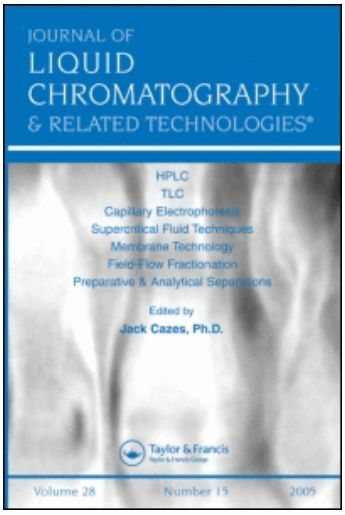

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SEPARATION OF PEPTIDES BY LIQUID CHROMATOGRAPHY ON POLYALKYLENE COLUMNS

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ABSTRACT

Polyethylene was examined as stationary phases for reversed-phase liquid chromatography of peptides. The selectivity for the separation of substance P and GnRH diastereoisomers on polyethylene stationary phases was compared with that for separation on alkyl-bonded silica and porous poly(styrene divinylbenzene). In contrast to alkyl-bonded silica polyethylene and polypropylene columns, having hydrophobic surfaces without polar groups, are stable over the wide pH range of 1-14, allowing the effective regeneration of the stationary phases, especially for peptides and proteins in preparative work. It is shown that GnRH, substance P, magainine-II-amide, fibrinogen peptides prepared by solid-phase method and semisynthetic insulins can be purified to a high degree, wich demonstrates the usefulness of polyethylene columns for the purification of peptides.

INTRODUCTION

Reversed-phase liquid chromatography has become an efficient method for the separation of biological

active substances such as peptides and proteins. Currently more than 70% of HPLC separations are carried out in the reversed-phase mode by utilizing alkyl-bonded silica as stationary phases (1).
However, the presence of surface silanols wich act as weak acids and are ionized above pH 3.5-4.0 (2) causes interactions with basic residues of peptides and have an adverse effect on resolution. The chemical instability at high pH prevents the effective regeneration of the stationary phases, especially in preparative work.
Organic polymer-based stationary phases (3-7), as well as the polymer-coated silica phases (8-9) have been developed to increase the chemical stability of the stationary phases and decrease electronic interactions. Polyethylene and polypropylene have homogeneous hydrophobic surfaces without polar groups and were examined for their use as stationary phases for reversed-phase liquid chromatography (10). The excellent chemical stability over the wide pH range of 1-14 and the absence of residual polar groups on the adsorbent surface make the polyethylene stationary phases appropriate for preparative separations.
In the present work, the retention behaviour of synthetic peptides on polyethylene columns was examined. The selectivity, resolution power and stability with regard to the purification of several peptides will be described.

MATERIALS AND METHODS

Polyethylene was supplied by E. Merck, Darmstadt and sieved to 20-40 µm and 40-60 µm irregular particles. The dry polymer particles were dispersed in methanol

and the slurry was packed into a column at a pressure of up to 1.5 MPa.

Analytical separations of peptides were carried out on 150 x 4 mm I.D. column containing the 20-40 µm particles at an eluent flow-rate of 0.5 ml/min.

Preparative peptide separations were caried out on a 500 x 25 mm I.D. glass column (Omnifit-Macherey-Nagel, Düren) containing the 40-60 µm polyethylene particles at an eluent flow-rate of 5 ml/min.

The instrumentation consisted of two LC-8A pumps, a SCL-8A system controller, a SPD-6A UV detector operated at 220 nm and a C-R4A Chromatopac integrator purchased from Shimadzu Europa, Duisburg.

Sample concentrations were 1-10 mg/ml of peptides. A Rheodyne Model 7125 injection valve (20-and 2000 µl loops) was used for sample injection.

Separations were performed at ambient temperature. Capacity factors (k') were calculated from the retention time, t_R: $k'=(t_R-t_0)/t_0$. Data for t_0 were obtained by injecting a liquid mixture with a volume composition different from that of the eluent.

