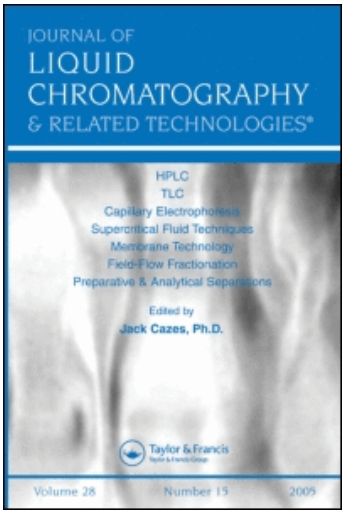

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High-Performance Liquid Chromatography of the Tetradecapeptide Somatostatin

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE TETRADECAPEPTIDE SOMATOSTATIN

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ABSTRACT

Optimum reversed-phase systems for qualitative and quantitative determinations of somatostatin are evaluated. Temperature, pH, buffer concentration, type and concentration of organic modifier and the presence of ion-pairing agents more or less influence the retention and the separating efficiency. The retention behaviour of some analogues is described to illustrate the selectivity of the system. Down to 10-20 ng of somatostatin can be determined, even in the presence of a large excess of albumin, by using 210 nm as the detection wavelength.

INTRODUCTION

Large peptides, especially those synthesized by the solid-phase technique, are often very heterogeneous, so that purification, characterization and quantitation of such peptides present great problems. High performance liquid chromatography (HPLC) has proved to be superior to other techniques such as isotachophoresis, thin-layer chromatography and radioimmunoassay. Several reports on reversed-phase HPLC have appeared in the literature

dealing with the separation of peptides, ranging from dipeptides to proteins (1-8).

It is difficult to predict the net effect of a change in composition and/or the sequence of amino acids in the peptide. For optimum chromatographic results it is therefore necessary to study each parameter, such as column, temperature, pH, buffer concentration and type and concentration of organic modifier, separately.

This article presents the results of the development of chromatographic systems for qualitative and quantitative determinations of the cyclic tetradecapeptide somatostatin (Figure 1). Some of the biological activities of this peptide hormone are suppression of growth hormone release from the pituitary gland as well as lowering of the glucose level (9).

EXPERIMENTAL

Apparatus

A Waters Model 6000 pump (Waters Associates, Milford, Mass., U.S.A.) or a Milton Roy Minipump (LDC, Riviera Beach, Florida, U.S.A.) was used and operated at a flow rate of about 1 ml/min and at a pressure drop of about 2500 p.s.i. The column effluent was monitored at 210 nm or 280 nm (0.01-0.04 AUFS) by a Perkin Elmer LC 55 variable wavelength detector (Perkin-Elmer Corporation, Norwalk, Connecticut, U.S.A.) with an 8 µl flow-through cell.

The stainless steel column was 150 mm long (I.D. 4.6 mm). It was packed with LiChrosorb RP-8, 5 µm particles (E. Merck, Darmstadt, G.F.R.), by the balanced-density slurry-packing technique. The column used had 2500 theoretical plates (HETP = 0.06 mm) when

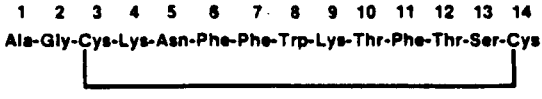

FIGURE 1. Sequence of somatostatin (molecular weight 1639).

tested with phenetole (k' = 3.2) and methanol/water (60+40) as eluent with a flow rate of 1 ml/min. In one instance a commercial μBondapak C_{18} column, 300 x 4 mm, 10 μm particles (Waters Associates, Milford, Mass., U.S.A.) was used. When increased temperature was used, the column was water-jacketed and the temperature was controlled by a Haake thermostat (Haake, Karlsruhe, G.F.R.).

The sample was injected with a Waters U6K injector (Waters Associates, Milford, Mass., U.S.A).

Reagents and Chemicals

Acetonitrile was of HPLC grade S from Rathburn Chemicals (Walkerburn, Scotland) and ethanol, 99.5 %, was of pharmacopoeial purity grade.

Sodium salts of phosphate and acetate (E. Merck, Darmstadt, G.F.R.) were used to prepare the buffers. Different buffer concentrations and ionic strengths were used.

