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PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES ON A NEW REVERSED-PHASE PACKING MATERIAL, KROMASIL™ C_{18}

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SUMMARY

Preparative reversed-phase high-performance liquid chromatography has found wide use in the production of peptides for pharmaceutical formulations. Purity of the substance and overall economy of the chromatographic system are the most important criterias. In this sense optimized, silica particles and production process with capability to separately control parameters important to chromatography, are essential to high-performance chromatography. Kromasil™ C_{18} packing material was tested and evaluated in respect of its selectivity, flow and pressure properties, resolution, load capacity, recovery, adsorption effects, mechanical strength and chemical degradation.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) of unprotected peptides for their identification and analysis was introduced in the mid 1970s by several authors[1–4]. As a result of the rapid progress over the past 10 years, RP-HPLC has become a valuable method for the preparation of production quantities of biologically active substances as peptides and proteins. Optimized silica packings have made the most important contribution to column efficiency in HPLC. In the production of Kromasil™, a new high-performance silica for liquid chromatography[5], efforts have been focused on surface chemistry, chemical purity, chemical and mechanical stability, pore size and pore-size distribution, surface area, pore volume and particle form and size. The optimization of these parameters together with the proper choice of the mobile phase are essential for separating power and load capacity, long-term stability, high recovery and prevention of denaturation of sensitive biomolecules as proteins.

The main impurities arising from the synthesis of neurohypophysial hormones, such as vasopressin and oxytocin and their analogues, are dimers/polymers and closely related peptides such as deamidated products and isomers. Some of these impurities can be removed by conventional gel and ion-exchange chromatography[6], but for isomers a more selective method such as reversed-phase chromatography is necessary.

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TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF KROMASIL C_{18}

Property measured	*Value*	*Technique*
Particle shape	Spherical	Electron microscopy
Particle size (mean volume)	12.5 μm	Optical microscopy
Particle-size distribution	d_p^{90}/d_p^{10} 1.65	Optical microscopy
Surface area	360 m^2/g	Gas sorption[7]
Pore size (mean)	110 Å	Gas sorption[7]
Pore volume	1.0 ml/g	Gas sorption[7]
Carbon coverage C_{18} monolayer	3.1 $\mu mol/m^2$	Elemental analysis
Endcapped	Yes	
Trace metal content	Na 20 ppm Al <30 ppm Fe 11 ppm	Atomic absorption spectroscopy

MATERIALS AND METHODS

Kromasil C_{18}, 12.5 μm (EKA Nobel, Surte, Sweden) having the physical and chemical characteristics shown in Table I, was slurry-packed into a 250 mm × 10 mm stainless-steel column. Lichroprep C_{18}, 5–20 μm (Merck, Darmstadt, F.R.G.), was packed in the same way. Surface areas and pore-size distributions were measured by the conventional Brunauer–Emmett–Teller (BET) method[7]. The instrumentation consisted of a Digisorb 2600 (Micromeritics, Norcross, GA, U.S.A.). The vasopressin and oxytocin peptides, 8-Arg-vasotocin (AVT), 8-Arg-vasopressin (AVP), d-4-Asn-8-D-Arg-vasopressin (4-Asn-dDAVP), d-8-D-Arg-vasopressin (dDAVP) and oxytocin (OT) were synthesized by fragment condensation (Ferring Pharmaceuticals, Malmö, Sweden). The pumping system was a Waters 600 gradient module with a WISP