Substance P, magainin-II-amide and fibrinogen peptides prepared by solid-phase method using Fmoc-strategy were obtained from the Department of Peptide Chemistry. GnRH peptides were synthesized by enzymatic fragment condensation (11). Deshexapeptide(B25-30) insulinamide was prepared by enzyme-catalyzed semisynthesis (12).

RESULTS AND DISCUSSION

Retention behaviour of peptides

To estimate the hydrophobic character of the polyethylene stationary phases, the capacity factors of

aromatic hydrocarbons were determined (10). The results indicate a similar behaviour of the polyethylene and the alkyl-bonded silica. However, the capacity factors of the polymer phases were lower than those of alkyl-bonded silica. The absence of polar groups on the adsorbent surface causes a strong decrease of retention times, especially for basic compounds which are mainly retained by silanophilic interactions.

Human insulin, the acidic dipeptide Z-Phe-Phe and the basic undecapeptide Substance P, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, were chromatographed with aqueous methanol containing 0.1% TFA and the capacity factors were plotted as a function of the methanol concentration in the eluent, as shown in Fig. 1.

The dependence of log k' on methanol concentration was very close to linear and the capacity factors of polyethylene were lower then those of PLRP-S, a copolymer of styrene and divinylbenzene. The increase in capacity factor of the dipeptide Z-Phe-Phe on the polystyrene stationary phases is probably due to the additional π-π interactions with aromatic dipeptide.

To demonstrate the separation capabilities of the polyethylene stationary phases for peptide separation, the selectivity for the separation of D-Phe(6)-GnRH (p-Glu-His-Trp-Ser-Tyr-D-Phe-Leu-Arg-Pro-Gly-NH_2) and Substance P diastereoisomers were determined (Table I). The data for α, as shown in Table I, confirm the higher selectivity of the polyethylene stationary phases in comparison with the alkyl-bonded silica and PLRP-S. The differences of α could be explained by the absence of strong binding sites on polyethylene, such as silanols and π-systems.

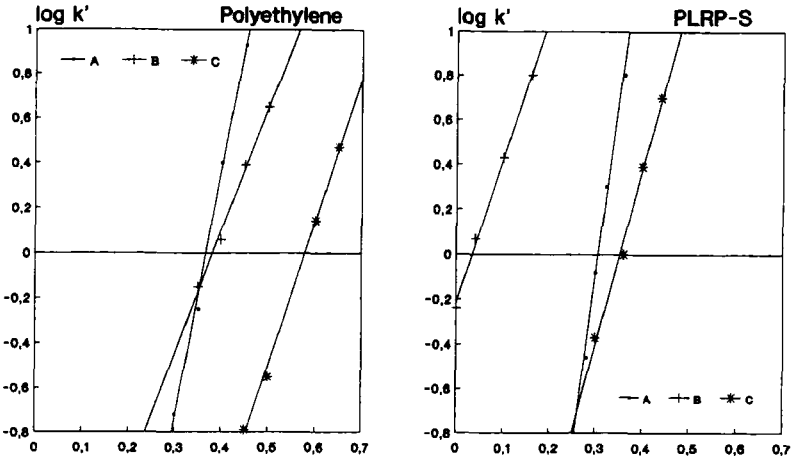

FIGURE 1. Change in the capacity factors with increasing volume content of water in methanol on polyethylene 20-40 µm and PLRP-S 15-25 µm as stationary phases.
(A) Human insulin, (B) Z-Phe-Phe, (C) Substance P.

TABLE 1

Selectivity α (k'_2/k'_1) for the Separation of Diastereoisomers. Mobile Phase: Acetonitrile / 0.07 M KH_2PO_4, pH 2.5.