1-Propanesulphonic acid and the sodium salts of 1-butane-, 1-pentane-, 1-hexane and 1-heptanesulphonic acids were from Eastman Kodak Co. (Rochester, N.Y., U.S.A.). D(+)-Camphorsulphonic acid was from E. Merck (Darmstadt, G.F.R.) and sodium dodecyl sulphate (SDS), specially pure, was from BDH Chemicals Ltd. (Poole, England).

The eluents were mixtures of acetonitrile and buffers, in some cases containing the sulphonates or SDS. The pH values of the eluent mixtures (measured directly) are indicated in this article. The concentration of buffer salts and ion-pairing agents are expressed as mol/l of the eluent mixture.

Somatostatin and its analogues were synthesized by the solid-phase technique at Kabi AB (Stockholm, Sweden) or Bachem (Torrance, California, U.S.A.).

RESULTS AND DISCUSSION

Chromatographic Conditions

Support. Different alkylsilane-bonded silica gel supports (C_{18}, C_8 and C_2) as well as NH_2-bonded and underivatized silica gels were tested. The hydrophilic supports gave unreproducible chromatograms with asymmetrical peaks and low yields, probably due to adsorption.

Capacity ratios (k') and separating efficiencies (HETP) were compared on columns containing LiChrosorb RP-2, RP-8 and RP-18. There were no significant differences in k' but HETP increased in the series RP-8 < RP-18 < RP-2.

Temperature. The temperature was varied between 20°C and 50°C and, as can be seen from Figure 2, a linear relationship was obtained between the k' values and column temperature. An increase in temperature led to decreased k' values and a slight increase in efficiency was observed. O'Hare and Nice (8) reported similar effects for $ACTH_{1-24}$ but a somewhat decreased efficiency at temperatures above 40°C.

We have found that the advantages of using increased temperatures are of minor importance compared to the effects of other chromatographic parameters such as variation in the properties of the eluent.

Organic Modifier in the Eluent. Ethanol, methanol and acetonitrile were used as organic modifiers in the eluent. No differences with regard to peak symmetry or selectivity of the system were noticed between ethanol and methanol. The selectivity was increased, however, by using acetonitrile instead of the alcohols.

The concentration of acetonitrile in the eluent was varied between 20 % and 70 %, v/v, which corresponds to a k' range of 50 to 0. The concentration had a drastic effect on the k' values, a phenomenon which has also been reported by Bennett et al. (5), Glasel (10), Krummen et al. (11), Rivier (3), Stoklosa et al. (2)

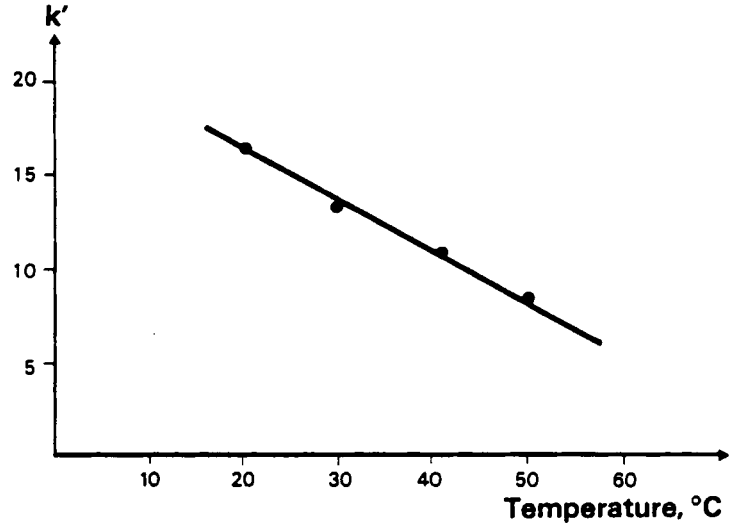

FIGURE 2. Influence of column temperature on the capacity ratio (k').
Column: LiChrosorb RP-8, 10 µm particles, length 2 x 150 mm, I.D. 3.9 mm. *Eluent:* 25.5 %, v/v, of acetonitrile in phosphate buffer (ionic strength = 0.1), pH 7.4.

and O'Hare and Nice (8), who studied peptides ranging from pentapeptides to proteins. We found that an increase in the content of acetonitrile by 5 % (e.g. from 25 % to 30 %, v/v) resulted in an approximately sixfold decrease in k' of somatostatin. Figure 3 shows the rectilinear relationship between log k' and percent of acetonitrile over the range of 20 % to 40 %, v/v.

