of hydrophilic active sites of proteins or protein fragments, involved for example in antigenic recognition.

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