UNEXPECTED ELUTION BEHAVIOUR OF PEPTIDES WITH VARIOUS REVERSED-PHASE COLUMNS

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SUMMARY

Gastrin and cholecystokinin peptides were separated by reversed-phase chromatography on conventional bristle-type and polymer-coated stationary phases. The retention of the sulphated and non-sulphated isomeric forms of both peptides is governed by the structure of the peptide, the net charge and additional polar interactions with the stationary phases. Polymer-coated phases are optimum for separations according to chain length, whereas polar interactions are required for the separation of sulphated and non-sulphated peptides of identical chain length.

INTRODUCTION

Gastrin and cholecystokinin (CCK), peptides of the CCK family, are present in both the gut and brain tissues and the corculation[1,2]. Both peptides are structurally related. They have the same carboxyl-terminal pentapeptide, and a further structural similarity is a sulphated tyrosine at the C-terminus[3,4]. The release of gastrin and cholecystokinin peptides into the peripheral blood occurs in response to a protein- and fat-rich meal. In the gut, the physiological role of CCK is to regulate gall-bladder contraction and pancreatic exocrine secretion, whereas gastrin stimulates the gastric acid secretion[5]. Various multiple molecular forms of gastrin and cholecystokinin peptides, varying in peptide length and charge, have been identified and separated by means of gel-filtration column chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC), followed by radioimmunoassay[1,6]. Further, the octapeptide of CCK (CCK8) and the CCK variant containing 33 amino acids (CCK33), and also the heptadecapeptide (G 17) and the 34 amino acid sequence (G 34) of gastrin, exist naturally in both a sulphated and non-sulphated form[1,7]. Because the concentrations of these peptides in body fluids are below the usual detection sensitivity, optimization of separation must be performed using standards. The

resolution should be high in order to obtain fractions at expected elution windows and to use the fractions for the more sensitive immunological assays.

There have been long and lively discussions about the effects of stationary-phase properties on peptide and protein retention[8,9]. The use of silica-based materials in the RP-HPLC of peptides and proteins has at least two inherent disadvantages. First, the presence of silanols at the surface, which have not been covered or shielded by the "classical" silanization process, is responsible for irreversible adsorption of basic solutes or elution with asymmetric peak shapes[10]. This has been extensively discussed for RP-HPLC of low-molecular-weight solutes[11]. Anomalous behaviour of peptides, i.e., increase in retention at high acetonitrile concentrations[12], could be attributed to such silanophilic interactions. It has been shown that with "good" stationary phases, where surface silanols have been completely shielded, this behaviour could no longer be observed[13]. The second problem is the stability of the silica matrix or the cleavage of the siloxane bonds to the organosilane, resulting in a loss of carbon and non-reproducible retention times, owing to the harsh elution conditions required for peptide separations[14]. Standard peptide separation conditions are gradient elution at low pH (< 3) or addition of ion-pairing reagents, such as trifluoroacetic acid (TFA). Several strategies have been described to overcome these restrictions. The influence of silica gel structure on hydrolysis[15] was studied and the use of new silanization reagents[16] for preparing more stable modified silicas was discussed. The use of cross-linked polymeric stationary phases has been discussed[17,18]. They are stable toward hydrolysis[9] and show different selectivities, but do not generate the efficiency of silica-based stationary phases of identical particle diameter[19].

Polymer-coated or polymer-encapsulated silicas seem to combine the advantages of microparticulate silica (defined pore structure, mechanical rigidity) and the polymeric phases by totally shielding of surface silanols. Different approaches led to this new type of stationary phase by either coating the silica with a convenient prepolymer, which is then immobilized at the silica surface[20,21], or by allowing the silica to react with an olefinic silane and polymerizing an olefin coated onto the surface[22]. Both methods give stationary phases with excellent retention properties for basic solutes and show normal elution behaviour of proteins.