D-Phe(6)-GnRH

Stationary phase	$k'_{D\text{-}Trp3}$	$k'_{L\text{-}Trp3}$	α
Spherisorb ODS	6.85	8.28	1.21
PLRP-S	1.95	2.42	1.24
Polyethylene	2.80	3.61	1.29

Substance P

Stationary phase	$k'_{L\text{-}Phe7}$	$k'_{D\text{-}Phe7}$	α
Spherisorb ODS	1.49	2.35	1.58
PLRP-S	1.93	3.32	1.72
Polyethylene	1.67	3.21	1.92

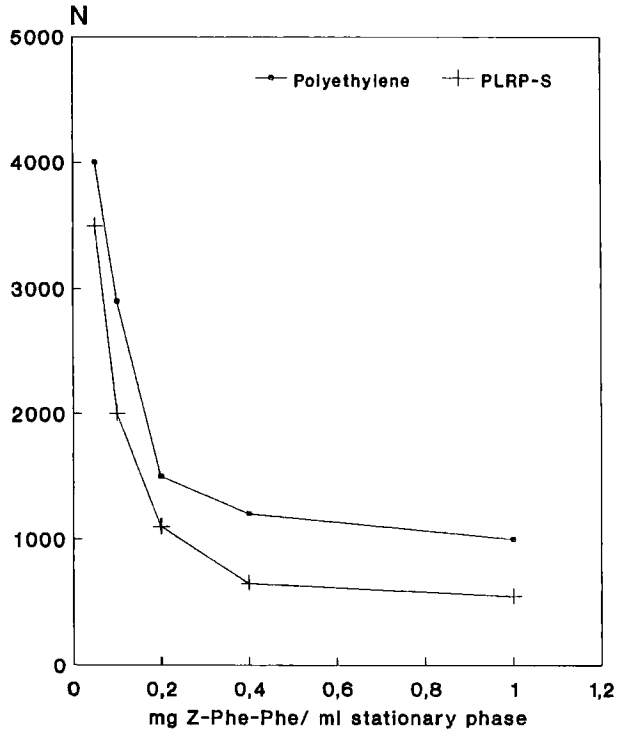

FIGURE 2. Influence of dipeptide sample load on the efficiency of polymer columns.
Mobile phase: PLRP-S 95% methanol/ 0.1% TFA, polyethylene 60% methanol/ 0.1% TFA.

Preparative chromatography of peptides

To assess the sample capacity of the preparative polyethylene column for peptide separation, the influence of the amount of sample injected on the number of theoretical plates, N, was determined (Fig. 2.) with the dipeptide Z-Phe-Phe using methanol-water-0.1% trifluoracetic acid as eluent.

The efficiency of both columns decrease with an increasing sample load from 0.05 to 0.4 mg /ml volume of the stationary phases and then remains close to con-

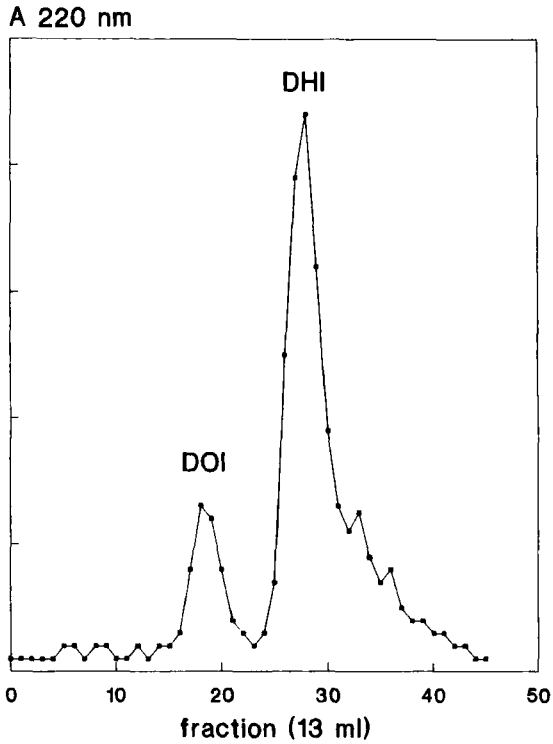

FIGURE 3. Chromatogram of the separation of des-hexapeptide (B25-30) insulinamide (DHI) and des-octapeptide(B23-30) insulin (DOI) on a 500 x 25 mm I.D. polyethylene column. Flow rate: 5 ml/min. Linear gradient from 20 to 35% acetonitrile in water containing 0.1 M KH_2PO_4, pH 3.0.

stant. The sample capacity of the 20-40 µm polyethylene stationary phases was higher than those of the 15-25 µm PLRP-S column. Polyethylene can be used with sample loads up to 1 mg of peptide per ml of stationary phase.

