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Use of polymeric reversed-phase columns for the characterization of polypeptides extracted from human pancreata

II. Effect of the stationary phase

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ABSTRACT

The potential value of eight commercial available polymer-based reversed-phase (RP) columns for peptide and protein separations was evaluated using crude acetic acid extracts of normal and diabetic human pancreata and mixtures of pure polypeptides as samples. All columns were characterized with acetic acid gradients in water as mobile phase, and different chromatographic profiles were obtained depending on the type of polymer column (bare or derivatized) and the type of ligand. Some of the columns were virtually free from effects related to the polymer skeleton whereas in others the separation was influenced by both the ligand and the polymeric backbone. Two selected polymeric RP columns were, together with a silica-based C_4 column, further characterized with acetonitrile gradients in trifluoroacetic acid (TFA), and the separation temperature was found to have a drastic effect on the separation efficiency for proteins with mol. wt. > 6000 dalton. No such effect was seen for polypeptides with mol. wt. < 6000 dalton.

Mixtures of pure peptides and proteins were separated using acetic acid gradients in water, acetonitrile or isopropanol, and normally the highest efficiency was found with the use of acetonitrile as mobile phase modifier. Isopropanol was less suitable as an organic modifier. The separation of the β-lactoglobulin A- and B-chains may be used to give a rapid estimate of the chromatographic usability of polymer-based RP-columns for peptide and protein separations in acetic acid gradients in water and in acetonitrile gradients. Recoveries for insulin, proinsulin, growth hormone, ovalbumin and human serum albumin were measured for several polymer-based RP columns eluted with acetic acid gradients in water and with acetonitrile-based mobile phases. The highest recoveries of serum albumin and ovalbumin were found after elution with acetic acid gradients in water.

INTRODUCTION

Although the first commercially available silica-based reversed-phase high-performance liquid chromatographic (RP-HPLC) columns were introduced more than 15 years ago, this type of stationary phase is still the main choice for RP-HPLC-based polypeptide analyses. More than 65% of the total number of HPLC analyses are performed on such columns [1], and the choice of mobile phase additives for these analyses (*e.g.*, insulin analyses) is invariably from one of three groupings: ion-pairing

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agents [alkylammonium salts, trifluoroacetic acid (TFA), phosphoric acid], salts at acidic or neutral pH (acetates, phosphates, sulphates) or chaotropic agents (*e.g.* perchlorates) in combination with acetonitrile [2].

Several polymer-based RP-HPLC columns have been available for some time, but although they in theory should offer some advantages over similar silica-based columns (higher chemical stability, no potential interfering silanol group [3]), their use for polypeptide analyses has been rare [4].

We have recently shown that polymeric RP columns eluted with acetic acid gradients (without any "organic modifier") are an interesting alternative for polypeptide analyses [5], especially for crude extracts of biological materials [6]. The separation patterns of acetic acid extracts of diabetic and normal human pancreata obtained after elution with acetic acid gradients were comparable to those resulting from the use of acetonitrile gradients in TFA or triethylammonium phosphate (TEAP), and similar results were obtained on a divinylbenzene (DVB)-based RP column and a silica C_4 column [6,7].

As the polypeptide selectivity of the DVB column could be changed in parallel with that of the silica C_4 column (*i.e.*, by changes in the mobile phase composition), and as the separation patterns after acetic acid gradient elution of a DVB-based and a styrene-based RP column were strikingly alike [7], a number of commercially available polymeric RP columns were compared with respect to their separation capacity for crude pancreatic extracts. In this work we examined several polymeric "phenyl" and "alkyl" columns with 120-min acetic acid gradients, and a few of these stationary phases were further characterized (together with a silica C_4 column) with similar samples using acetonitrile gradients in TFA.

EXPERIMENTAL

The HPLC equipment, columns and samples used were essentially as described in Part I [7]. Detailed descriptions of samples and stationary and mobile phases are given in the figure legends.