For optimization of the separation of gastrin and CCK, commercially available peptides, such as the sulphated and non-sulphated forms of cholecystokinin and gastrin, have been used. The influence of stationary phase properties on absolute and relative retention has been evaluated. In addition to conventional bristle-type RP systems, new polymer-coated reversed-phase materials have been included in the study[21,22]. Because they should exhibit only hydrophobic and very few silanophilic properties.

EXPERIMENTAL

A modular liquid chromatograph, consisting of two M 510 pumps (Waters Assoc., Milford, MA, U.S.A.), a Waters Chromatography Data Station 840, a multiwavelength detector (Knauer, Berlin, F.R.G.), detection wavelength 220 nm, and a Rheodyne 7125 sample injector (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop, was used.

Different stationary phases with classical modification by organosilanes or with

TABLE I

COLUMNS USED

Material	Particle size, (μm)	Pore size (nm)	Column length × I.D. (cm)	Distributor
Macherey, Nagel & Co.:				
Nucleosil C_8	5	12	12.5 × 0.4	Bischoff, Leonberg, F.R.G.
Nucleosil C_{18}	5	12	12.5 × 0.4	Bischoff
Nucleosil C_8	5	30	12.5 × 0.4	Bischoff
Merck:				
Lichrospher C_{18}	10	30	25 × 0.4	Bischoff
Vydac C_4 214 TPB	10	30	25 × 0.4	Macherey, Nagel & Co., Düren, F.R.G.
PolyEncap Silica:LiChrosorb	10	10	25 × 0.4	Laboratory prepared[a]
Vinyl ether C_{18} Silica: LiChrosorb	10	10	12.5 × 0.4	Laboratory prepared[a]
MH 1	5	12	25 × 0.4	Gynkotek, Germering, F.R.G.
Silica: Nucleosil				
PolyEncap Silica: Nucleosil	5	30	12.5 × 0.4	Bischoff

[a] Prepared in our laboratory, as described in ref. 22.

polymer-coating were used. The columns, their dimensions and the distributors are summarized in Table I. The Vydac C_4 phase was purchased as bulk material and packed into columns in our laboratory with a standard slurry-packing technique. The synthesis of the PolyEncap and the octadecyl vinyl ether phases have been described recently[22].

Mixtures of water (purified with a Milli-Q equipment; Millipore, Eschborn, F.R.G.) and acetonitrile of HPLC grade (ACN) (Baker, Gross-Gerau, F.R.G.; were used isocratically (30% ACN) or in gradients (28% to 40% ACN in 30 min for 12.5-cm columns and 60 min for 25-cm columns). All eluents contained 0.1% TFA. The ionic strength was adjusted with potassium chloride.

Cholecystokinin (Pancreomycin) octapeptide (CCK8), sulphated and non-sulphated, and gastrin-17 (peptide with 17 amino acids), sulphated and non-sulphated, were purchased from Sigma (Munich, F.R.G.). The amino acid sequence of CCK8 is Asp–Tyr–Met–Gly–Trp–Met–Asp–Phe–NH_2. Sulphatation occurs on tyrosine. The sequence of gastrin-17 is p-Glu–Gly–Pro–Trp–Leu–(Glu)$_5$–Ala–Tyr–Gly–Trp–Met–Asp–Phe–NH_2. Sulphatation also occurs on tyrosine.