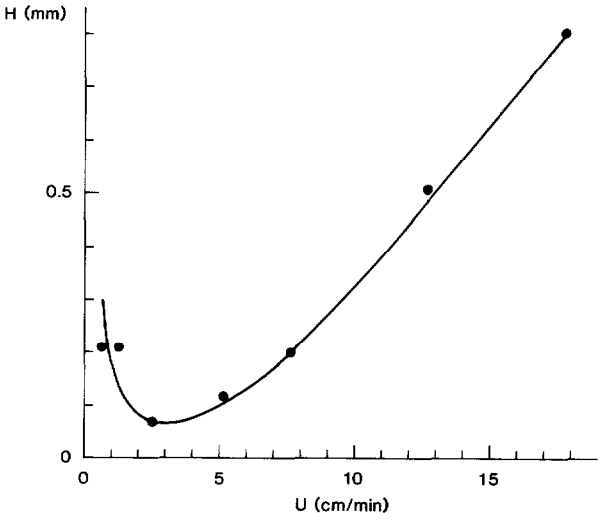


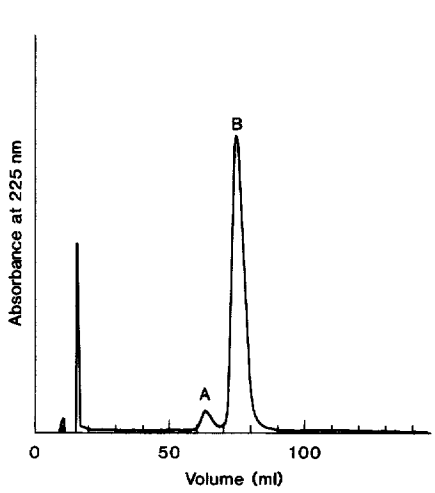
Fig. 1. Dependence of band broadening on flow-rate. Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–0.1% aqueous trifluoroacetic acid (TFA) (1:4).

autoinjector and a Waters Lambda 410 UV detector (Waters Assoc., Milford, MA, U.S.A.). The solvents were of HPLC grade, filtered through a 0.45-μm filter and sparged with helium. The flow-rate was 4 ml/min (5.1 cm/min) (Fig. 1). Chromatography was performed at ambient temperature.

RESULTS AND DISCUSSION

Resolution and selectivity

As test material for the chromatographic evaluation, a crude preparation of d-8-D-Arg-vasopressin (dDAVP) was chosen (Fig. 2). Apart from dDAVP, B (k' 5.9), it also contained an impurity, A (k' 4.7), arising from the synthesis. The impurity could not be removed by either gel or ion-exchange chromatography. The resolution, R_s, of the two peaks, A and B (Fig. 2), was plotted against the linear flow-rate (Fig. 3). The negative effect of bandbroadening caused by diffusion along the column in this system is reduced by the mass transfer in the stationary phase and therefore the R_s value increases as the flow-rate decreases. Spherical particles, in combination with a narrow size distribution and an high mechanical strength, result in low back pressure in the column (Fig. 3).



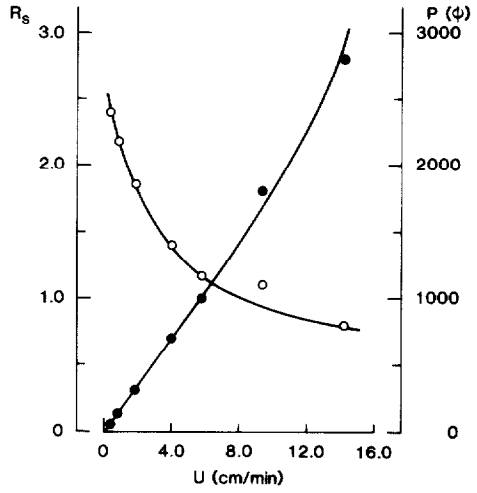


Fig. 2. Resolution of d-8-D-Arg-vasopressin (dDAVP) (B) and an impurity (A). Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–0.1% aqueous TFA (1:4). The same selectivity was observed on the same support with 0.1 *M* ammonium acetate as the eluent.

Fig. 3. Resolution, R_s, of peaks A and B (Fig. 2) *versus* linear flow rate, U (○—○) and the pressure drop, ψ, *versus* linear flow rate (●—●). Support: Kromasil C_{18} (12.5 μm).