Figure 4 shows the corresponding plot obtained with an eluent containing ethanol, used in experiments with µBondapak C_{18} as the support. An increase in the content of ethanol by 5 % (e.g. from 30 % to 35 %, v/v) resulted in an approximately 2.5-fold decrease in k' of somatostatin. The data have also been plotted as linear relationships between log k' and the logarithm of the molar concentration of the organic modifier (solvating component), as reported by Eksborg (12) and Jansson et al. (13). The slopes of the lines were -9 and -7 with acetonitrile and ethanol, respectively.

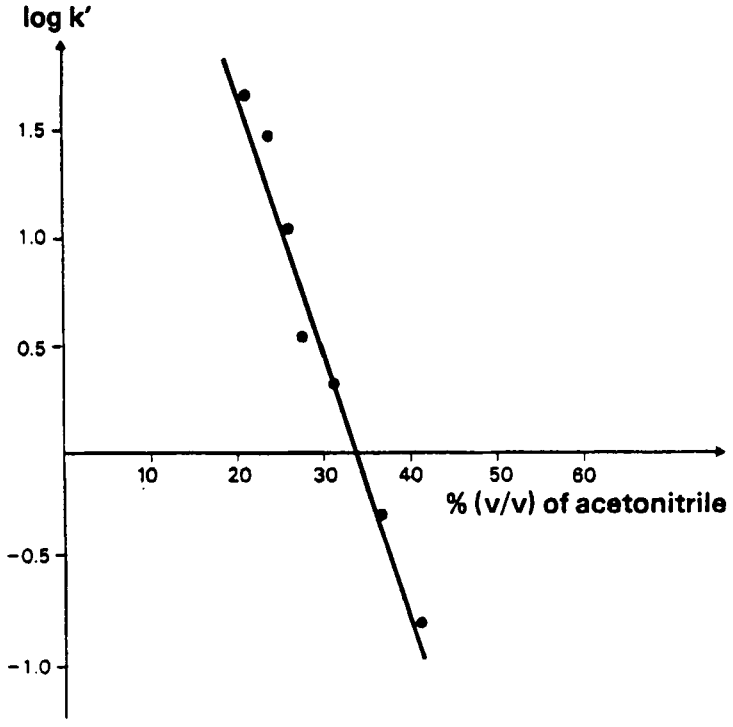

FIGURE 3. Influence of the concentration of acetonitrile on the capacity ratio (k').
Column: LiChrosorb RP-8, 5 µm particles, length 150 mm, I.D. 4.6 mm. *Eluent*: Acetonitrile in phosphate buffer (ionic strength = 0.1), pH 4.5.

By increasing the concentration of acetonitrile from 20 % to 35 %, v/v, (pH of the eluent = 4.5), the k' value decreased from 50 to 0.5. The relationships between the observed HETP values and k' are indicated in Figure 5. The increase in HETP at low k' values is obvious, while the more retained peaks have a fairly low and constant HETP. Symmetrical peaks were obtained in all cases with an HETP of 0.09 at k' = 12. This is the k' value chosen in the studies on the homogeneity of different batches of somatostatin. The asymmetry factors of the peaks at k' = 30 and 50 have been calculated to be 1.2 and at k' = 10, 1.0.

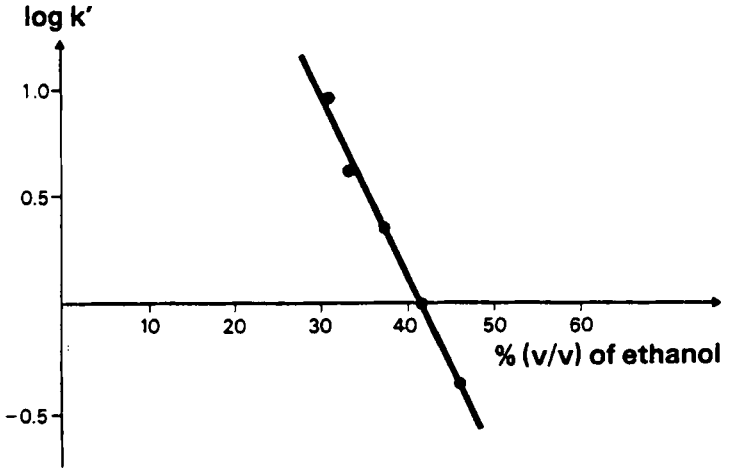

FIGURE 4. Influence of the concentration of ethanol on the capacity ratio (k').
Column: µBondapak C_{18}.
Eluent: Ethanol in acetate buffer, 0.01M, pH 3.5.