RESULTS AND DISCUSSION

Peptide separations with gradient elution

For protein and peptide analysis, several brands of specially designed reversed-phase columns are now available. One of the most widely applied is the Vydac RP column with C_4 bristles and this system was used for the first experiment. As can be seen in Fig. 1, under standard gradient conditions the two CCK8 peptides (1 and 2)
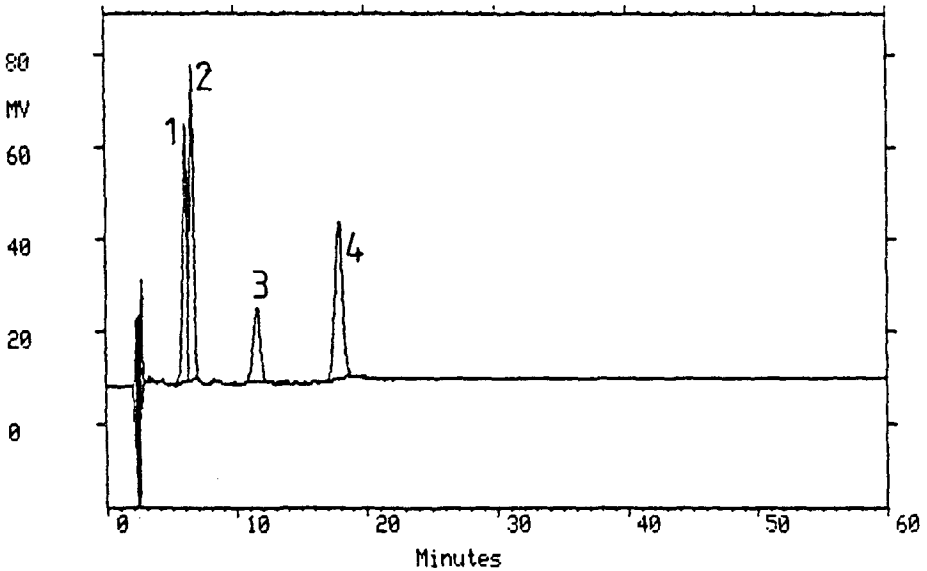
Fig. 1. Separation of gastrin and CCK8 peptides on Vydac C_4 30 nm. Solutes, 1 = CCK8 sulphated; 2 = CCK8 desulphated; 3 = gastrin-17 sulphated; 4 = gastrin-17 desulphated. Column, Vydac C_4 30 nm (see Table I); eluent, gradient from 28% aqueous ACN–0.1% TFA to 40% ACN–0.1% TFA in 60 min.
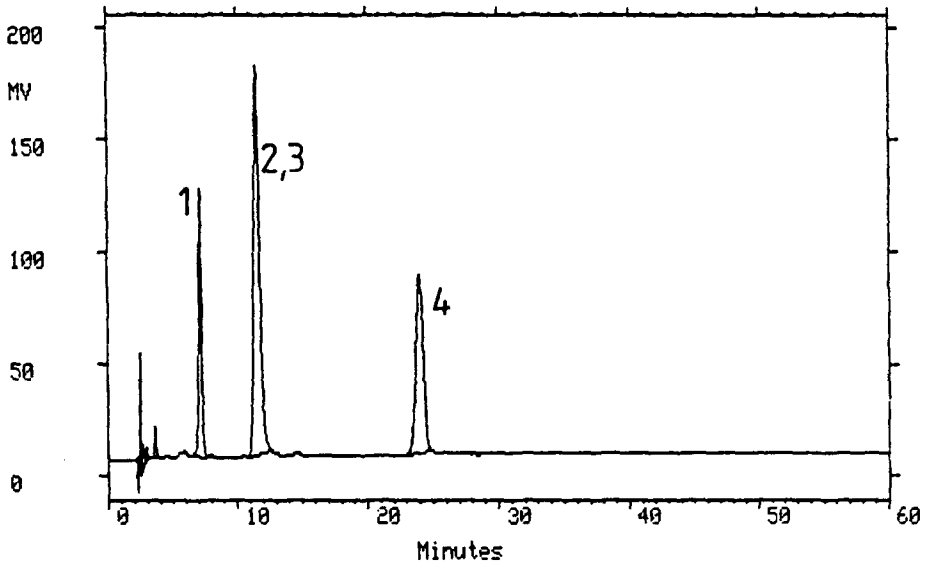

Fig. 2. Separation of standard peptides on LiChrospher C_{18} 30 nm. Column, see Table I. Samples and conditions as in Fig. 1.

are not adequately separated for the desired collection of fractions. The elution order is as expected, the octapeptides being eluted before the non-sulphated ones. This finding led us to increase retention by increasing the carbon content of the stationary phase. The separation obtained under the standard gradient conditions with an RP C_{18} coating on silica with identical surface area and pore diameter (LiChrospher C_{18}, 30 nm) is shown in Fig. 2. The retention of the non-sulphated peptides (2 and 4) increased, as expected, with increasing carbon content of the stationary phase. However, the elution of the sulphated peptides (1 and 3) was hardly affected by the change in carbon content, thus leading to simultaneous elution of non-sulphated CCK8 with gastrin-17, sulphated. These observations can be explained with the superposition of at least two retention mechanisms: hydrophobic and ionic interactions.