The influence of the sample volume injected on the theoretical number of plates is given in Fig. 4. A normal sample volume in preparative HPLC is 2–5% of the column volume, but this depends on the sample, the selectivity of the compounds to be separated and the theoretical number of plates needed to separate a given sample. In the example (α = 1.25 on Kromasil C_{18}), the sample can be dissolved in 75% of the column volume and still show the desired selectivity. Compared to a commonly used

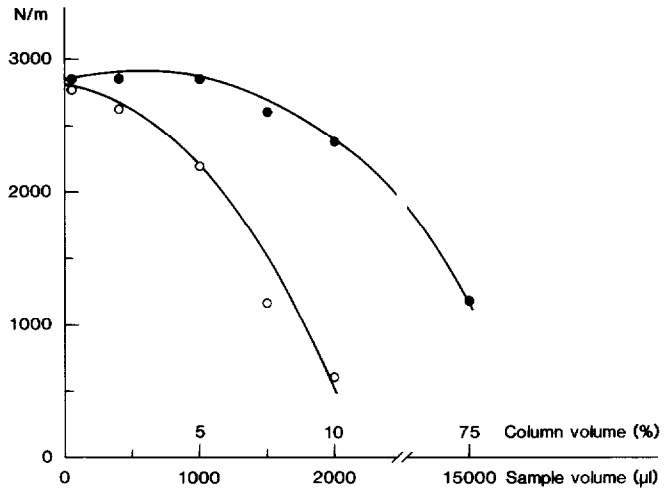


Fig. 4. The number of theoretical plates, N (m), *versus* sample volume or % of column volumes for Kromasil C_{18} (12.5 µm) (●—●) and Lichroprep C_{18} (5–20 µm) (○—○).

preparative RP material, Lichroprep C_{18}, Kromasil C_{18} is not so strongly influenced by the volume injected. The reason for this is the high specific surface area for Kromasil C_{18} and the high degree of carbon coverage. Also considering the amount in mg injected, the Kromasil C_{18} material shows an higher dynamic capacity than that of Lichroprep C_{18} (Fig. 5).

Selectivity

In the selectivity of five different vasopressin and oxytocin peptides, AVT, AVP, 4-Asn-dDVAP, dDAVP and OT, Kromasil C_{18} shows excellent performance and

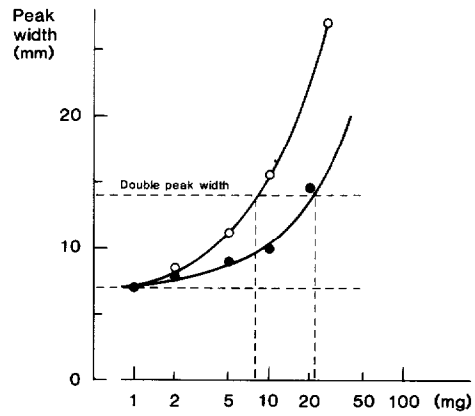


Fig. 5. Evaluation of the dynamic capacity of Kromasil C_{18} (12.5 µm) (●—●) and Lichroprep C_{18} (5–20 µm) (○—○). Solvent system: acetonitrile–0.1 M ammonium acetate (1:4).

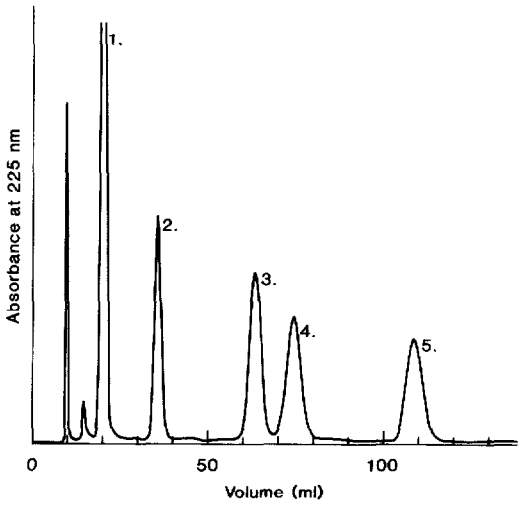


Fig. 6. Elution curve of a mixture of five nonapeptides: 8-Arg-vasotocin (1); 8-Arg-vasopressin (2); d-4-Asn-8-D-vasopressin (3); d-8-D-Arg-vasopressin (4) and oxytocin (5). Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–phosphate buffer (pH 7.0) (18:82, v/v).