The pH of the Eluent. Due to suppression of the ionization of the free amino groups in somatostatin, k' increased with increasing pH. This is illustrated in Figure 6a. The effect of pH (varied between 2 and 8) on the separating efficiency of the system is indicated in Figure 6b. Optimum results with HETP <0.1 mm are obtained over the pH range from 2 to 5. In order to extend the lifetime of the column, a pH of 4.5 was chosen for all routine qualitative and quantitative tests.

Buffer Concentration. The studies were performed with an eluent containing 25.5 %, v/v, of acetonitrile in phosphate or acetate buffers of pH 4.5. An increase in the buffer concentration (ionic strength) led to a decrease in the k' values. This is illustrated in Figure 7a. As can be seen from this figure the changes in k' are very pronounced below 0.1 mol/l of phosphate and 0.2 mol/l of acetate (including acetic acid). Such phenomena have been explained by Krummen and Frei (11) and Rivier and Bur-

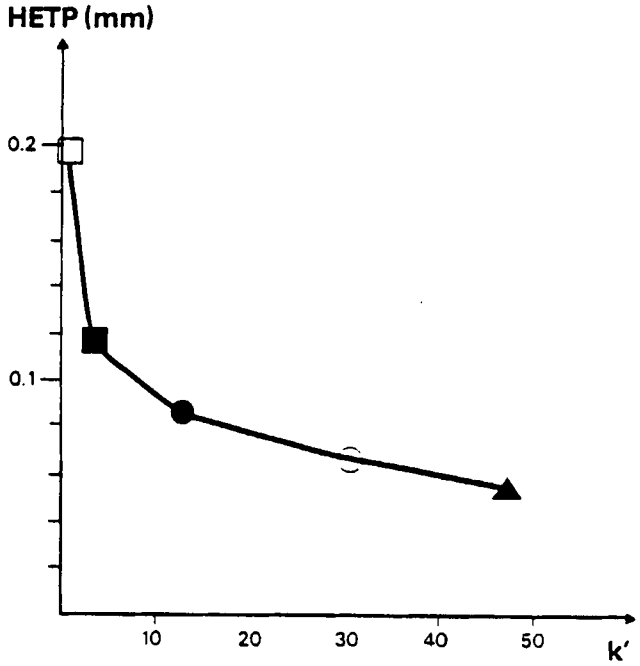

FIGURE 5. Influence of the concentration of acetonitrile on the separating efficiency (HETP).
Column: As in Figure 3. *Eluent*: Acetonitrile in phosphate buffer (ionic strength = 0.1), pH 4.5.
%, v/v, of acetonitrile: 35.8 (□), 30.6 (■), 25.5 (●), 22.9 (○), 20.4 (▲).

gus (14) as salting-in effects. Horvath et al. (15) claimed that the decrease in k' with increasing ionic strength is a result of increasing surface tension of the aqueous phase. Hancock et al. (16, 17) explained the effect as the formation of hydrophilic ion pairs. A more plausible explanation would be that an increase in the salt concentration would lead to increased shielding of the active sites of the support (unreacted silanol groups), resulting in decreased adsorption (retention).

The relationship between observed HETP values and k' values at different phosphate and acetate concentrations of the eluent are given in Figure 7b. Optimum efficiency is obtained at a phos-