Ionic interactions, which always play a role in the separation of nitrogen-containing pharmaceuticals, have been minimized in HPLC by the introduction of polymeric-coated stationary phases. With these columns, even strongly basic solutes, which are extremely sensitive to silanophilic interactions, are eluted with symmetrical peaks[22]. Fig. 3 shows the separation of the peptides from such a polymeric phase where octadecyl vinyl ether had been polymerized on the surface of a silica modified with vinylsilane[22]. Owing to the high carbon content, the retentions are relatively high, the sulphated and non-sulphated solutes cannot be separated and, surprisingly, the elution order for the sulphated and non-sulphated forms of CCK8 (1 and 2, respectively) has changed. Another difference from the separations shown in Figs. 1 and 2 was that here a silica base material with a specific surface area of 300 m^2/g was used. To differentiate between the influence of the surface area and the type of coating, a conventional reversed phase with the same surface area was also used. In Fig. 4 a standard separation with a Nucleosil C_{18} column is shown. With this column, the separation of all four peptides is possible and the elution order was identical with that on the wide-pore materials (30 nm).

In order to evaluate the different retention mechanisms and their influence on elution sequence more closely, additional isocratic studies of absolute and relative retention with stationary phases of different types were performed.

Polymer-coated vs. bristle-type phases

It is impossible to study the retention behaviour of proteins by isocratic elution. However, with the short-chain peptides used in this work, isocratic measurements of absolute and relative retentions are possible. Hence, it seems feasible to use these peptides for more general observations of protein retention in RP-HPLC. Figs. 1–4 show that sulphated and non-sulphated peptides exhibit different and surprising behaviour on conventional bristle-type and polymer-coated columns. Because of the low molecular weights and the greater variability of phases, silica with standard pore diameters of *ca.* 10 nm were used. Behaviour identical with that described for this material has been observed with wide-pore material (pore diameter 30 nm). Differences in absolute retention between the various stationary phases have been observed and could easily be related to the carbon content of the stationary phase and peptide chain length. However, the selectivities of the stationary phases are different for the separations according to peptide chain length and for sulphated and non-sulphated species. In Fig. 5 the relative retentions of CCK8 and gastrin-17 and their sulphated isomers are shown. In contrast to this selectivity according to chain length, Fig. 6
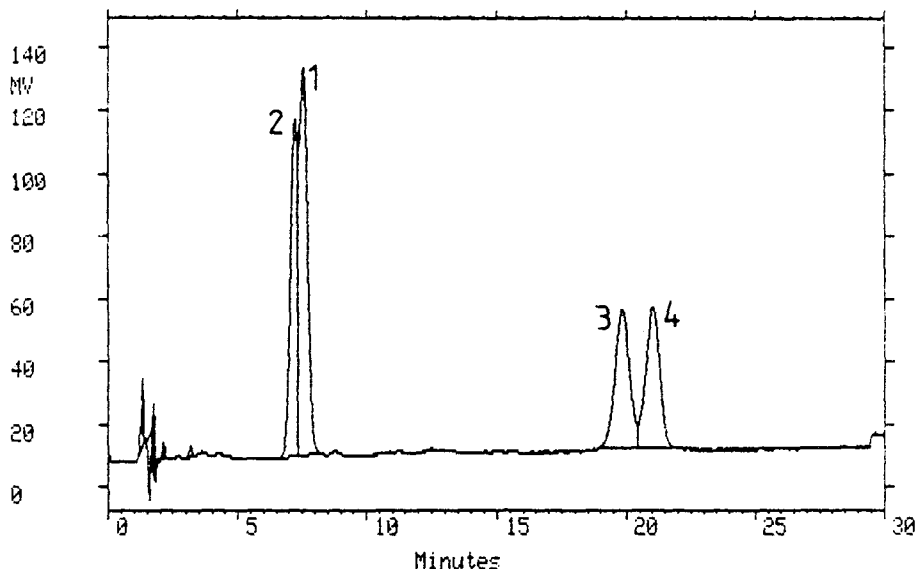
Fig. 3. Separation of standard peptides on C_{18} vinyl ether. Column, see Table I. Samples and conditions as in Fig. 1; gradient time, 30 min.