The preparative chromatograms of des-hexapeptide (B25-30) insulinamide, Fmoc-magainin-II-$(Lys)_{10}$-amide, D-Trp(6)-GnRH and the fibrinogen-γ'-chain tetrapeptide Arg-Gly-Asp-Phe are shown in Figs.3 to 6. The sepa-

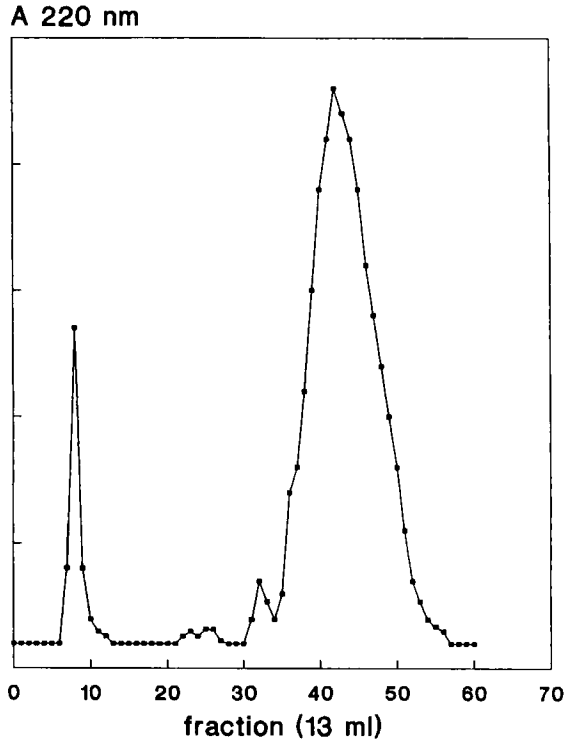

FIGURE 4. Elution of crude Fmoc-magainin-II-(Lys)$_{10}$-amide on a 500 x 25 mm I.D. polyethylene column. Flow rate: 5 ml/min. Linear gradient from 45 to 90% methanol in water containing 0.1% TFA.

rations were carried out on a 500 x 25 mm I.D. polyethylene column loading 100 mg of the peptide. Des-hexapeptide(B25-30) insulinamide prepared by enzymatic semisynthesis and the starting material des-octapeptide(B23-30) insulin are baseline separated on polyethylene using an acetonitrile gradient (Fig.3.). The more hydrohobic Fmoc-Magainin-II-(Lys)$_{10}$-amide prepared by solid phase method was purified using methanol-water-0.1% trifluoracetic acid as eluent (Fig.4.). The purification of the enzymatic fragment

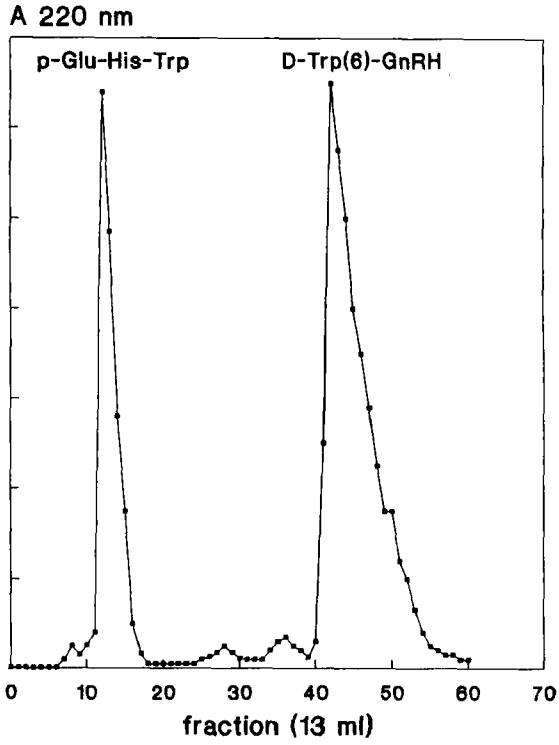

FIGURE 5. Chromatogram of the purification of D-Trp(6)-GnRH on a 500 x 25 mm I.D. polyethylene column. Flow rate: 5 ml/min. Linear gradient from 20 to 60 % methanol in water containing 0.1% TFA.