peak asymmetry factors in the range of 0.99–1.10 (Fig. 6). The Lichroprep C_{18} column cannot totally separate the same mixture, especially 4-Asn-dDAVP from dDAVP, and the asymmetry factor 1.0–2.0 indicates a tailing effect for the more basic peptides AVT and AVP (Fig. 7). Another example of the difference in resolving capacity is shown in Fig. 8. The more hydrophobic cyclic tetradecapeptide somatostatin was eluted with a k' value of 2.3 from Kromasil C_{18} (Fig. 8A), while k' increases to 5.7 on Lichroprep

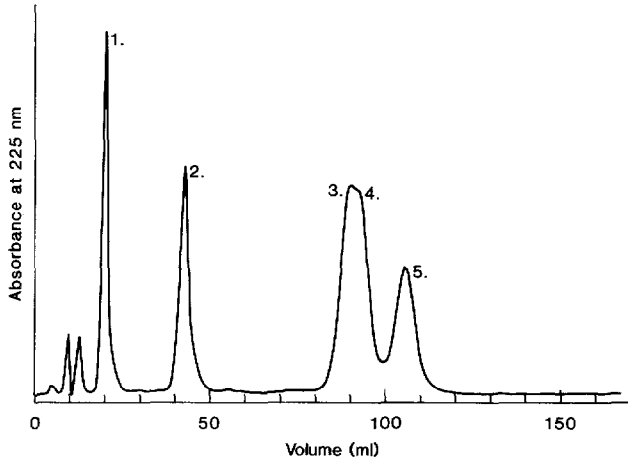


Fig. 7. Elution curve of a mixture of five nonapeptides as in Fig. 6. Support: Lichroprep C_{18} (5–20 μm). All conditions as in Fig. 6.

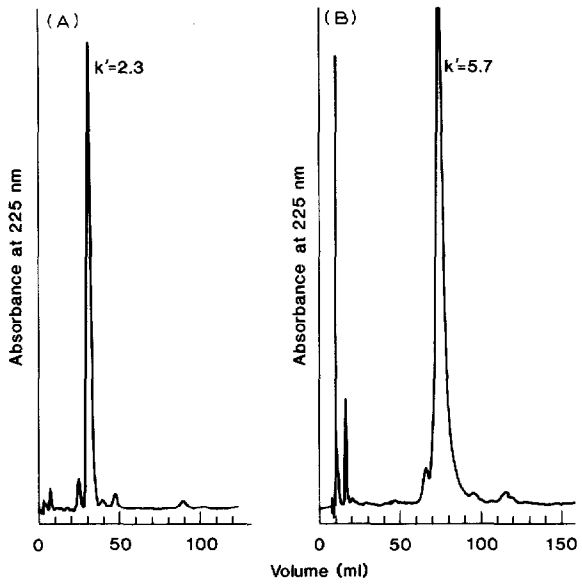


Fig. 8. Chromatography of somatostatin on Kromasil C_{18} (12.5 μm) (A) and Lichroprep C_{18} (5–20 μm) (B). Solvent system: acetonitrile–0.1 *M* ammonium acetate (30:70, v/v).

C_{18} and the chromatogram shows an extremely long tail and a decreased selectivity between the impurities and the main peak (Fig. 8B).