shows the polar selectivities of the sulphated and non-sulphated species of identical chain length. The polymer-coated stationary phases (e.g., C_{18} vinyl ether) show the highest hydrophobic selectivity. However, this column shows the lowest polar selec-
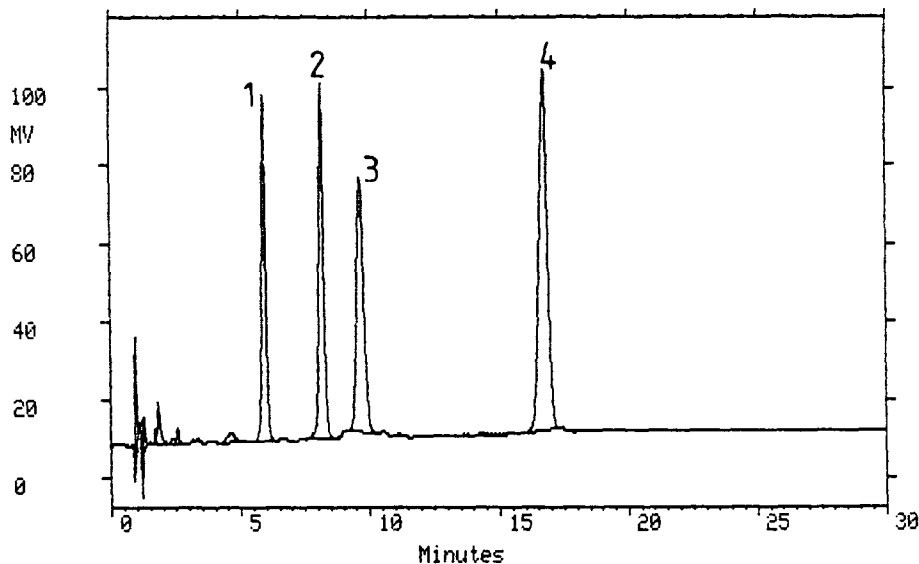

Fig. 4. Separation of standard peptides on MN Nucleosil C_{18} 12 nm. Column, see Table I. Samples and conditions as in Fig. 1; gradient time, 30 min.
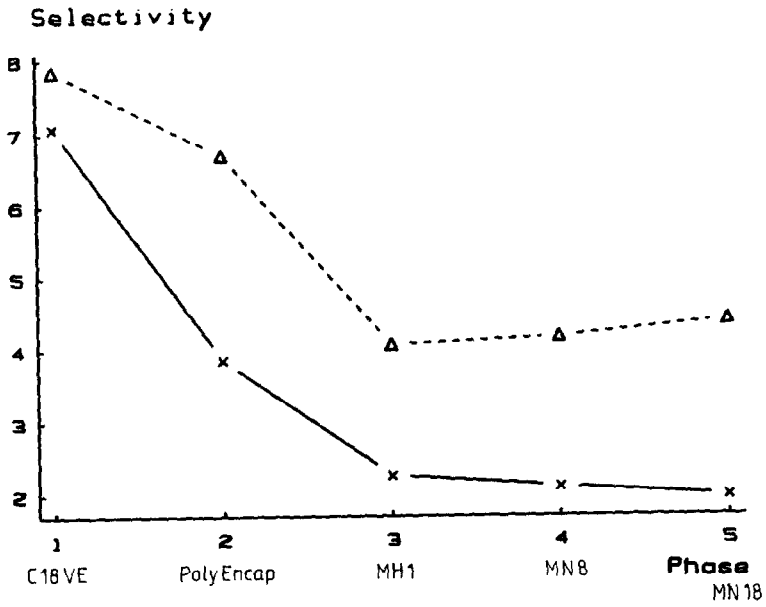
Selectivity