RESULTS

Fig. 1 shows the separation of crude acetic acid extracts from a normal and a diabetic human pancreas using three different polymeric RP columns [Dynospheres PD-102-RE (top, left), Chrompack 8P 300RP (top right) and TSK Phenyl 5PW RP+ (bottom)]. The three columns have aromatic groups as the principal type of bonded phase, but differ in other respects: the Dynospheres and Chrompack columns are polymerized (primarily) from DVB and styrene, respectively, whereas the TSK column has phenyl groups attached to a hydrophilic polymer backbone. The acetic acid gradients were similar in length and slope (increase of 0.45%/min of acetic acid). However, in order to obtain similar retention times for the principal sample components in the last part of the chromatograms of the normal pancreatic extracts (peak I material), the starting and ending points of the acetic acid gradient used for the elution of the TSK Phenyl column had to be adjusted from 37.5% to 90% acetic acid (used for the other two columns) to 20% to 72% acetic acid. On elution with the 37.5% to 90% acetic acid gradient, the chromatograms from the TSK Phenyl column

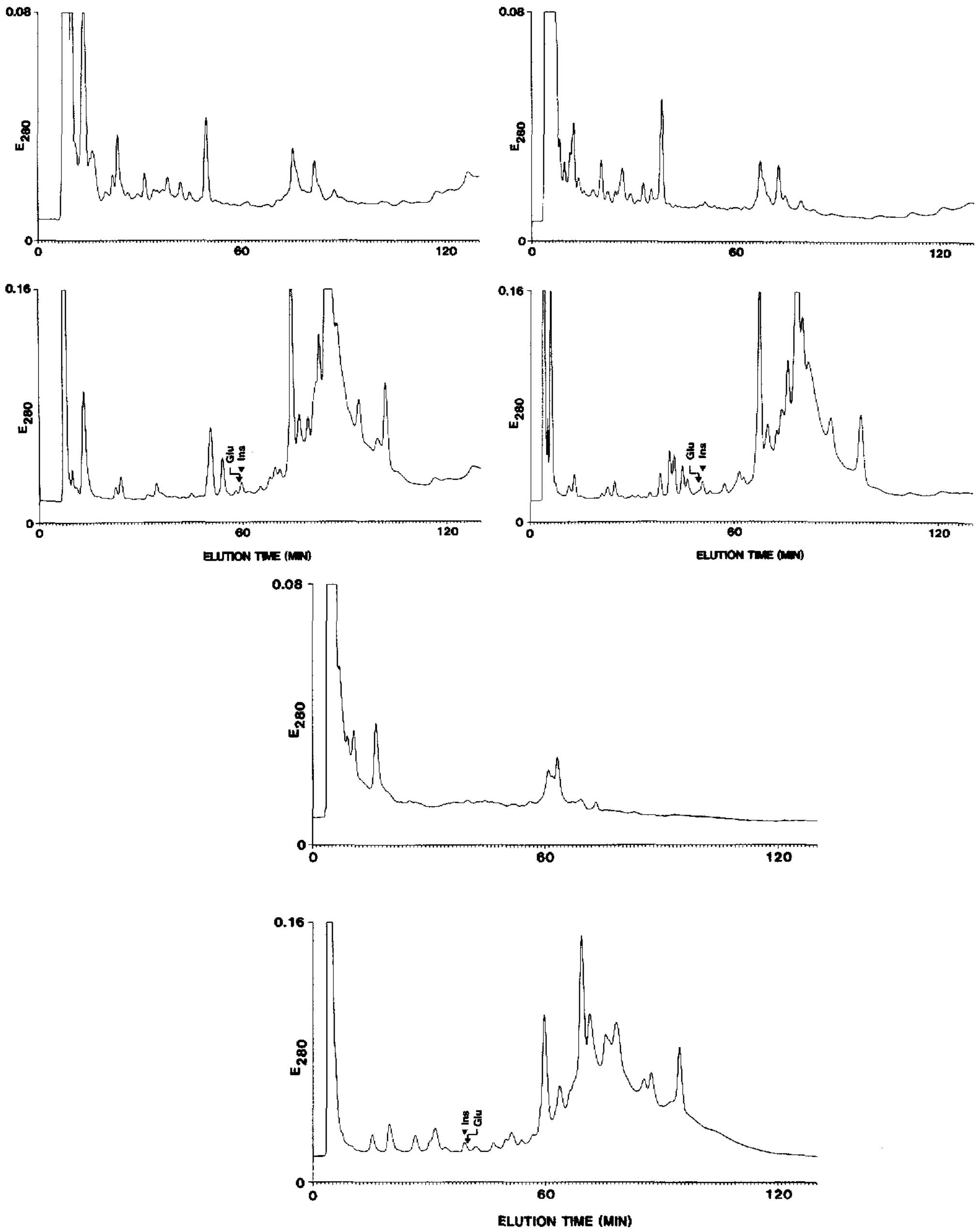


Fig. 1. RP-HPLC of 50 µl of crude acetic acid extract of a diabetic human pancreas (top) and a normal human pancreas (bottom) using a 250 × 4.6 mm I.D. Dynospheres PD-102-RE column (top left), a 150 × 4.6 mm I.D. Chrompack 8P 300 RP column (top right) or two 75 × 4.6 mm I.D. TSK Phenyl 5PW RP+ columns in series (bottom) eluted with an acetic acid gradient [from 34% to 90% acetic acid (top) or from 20% to 72% acetic acid (bottom) linearly for 120 min followed by 10 min isocratically at 90% acetic acid]. Flow-rate, 0.5 ml/min. Elution positions of authentic human insulin (Ins) and glucagon (Glu) are marked.

were unacceptably compressed and lacked all details otherwise revealed in the initial half of the chromatograms, and insulin and glucagon were not resolved from the front peak (data not shown).

The chromatograms for the Dynospheres and the Chrompack columns were remarkably similar: the chromatograms of the diabetic extract were almost replicas of each other, and they differed markedly from that obtained on the TSK Phenyl column. Further, the sharp peaks obtained on both columns in the first half of the chromatograms of extracts of a normal pancreas disappeared in the chromatogram from the TSK Phenyl column. However, the best separation of the individual components in the peak I material (composed primarily of albumin, globulin and the digestion enzymes from the exocrine pancreas [6]), eluted in the last part of the three chromatograms, was obtained with use of the TSK Phenyl 5PW RP+ column.