Undesired interaction between the stationary phase and the solute is a common

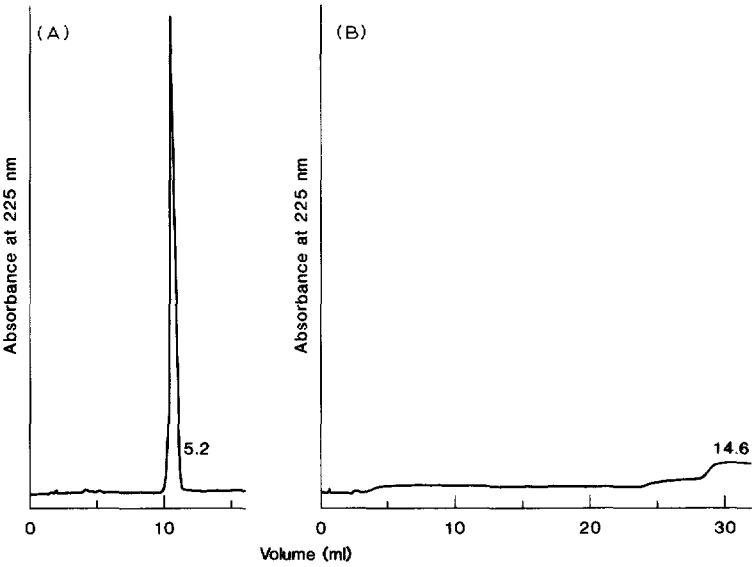


Fig. 9. Chromatography of N,N-diethylaniline on Kromasil C_{18} (A) and Lichroprep C_{18} (B). Chromatographic conditions: columns 200 mm × 4.6 mm; solvent system acetonitrile–water (70:30, v/v); flow-rate 2 ml/min.

problem in liquid chromatography. This interaction is probably due to residual silanols or other adsorption sites, *e.g.*, traces of metals[8,9]. The chemical purity of the silica matrix and high carbon coverage contribute to minimizing these effects. This can be seen by chromatographic a basic substance such as N,N-diethylaniline (Fig. 9). Adsorption of the basic compound was tested on Kromasil C_{18} and Lichroprep C_{18}. The N,N-diethylaniline was easily eluted from the Kromasil column, after the first injection (20 nmol) (Fig. 9A), but was totally adsorbed on the Lichroprep column, and after seven injections each of 20 nmol the aniline appeared as a broad hill (Fig. 9B).

The same difference in retention and selectivity was observed with the Arg-containing peptide, dDAVP (Fig. 10). The columns were washed with ten column volumes of 0.1% aqueous trifluoroacetic acid (TFA) before equilibration in the mobile phase. On Lichroprep C_{18} the peptide was strongly retained and gave rise to a distorted peak and an asymmetry factor of 3.6, without separation of the main peak from the impurity. However, the peptide was eluted normally from Kromasil C_{18} with an α value of 1.45.

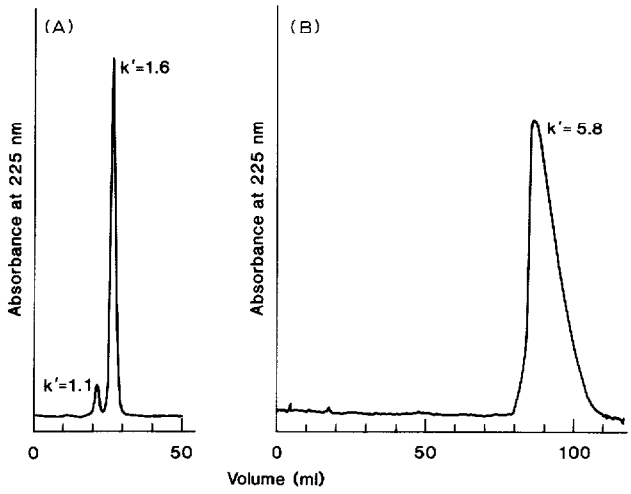


Fig. 10. Chromatography of dDAVP on Kromasil C_{18} (A) and Lichroprep C_{18} (B) after washing with 0.1% aqueous TFA (ten column volumes) and equilibration in acetonitrile–0.1% aqueous TFA (1:4, v/v).