Fig. 5. Hydrophobic selectivity of stationary phases. Columns, see Table I. Isocratic measurements with 30% aqueous ACN–0.1% TFA. ×, Gastrin sulphated–CCK sulphated; △, gastrin–CCK.
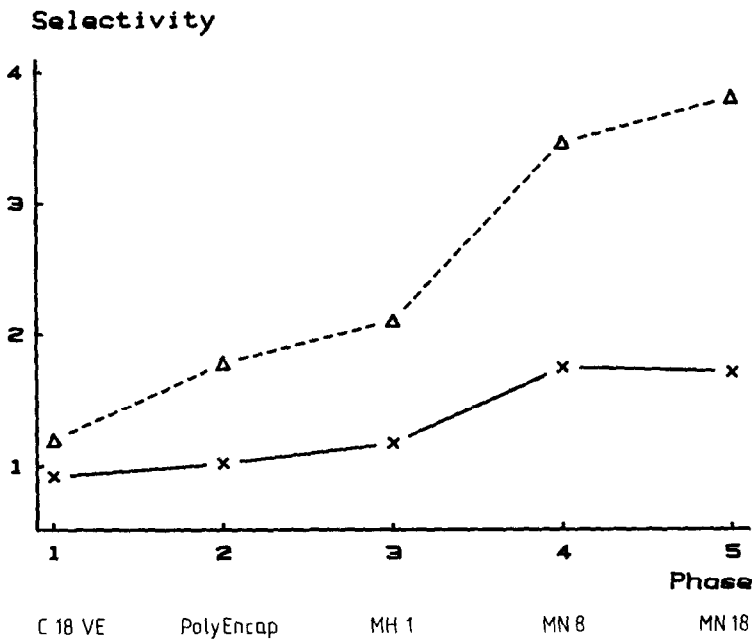
Selectivity

Fig. 6. Polar selectivity of stationary phases. Conditions as in Fig. 5. ×, CCK8–CCK8 sulphated gastrin-17–gastrin-17 sulphated.

tivity for the separation of sulphated and non-sulphated peptides, as can be seen in Figs. 3 and 6. In contrast, the opposite is true for the conventional bristle-type stationary phases MN 8 (Nucleosil C_8), where the hydrophobic selectivity is much lower. The two other polymer-coated stationary phases also included in Figs. 5 and 6 are in between these two extremes. Owing to its more polar surface groups the stationary phase (PolyEncap) where a butyl acrylate had been polymerized on silica modified with vinylsilane[22], shows, despite the small change in carbon content from C18 vinyl ether (less than 3%), less hydrophobic selectivity and a slightly enhanced polar selectivity. The other polymer-coated stationary phase (MH 1) resembles the conventional bristle-type phases much more. This can be explained by the preparation technique used for this type of stationary phase. In this instance, a trimethylsilylated silica is covered with a polysiloxane containing octadecyl groups[21].

From these findings, it may be concluded that stationary phases for peptide and protein separations require not only optimum coverage of surface silanols, usually determined by measurement of the peak symmetry of basic solutes, but also additional centres for polar interactions to permit the separation of isomers differing only in a polar functional group.

Influence of ionic strength

In protein and peptide separations with silica-based stationary phases, silanophilic interaction with unmodified surface silanols is observed in addition to the main retention mechanism, *i.e.*, hydrophobic interaction. It has been demonstrated[23] that these silanophilic interactions, like ion exchange of basic peptides and ion exclusion of acidic peptides, can be minimized by increasing the ionic strength of the buffer solution by the addition of salts, such as potassium chloride. As can be seen in Fig. 7, the retention of the non-sulphated CCK 8 decreases with increasing ionic strength, whereas the retention of the sulphated isomer is only slightly affected by increasing the ionic strength, especially up to 0.15 M potassium chloride solutions. At first glance, this is not understandable, because for the sulphated species ion exclusion should be expected. However, if one calculates the net charge for the two isomers at pH 2.5, the sulphated CCK has a net charge of zero (positively charged, terminal amino group and a negatively charged sulphate group), whereas the non-sulphated CCK has a net charge of $+1$ (positively charged terminal amino group only). Consequently, this species (non-sulphated) can interact with surface silanols by an ion-exchange mechanism. Enhancement of retention by increasing salt concentration, usually applied in hydrophobic-interaction chromatography (HIC), could not be observed for this small peptide up to a concentration of 1 M.