condensation product D-Trp(6)-GnRH, pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH2, was achieved by separation of pGlu-His-Trp-OH, generated by hydrolysis of the unreacted tripeptide ester (Fig.5.). The purity of crude synthetic GnRH and of the purified peptide were determined by reversed-phase HPLC to be 64,3 and 95,1%, respectively. The tetrapeptide Arg-Gly-Asp-Phe (84.8% HPLC) was purified using a mobile phase consisting of 2% of methanol and 0.1% of trifluoracetic acid and isocratic conditions (Fig.6.) to give a peptide wich

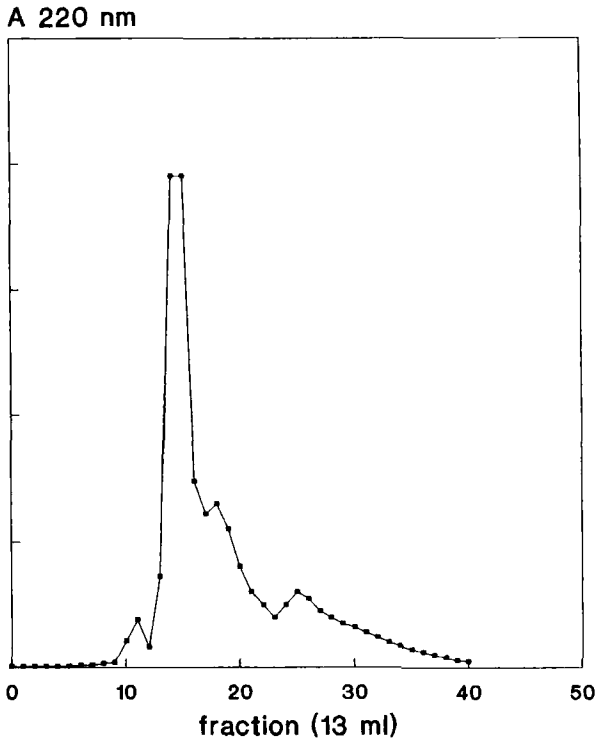

FIGURE 6. Isocratic elution of crude Arg-Gly-Asp-Phe on a 500 x 25 mm I.D. polyethylene column. Flow rate: 5 ml/min. Mobile phase: Methanol-water (2:98,v/v) containig 0.1% TFA.

was determined by analytical HPLC to be > 99%. The recoveries were 90-98%. More lipophilic compounds adsorbed on the stationary phase were removed by washing with organic solvent.

The results indicate a similar behaviour of the polyethylene column and the polypropylene stationary phases (10). To regenerate the preparative column, irreversibly adsorbed biopolymers can be removed with 1 M sodium hydroxid solution. The polyalkylene stationary phases were stable under these conditions. Therefore,

polyalkylenes are particularly suitable for preparative separations of peptides and proteins that require a chemically stable stationary phase but not a high number of theoretical plates. The disadvantages of polyethylene and polypropylene used as stationary phases include the lower pressure stability compared with silica phases. 1.5 MPa should not be exceeded because distortion of particles may occur, leading to reduced efficiency of the polymer column.

CONCLUSIONS

The polyalkylene stationary phases have homogeneous hydrophobic surfaces without strong binding sites. The retention behaviour of peptides indicates a higher selectivity of the polyethylene stationary phases in comparison with alkyl-bonded silica and with PLRP-S, a copolymer of styrene and divinylbenzene. The polyalkylenes are stable over the wide pH range of 1-14, allowing the effective regeneration of the stationary phases, especially for peptides and proteins in preparative work. The high peptide recoveries, the chemical stability of the phases and the sufficient performance notwithstanding the lower pressure stability recommend the use of polyalkylenes as stationary phases for preparative chromatography.

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