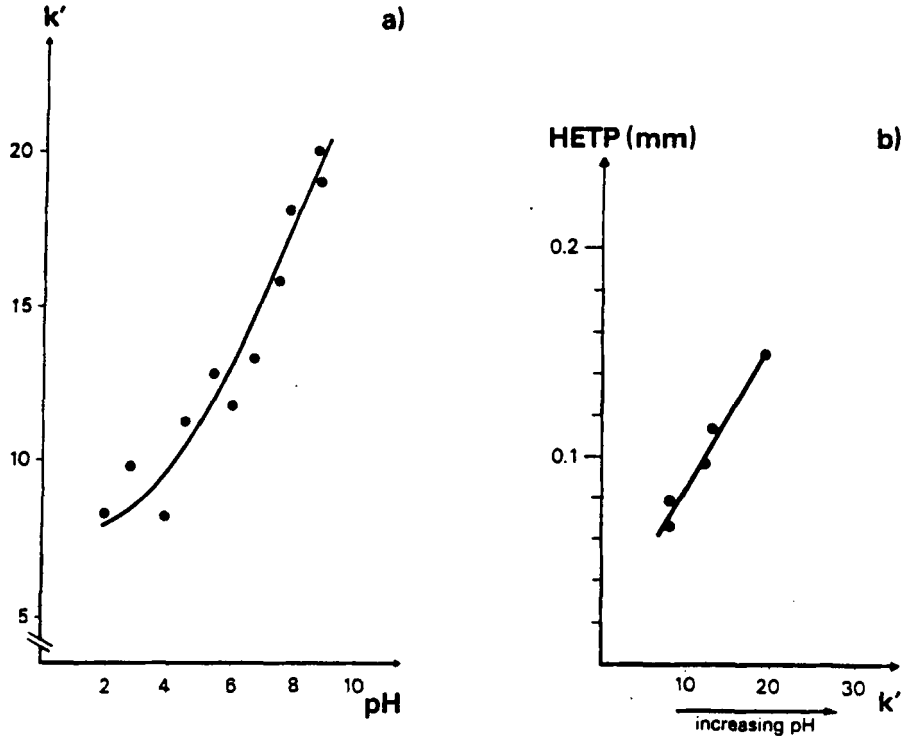

FIGURE 6. Influence of pH on the capacity ratio (k') and the separating efficiency (HETP).
Column: As in Figure 3. *Eluent*: 25.5 %, v/v, of acetonitrile in phosphate buffer (ionic strength = 0.1).

phate concentration of 0.1-0.25 mol/l and at an acetate concentration of approximately 0.2-0.5 mol/l in the eluent. This corresponds to ionic strengths of 0.1-0.3 for both buffers. At higher and lower concentrations (lower and higher k' values) HETP is increased and it is evident that a certain concentration is necessary for good efficiency.

Ion-pairing Agents. The retention of somatostatin was varied by adding such anions as propane-, butane-, pentane-, hexane-, heptane- and camphorsulphonates and sodium dodecyl sulphate (SDS)