Separations of similar acetic acid extracts, using four different alkyl-polymer columns, are shown in Fig. 2. Three of these (the Asahipak columns) are composed of an identical poly(vinyl alcohol) matrix, to which C_4, C_8 or C_{18} groups are anchored (top left, top right and bottom left, respectively), whereas the fourth, a Tosoh octadecyl 4PW column (bottom right), has C_{18} groups attached to the same hydrophilic backbone as the TSK Phenyl 5PW RP+ column. The acetic acid gradients were of similar length and slope, but in order to obtain retention times comparable to the other three, the starting and ending points of the gradient used for the Asahipak C_4 column were reduced so as to be similar to that described above for the TSK Phenyl 5PW RP+ column.

A decreasing hydrophobicity of the bonded phases in the series C_{18} to C_4 was clearly demonstrated in the components eluted in the first half of the chromatogram of the normal pancreas [by and large corresponding to peak II material with molecular weight (MW) $<$ 6000 dalton], which were best separated on the C_{18} column (bottom left). In parallel with this, the components in the last half of the chromatograms (peak I material, MW $>$ 6000 dalton) displayed the greatest number of details on the C_4 column (top left). This column also seemed to have the best selectivity for the diabetic extract (top left, upper trace). However, the chromatograms of this extract obtained from all three columns were less detailed than those obtained using the Dynospheres or Chrompack columns (Fig. 1, top).

The separation of both extracts on the Tosoh C_{18} 4PW column (Fig. 2, bottom right) bore some resemblance to that on the TSK Phenyl 5PW RP+ column (Fig. 1, bottom): lack of most of the details from the diabetic extract and a reduced peak capacity in the first half of the chromatogram of the normal extract. However, the components with MW $>$ 6000 dalton were less well separated on this column than on the TSK Phenyl 5PW RP+ column.

The separation capacities of the seven different polymeric columns towards the extremely complex, lipid-containing pancreatic extracts were obviously very different. In order to compare the individual variations obtained with acetic acid gradients with "classical" RP-HPLC polypeptide analyses, crude extract and peak I and peak II components were separated on a Dynospheres column (Fig. 3, left), a Nucleosil C_4 column (Fig. 3, right) and an Asahipak C_8 column (Fig. 4, right) eluted with an acetonitrile gradient in TFA.