In Fig. 8, the influence of ionic strength on retention is shown for a polymer-coated stationary phase. Here, the retention increases for both isomers at low ionic strength. In their preparation surface silanols are totally covered by a polymeric layer. The increase in eluent hydrophilicity due to increasing potassium chloride concentration increases the retention of both solutes. The retention of the non-sulphated isomer with larger hydrophobicity increases continuously, whereas that of the sulphated species remains constant. The polar ester functionalities in the polymeric layer may be the reason for this behaviour.

The differences between the two types of stationary phases can be seen much more clearly from the dependence of relative retentions on ionic strength, as shown in
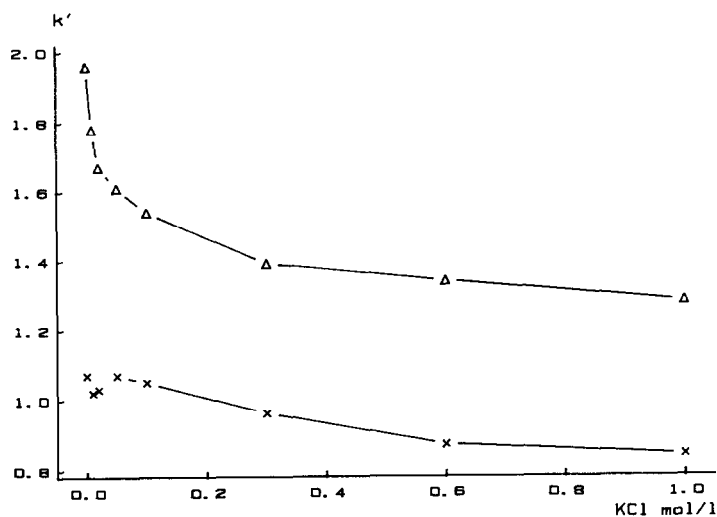
Fig. 7. Influence of ionic strength on peptide retention with a bristle-type stationary phase. Column, Lichrospher C_{18}, 30 nm (see Table I). Other conditions as in Fig. 5. ×, CCK8 sulphated; △, CCK desulphated.

Fig. 9. The selectivity with the conventional bristle-type stationary phase decreases as ionic strength increases, because for the non-sulphated CCK (one positive charge) the additional contribution of ion exchange for the non-sulphated CCK (one positive charge) decreases with increasing ionic strength. At a salt concentration above 0.5 M, additional contributions to the retention mechanism are excluded, and selectivity is
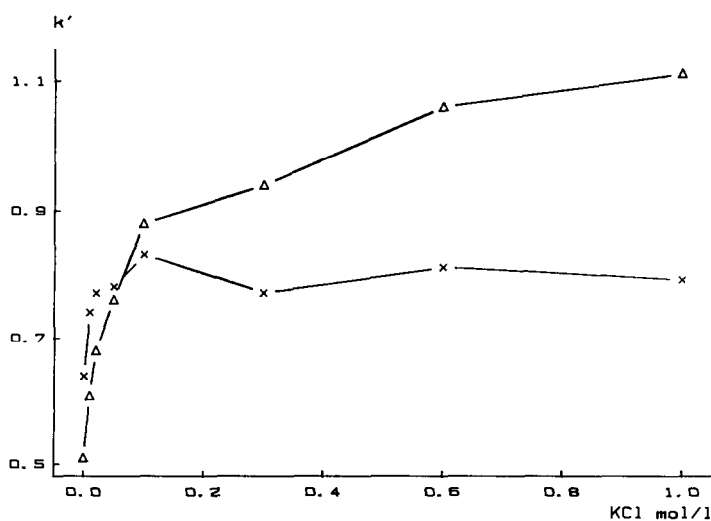