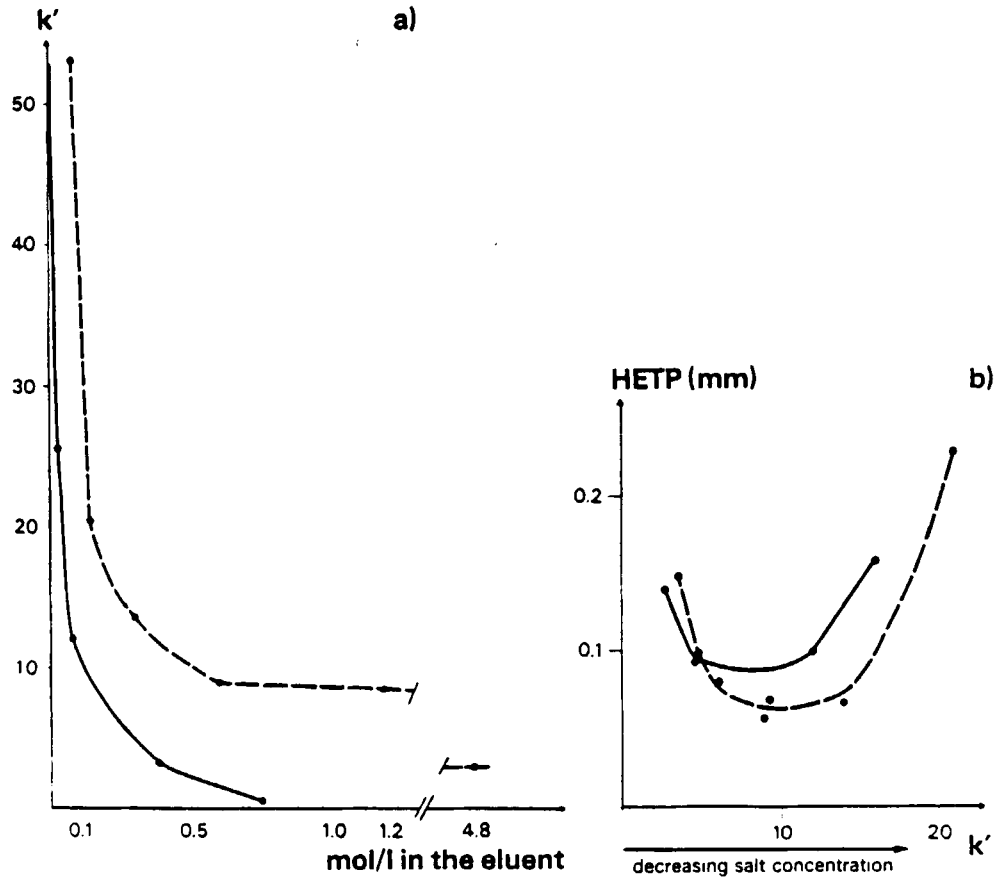

FIGURE 7. Influence of concentration of buffer salts on the capacity ratio (k') and the separating efficiency (HETP). *Column:* As in Figure 3. *Eluent:* 25.5 %, v/v, of acetonitrile in buffers, pH 4.5. Phosphate (———), acetate including acetic acid (-----).

to the eluent (pH 4.5) containing acetonitrile and phosphate buffer. The results have been plotted as k' values against concentrations of the sulphonates in Figure 8 and against concentration of SDS in Figure 9. In the former case reasonable k' values

were obtained with 25 %, v/v, of acetonitrile in the eluent, while the concentration had to be increased to 35 %, v/v, when SDS was used.

In all cases the k' values increased with increasing concentrations of anion. This effect was most pronounced when the more hydrophobic anions (with longer carbon chains) were used.

The k' values increased in the order butanesulphonate < pentanesulphonate < hexanesulphonate < heptanesulphonate < SDS, i.e., in the order of increasing length of the carbon chain. Deviating results were obtained with propane- and camphorsulphonates. The former gave comparatively high k' values and the latter (containing ten alicyclic carbon atoms) gave comparatively low k' values.

The addition of SDS led to a drastic increase in the retention of somatostatin and had a hundred times greater effect than the sulphonates. The retention seemed to be more affected by the addition of these anions when the eluent contained 25 %, v/v, instead of 35 %, v/v, of acetonitrile.

The results indicated that ion-pairing is at least partly responsible for the increased k' values, but further retention mechanisms cannot be excluded. The theory and applications of ion-pair chromatography have been thoroughly discussed by Schill and Wahlund (18) and Knox (19).

When the separating efficiency (HETP) was plotted against k' at different concentrations, as well as types of ion-pairing agents, profiles similar to that in Figure 5 were obtained. Thus increased k' values (and improved separating efficiency) can be achieved equally well by decreasing the concentration of the organic modifier. This is further illustrated in Figure 10, which shows chromatograms obtained with and without an ion-pairing agent present. There are no significant differences in separating efficiencies of the two systems (both giving k' values of about 2 and an HETP of about 0.12). It is possible that the overall effect