Although the chromatograms of the normal extract separated with acetic acid or acetonitrile–TFA were comparable in outline, it is evident that the latter mobile

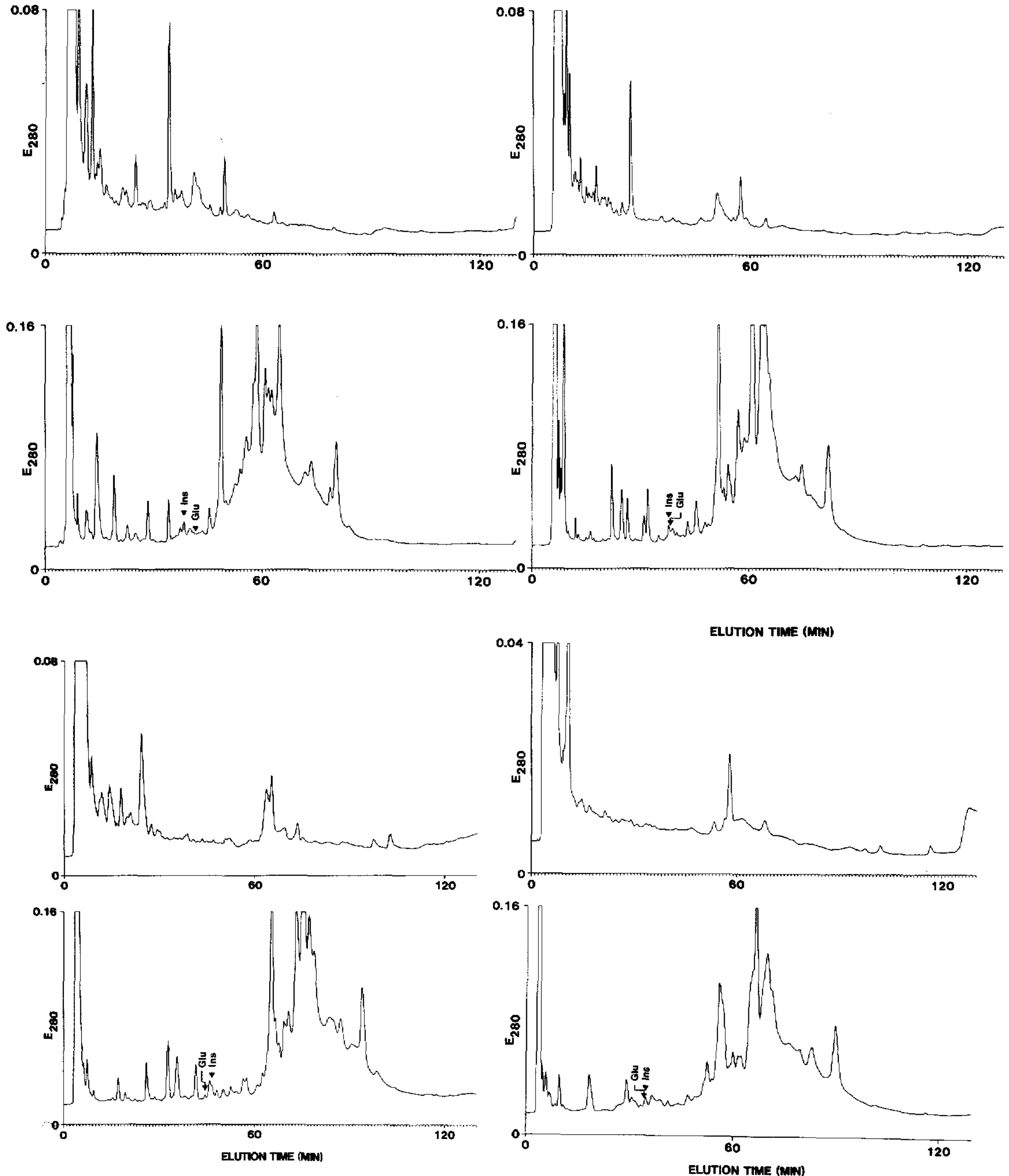


Fig. 2. RP-HPLC of 50 μl of crude acetic acid extract of a diabetic human pancreas (top) and a normal human pancreas (bottom) using a 250 × 4.6 mm I.D. Asahipak C4P column (top left), a 250 × 4.6 mm I.D. Asahipak C8P column (top right), a 150 × 4.6 mm I.D. Asahipak ODP column (bottom left) and a 150 × 4.6 mm I.D. Tosoh octadecyl 4PW column (bottom right) eluted with an acetic acid gradient [from 34% to 9% acetic acid (Asahipak C18 and C8, Tosoh octadecyl 4PW) or from 20% to 72% acetic acid (Asahipak C4) linearly for 120 min followed by 10 min isocratically at 90% acetic acid]. Flow-rate, 0.5 ml/min. Elution positions of authentic human insulin (Ins) and glucagon (Glu) are marked.