Fig. 8. Influence of ionic strength on peptide retention with a polymer-coated phase. Column, PolyEncap 30 nm (see Table I). Other conditions as in Fig. 5. ×, CCK8 sulphated; △, CCK desulphated.
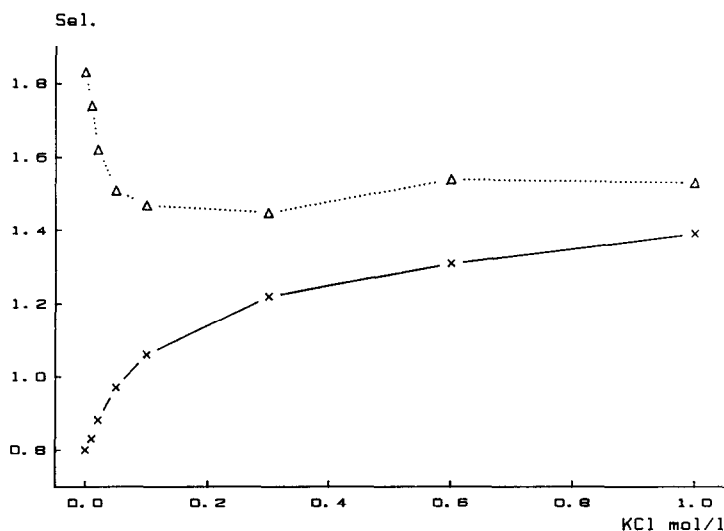
Fig. 9. Influence of ionic strength on selectivity (CCK8 desulphated–CCK8 sulphated) with different types of stationary phases. Columns: ×, PolyEncap 30 nm; △, LiChrospher C_{18}, 30 nm; see Table I. Other conditions as in Fig. 5.

determined solely by differences in peptide hydrophobicity. It has also been observed in size-exclusion chromatography of proteins[23] that a salt concentration of 0.5 M is necessary for suppression of electrostatic interactions.

With a polymer-coated stationary phase at low ionic strength the sulphated peptide is more strongly retarded than the non-sulphated peptide. At a salt concentration of *ca.* 0.05 M the elution sequence changes, and the selectivity increases with increasing ionic strength. This is due to the stronger dependence of the retention of the non-sulphated peptide on ionic strength (see Fig. 8). At salt concentrations above 0.5 M the dependence of selectivity on ionic strength is identical with that observed for conventional bristle-type stationary phases, indicating an identical separation mechanism. It should be mentioned that all polymer-coated phases investigated behave identically, in spite of differences in functional groups and pore sizes (data not shown). This demonstrates the advantages of polymer-coated stationary phases due to the absence of additional retention mechanisms.

CONCLUSIONS

The complexity of peptide and protein structures, together with the heterogeneity of stationary phases for HPLC, can cause surprising retention behaviour. The charges of peptides cause, in addition to hydrophobic interactions, electrostatic interactions with polar surface groups on the stationary phases. At present, two different types of stationary phases are used for RP-HPLC of proteins: conventional bristle-type phases and polymeric-coated phases. The latter have been developed for the analysis of basic nitrogen-containing pharmaceuticals to reduce silanophilic interactions, which usually cause peak asymmetry. Both types of stationary phase exhibit

different retention behaviour in protein analysis. With "good" conventional bristle-type stationary phases (symmetrical peaks for peptides with one positive net charge) at low ionic strength (below 0.3), an ion-exchange mechanism contributes to peptide retention. With polymer-coated stationary phases the ion exchange does not contribute noticeably to retention. However, for the separation of isomeric peptides, differing by one sulphonic acid group, polar interactions with either surface silanols (bristle-type phases) or siloxanes (polysiloxane-coated phases) are necessary for separation.

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