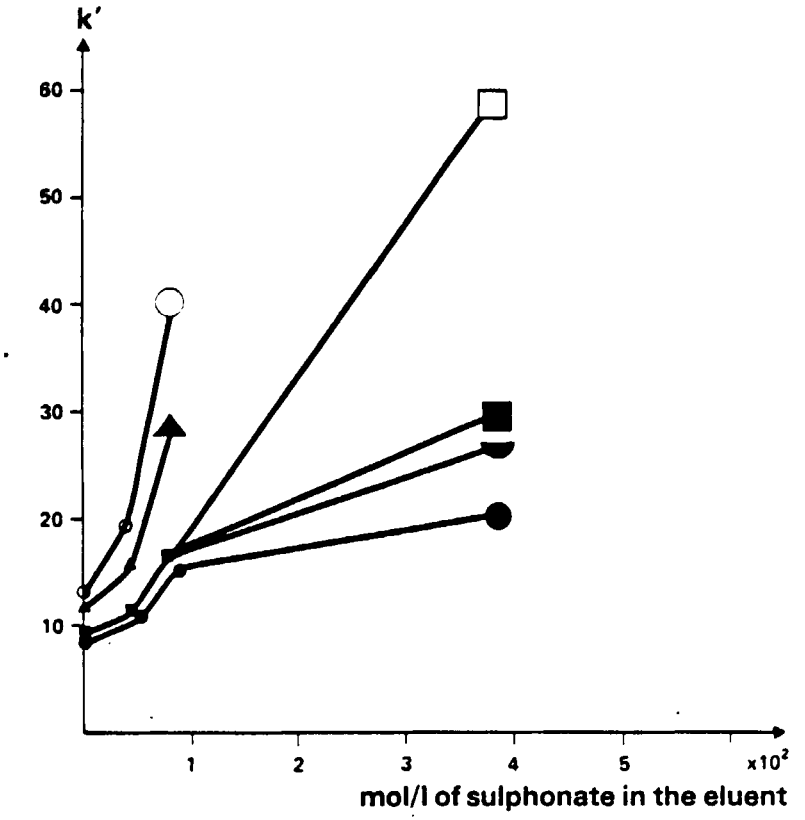

FIGURE 8. Influence of the sulphonate concentration on the capacity ratio (k').
Column: As in Figure 3. *Eluent:* 25.5 %, v/v, of acetonitrile in phosphate buffer (ionic strength = 0.1) containing sulphonates, pH 4.5.
Sulphonates: Propane (◒), butane (●), pentane (■), hexane (▲), heptane (○), camphor (□).

of ion-pairing agents is greater for smaller peptides than for the comparatively large somatostatin.

The results reported above indicate that an efficient chromatographic system for somatostatin could best be achieved by variations in the concentration of organic modifier while the pH should be kept between 2 and 5 and a certain salt concentration must be used.

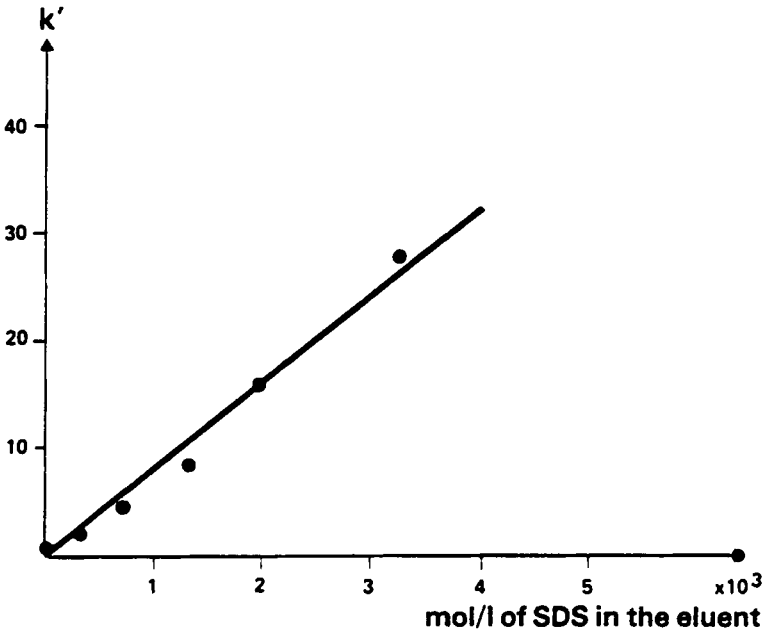

FIGURE 9. Influence of the SDS concentration on the capacity ratio (k').
Column: As in Figure 3. *Eluent:* 35.8 %, v/v, of acetonitrile in phosphate buffer (ionic strength = 0.1) containing SDS, pH 4.5.

Qualitative Analysis

The suitability of using HPLC in studies on the homogeneity of somatostatin is illustrated in Table I where the k' values of some analogues are given.

As can be seen from the table, all tested analogues were separated from somatostatin. A chromatogram of somatostatin synthesized by the solid-phase technique is given in Figure 11. To obtain the necessary selectivity of the system, the k' value of somatostatin should exceed 10 and phosphate buffers are preferred as they permit the use of 210 nm as the detection wavelength. Compared to 280 nm, this is a more universal wavelength for peptides.