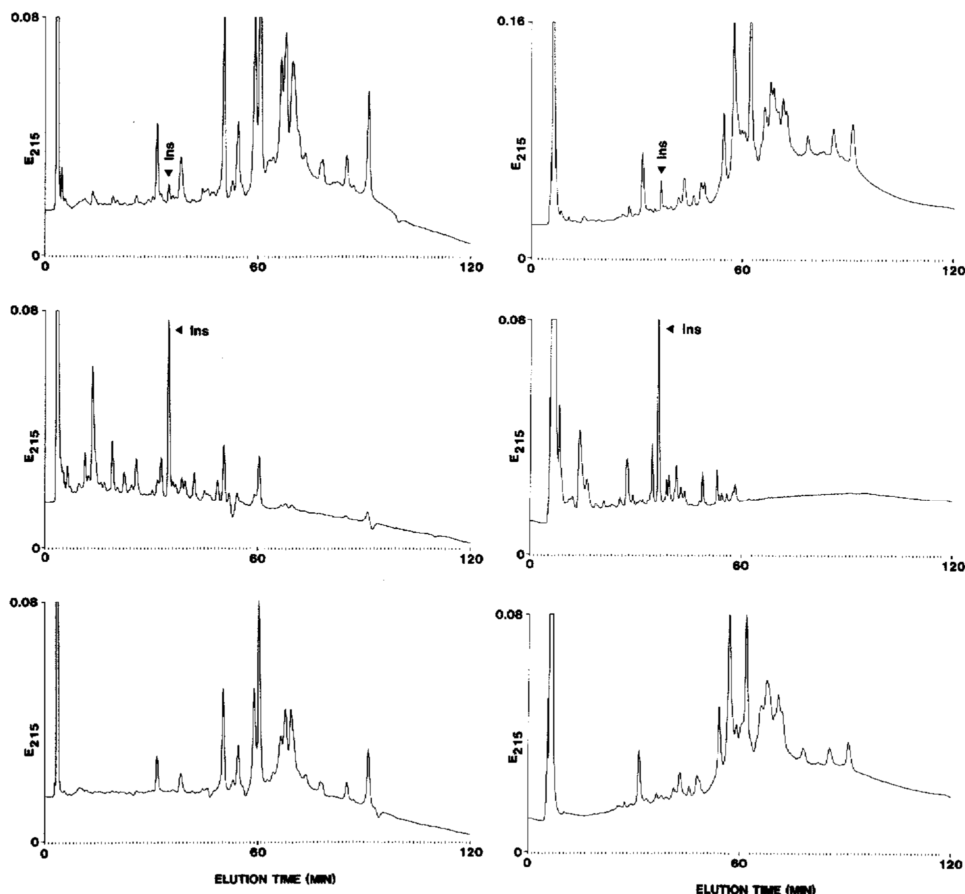


Fig. 3. RP-HPLC of 5 μl of crude acetic acid extract from a normal human pancreas (top) and 50 μl of the corresponding peak I (bottom) and peak II (middle) materials dissolved at 1 mg/ml in 3 *M* acetic acid using a 250 × 4.6 mm I.D. Dynospheres PD-102-RE column (left) or a 250 × 4.0 mm I.D. 5-μm Nucleosil 300 Å C_4 column (right) eluted with an acetonitrile gradient in TFA (from 0.075% TFA–20% acetonitrile to 0.070% TFA–60% acetonitrile linearly for 120 min). Flow-rate, 1.0 ml/min (Dynospheres) or 0.5 ml/min (Nucleosil). The elution position of authentic human insulin (added to the sample) is marked.

phase had a marked influence on especially the separation of the components with MW >6000 dalton, which were now much better separated on the Dynospheres column (compare Fig. 3, top left, with Fig. 1, top left). A more detailed separation of peak I material was not observed for the Asahipak column eluted with acetonitrile–TFA (compare Fig. 4, top right, with Fig. 2, top right).