Degradation

An important parameter in preparative RP-HPLC is the chemical and mechanical stability of the stationary phase. Since 0.1% aqueous TFA is a common eluent for the separation of peptides and proteins[10] it was of interest to determine the chemical stability under this condition. A recent investigation on C_1–C_4 organic ligands shows that there is a significant loss of bonded phase during elution with 0.1% aqueous TFA[11]. The organic material degraded under this condition or traces of metals eluted from the column may contaminate the active substance and cause denaturation and undesired effects[12]. The degradation of three different C_{18} bonded phase packings, Kromasil C_{18}, Lichroprep C_{18}, 5–20 μm, and Nucleosil 100-5, C_{18},

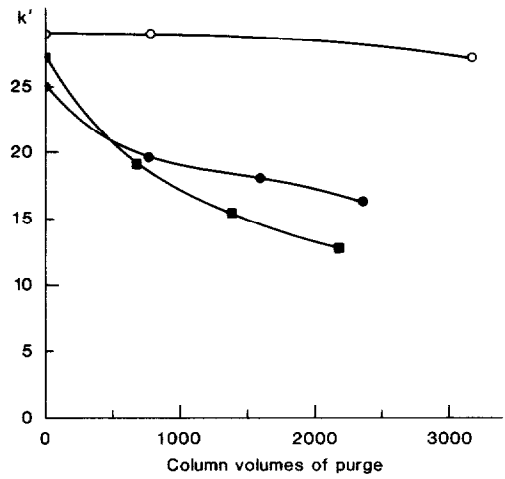


Fig. 11. Degradation of C-bonded phase packings after purging with 0.1% aqueous TFA at 70°C. ○—○, Kromasil C_{18}; ●—●, Nucleosil C_{18} and ■—■, Lichroprep C_{18}.

was investigated under accelerated conditions. The columns (200 mm × 4.6 mm) were purged with 0.1% aqueous TFA at 70°C, at a flow-rate of 2 ml/min. The capacity factor, k', for butylbenzene was determined. The purging process and the capacity factor measurement was repeated and curves showing k' *versus* column volumes were plotted as a measure of the hydrolytic stability (Fig. 11).

Mechanical stability

The mechanical stability of the stationary phase depends only on the naked silica itself. In analytical HPLC systems, the pumps work under constant pressure/flow

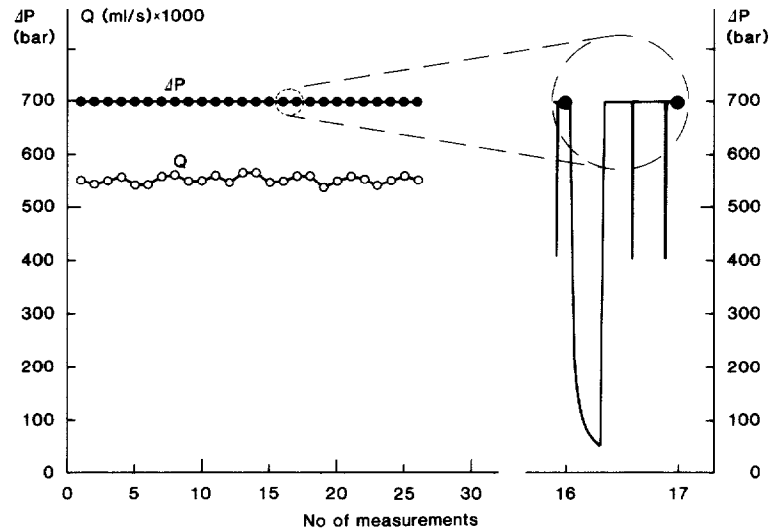


Fig. 12. Mechanical stability of unmodified Kromasil. The flow-rate, Q, was plotted *versus* the pressure drop along the column, ΔP. For further details see the text.