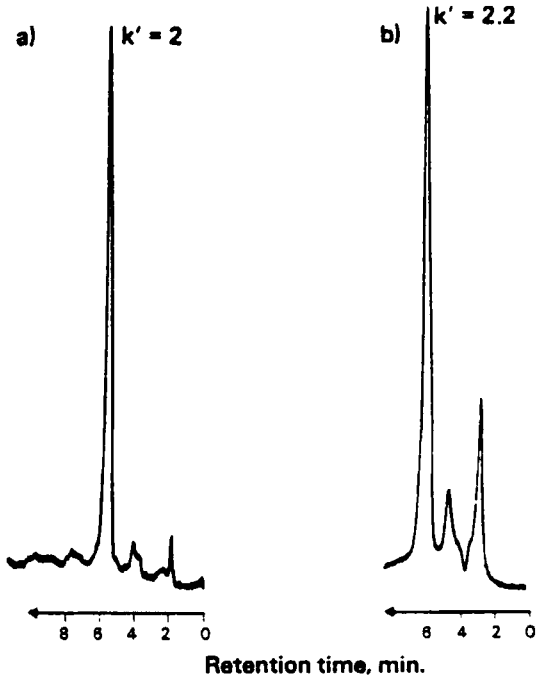

FIGURE 10. Influence of sulphonate on the separating efficiency. *Column:* As in Figure 3. *Detection wavelength:* 210 nm (0.02 AUFS). *Amount injected:* About 0.6 µg. (Samples of different purity degrees are shown in Figures a and b). *Flow rate:* 1 ml/min. *Chart speed:* 5 mm/min. *Eluent:* a) 30.6 %, v/v, of acetonitrile in phosphate buffer (ionic strength = 0.1), pH 4.5. b) 35.8 %, v/v, of acetonitrile in phosphate buffer (ionic strength = 0.1), pH 4.5. The mixture contains 0.03 mol/l of pentanesulphonate.

Quantitative Analysis

The system presented in Figure 10 is recommended for quantitative analysis. This system permits determinations of down to 10-20 ng of somatostatin. However, quantitative analyses in connection with stability studies would demand an extended k' range to obtain sufficient resolution between intact somatostatin and its possible degradation products (See Figure 11).

TABLE I

Relative Retentions of Some Analogues of Somatostatin

Chromatographic Conditions: See Figure 11

Compound	Relative Retention
Somatostatin	1.00
Somatostatin, linear	0.54
$Ser^{3,14}$- somatostatin, linear	0.32
Des Asn^{5}-somatostatin	0.70
Des Ala^{1}, Gly^{2}, Asn^{5}-somatostatin	0.69

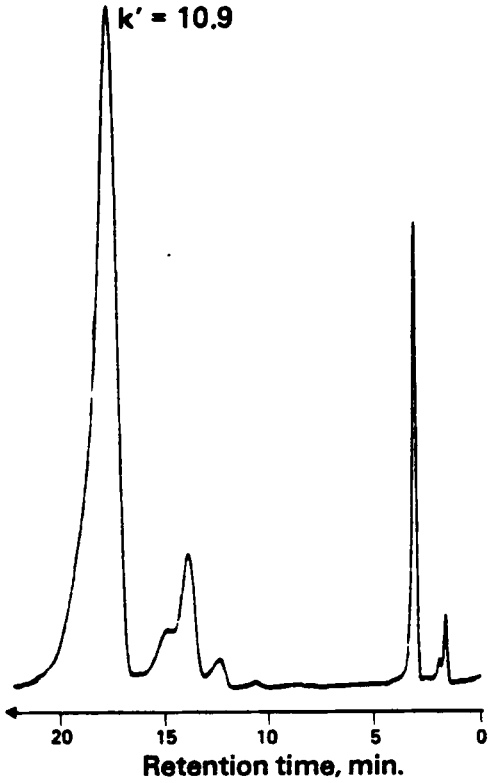

FIGURE 11. Study on the homogeneity of somatostatin. *Column:* As in Figure 3. *Eluent:* 25.5 %, v/v, of acetonitrile in phosphate buffer (ionic strength = 0.1), pH 4.5. *Detection wavelength:* 210 nm (0.16 AUFS). *Amount injected:* 4 µg. *Flow rate:* 1 ml/min. *Chart speed:* 5 mm/min.

No losses due to adsorption to the column have been observed in any of the systems. The chromatographic conditions presented in Figure 11 have been used for quantitation of somatostatin in the presence of a large excess of albumin with a precision of 2.5 % (CV). Although albumin was completely retained on the column, more than 50 injections could be made with maintained column efficiency and unchanged k' values (CV = 1.0 %).

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