With the exception of the effect of the difference in hydrophobicity of the bonded phases, the separation patterns of the crude extract, peak I and peak II materials using the Dynospheres column were comparable to those obtained on the Nucleosil C_4 column (Fig. 3, left and right). The separation of peak II material on the Asahipak column (Fig. 4, middle right) was superior to those obtained on the Dynospheres and Nucleosil columns (Fig. 3, middle left and right). The separation temperature had no

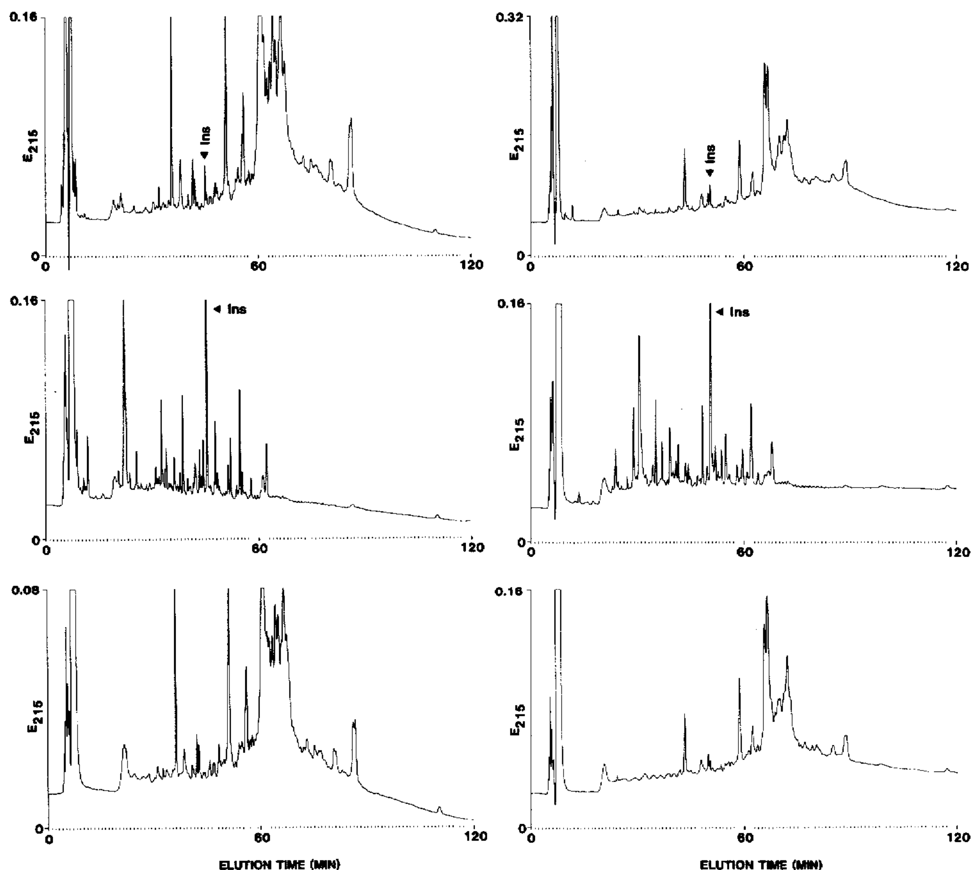


Fig. 4. RP-HPLC of 5 μl of crude acetic acid extract from a normal human pancreas (top) and 25 μl of the corresponding peak I (bottom) and peak II (middle) materials dissolved at 1 mg/ml in 3 *M* acetic acid using a 250 × 4.6 mm I.D. Asahipak C8P column eluted with an acetonitrile gradient in TFA (from 0.075% TFA–20% acetonitrile to 0.070% TFA–60% acetonitrile linearly for 120 min). Flow-rate, 0.5 ml/min. The elution position of authentic human insulin is marked. Separation temperature: room temperature (right) or 45°C (left).

major influence on this UV profile (Fig. 3, middle), whereas the separation of components with MW >6000 dalton was markedly improved when performed at 45°C (Fig. 4, bottom).