conditions. At the moment of injection, the pressure is drastically decreased, and this is followed by a rapid increase to the preselected level. In our investigation we reproduced this performance. A stainless-steel column (250 mm × 4.6 mm) was slurry-packed with 8-μm unmodified Kromasil and kept under constant pressure (700 bar). Every minute, the pressure was momentarily decreased to 20–50 bar, followed by an increase to 700 bar. The flow-rate was measured and plotted (Fig. 12). This procedure was repeated 26 times. After that, the column was emptied and the stationary phase was examined under a microscope (× 200 enlargement). None of the testing procedures indicated that any fines had been produced.

The change in chromatographic behaviour of Kromasil C_{18} over time under normal conditions was also investigated. Acetonitrile–0.1 *M* ammonium acetate (1:4) was chosen as the mobile phase system. More than 1400 column volumes passed through, which corresponds to 400 analyses. The selectivity between peaks A and B (Fig. 2) was calculated and plotted. No significant change in selectivity was observed (Fig. 13).

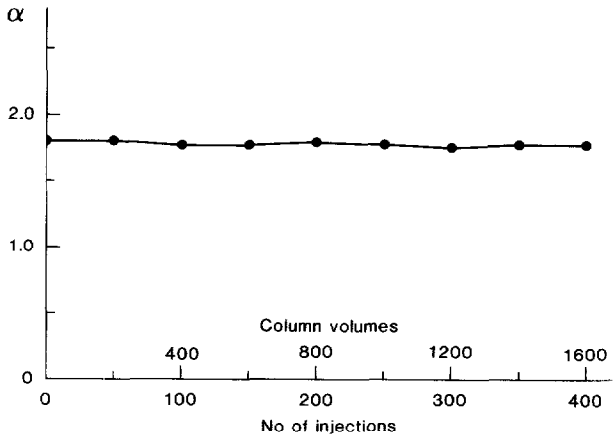


Fig. 13. The change in selectivity, α, of peaks A and B (Fig. 2) plotted *versus* time and column volumes. Support: Kromasil C_{18} (12.5 μm). Solvent system: acetonitrile–0.1 *M* ammonium acetate (1:4).

Recovery

In production-scale chromatography the yield of pure substance per unit time is an important parameter. It has been pointed out that one of the major causes of irreversible interaction in RP-HPLC is the interaction between the solute, the stationary phase and the mobile phase[13]. A 5-mg amount of pure peptide (dDAVP) was injected into the Kromasil C_{18} column under isocratic conditions, and when an increase in the absorbance at 280 nm was registered the eluate was collected manually and the volume measured. The absorbance at 274 nm of the diluted sample was correlated to a known concentration of the peptide dissolved in the mobile phase. The recovery of the peptide chromatographed under acidic (0.1% aqueous TFA) or neutral conditions (0.1 *M* ammonium acetate) was 98 and 95%, respectively. Corresponding figures for Lichroprep C_{18} were 84 and 94%, respectively. When the amount of

organic solvent in the mobile phase was increased no sample was eluted from the Kromasil C_{18} column with acidic or neutral eluents, nor from the Lichroprep C_{18} column with a neutral buffer. However, an increase in the UV signal was observed with the Lichroprep C_{18} column and the acidic 0.1% aqueous TFA.

CONCLUSION

This study has focused on parameters affecting preparative RP-HPLC: selectivity, flow and pressure properties, recovery and chemical and mechanical stability. These parameters are mainly determined by the structure of the naked silica and the method of preparation of chemically modified silica packings. We have shown that under strictly controlled conditions these are the important characteristics which together with a volatile buffer, acidic or neutral, determine whether the proposed purity of the peptide substance can be achieved.

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