Manufacturers of RP columns dedicated to polypeptide analyses often characterize their stationary phases with a number of pure "standard" proteins. As it could be of interest to know whether information about the behaviour of a limited number of pure polypeptides might allow reliable predictions of the potential value of the stationary phase for the separation of unknown peptides and proteins or highly complex samples, utilizing identical or alternative mobile phases, the polymeric columns described above were characterized with a number of "standard" proteins eluted with acetic acid gradients in water, acetonitrile or 2-propanol. The results for two C_{18} columns are shown in Fig. 5 (Tosoh octadecyl 4PW) and Fig. 6 (Asahipak ODP-50).

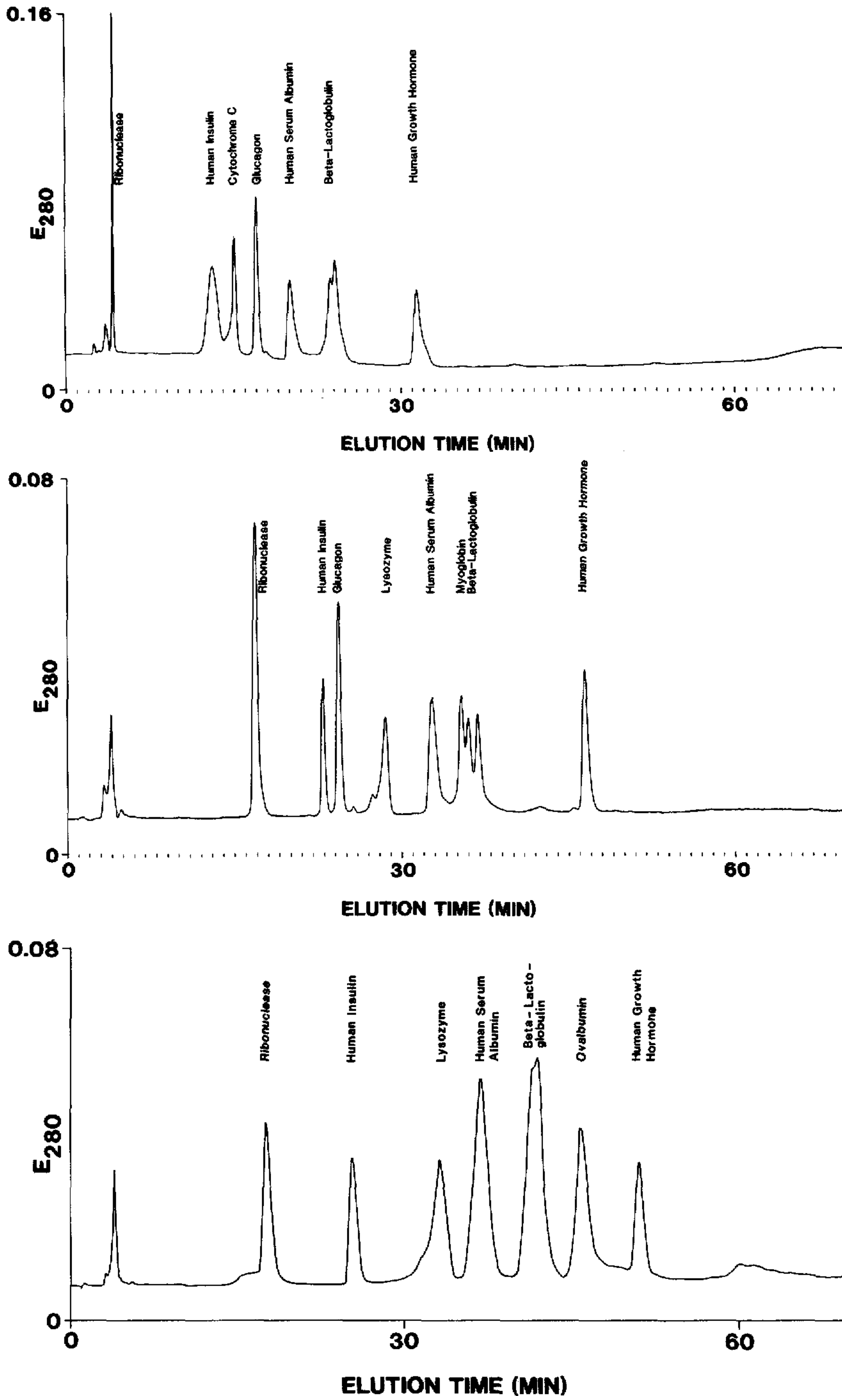


Fig. 5. RP-HPLC of a number of "standard" proteins using a 150 × 4.6 mm I.D. Tosoh octadecyl 4PW column eluted with a 2-propanol–acetic acid gradient (from 24% acetic acid–12% 2-propanol to 40% acetic acid–60% 2-propanol linearly for 60 min followed by 10 min isocratically at the final conditions; top), an acetonitrile–acetic acid gradient (from 24% acetic acid–12% acetonitrile to 40% acetic acid–60% acetonitrile linearly for 60 min followed by 10 min isocratically at the final conditions; middle) or an acetic acid gradient (from 34% to 90% acetic acid linearly for 60 min followed by 10 min isocratically at the final conditions; bottom). Flow-rate, 0.5 ml/min. Sample load, 50–100 μg of each "standard" protein.

In spite of individual variations, the majority of the polymeric RP columns analysed so far behaved in a manner identical with the Tosoh C_{18} column (Fig. 5): peak shape, selectivity and peak capacity were optimum with acetonitrile as organic modifier (middle), whereas broader peak shapes and (total or partial) loss of resolution occurred with acetic acid gradients (bottom).

Poor peak shapes of several of the more hydrophobic polypeptides were observed after elution with 2-propanol in acetic acid (Fig. 5, top, and especially Fig. 6, top). This was also observed with the use of the Chrompack column and the Asahipak C_4 and C_8 columns (data not shown).

The behaviour of the Asahipak C_{18} column was different from the average results described above (Fig. 6): equally sharp peaks were obtained for all polypeptides (including albumin, ovalbumin and lysozyme) in acetic acid–acetonitrile gradients and in acetic acid gradients in water (Fig. 6, bottom and middle), and also the resolution of the two closely related chains in β-lactoglobulin (a valuable measure for polypeptide selectivity) in acetic acid was superior to that obtained in acetonitrile. A comparable resolution of β-lactoglobulin in acetic acid gradients was only achieved with the TSK Phenyl 5PW RP+ and the Tosoh octadecyl NPR columns (Fig. 7). When the other polymeric RP columns were eluted with acetic acid gradients in water, they either lacked the ability to separate the two chains or the separation was just indicated (data not shown).

The recoveries obtained for some of the polypeptides and proteins used in the characterization of the polymeric RP columns, are listed in Table I.

DISCUSSION

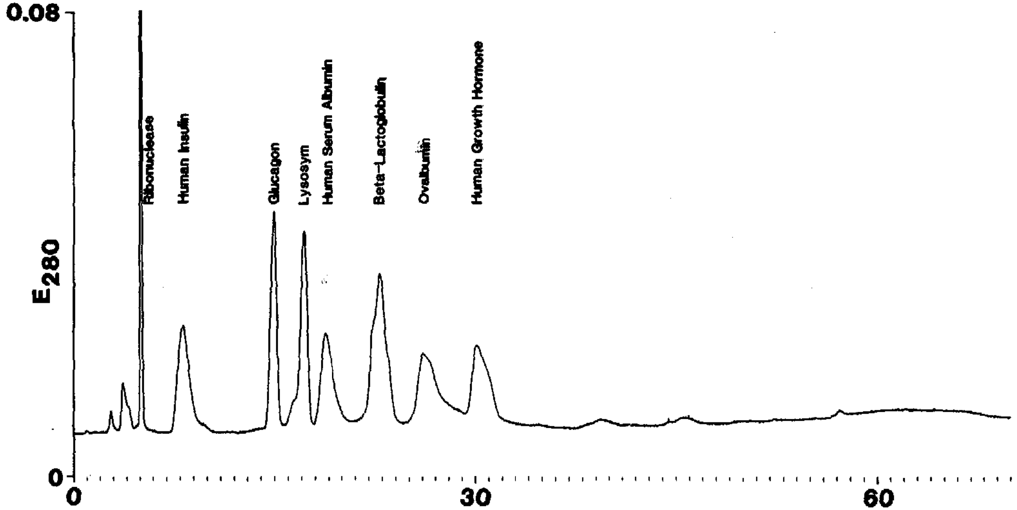
Reversed-phase analyses are still the most popular type of HPLC analyses and the preferred column for such analyses, in both Europe and the U.S.A., is a 5–10-μm silica-based C_{18} column [8].

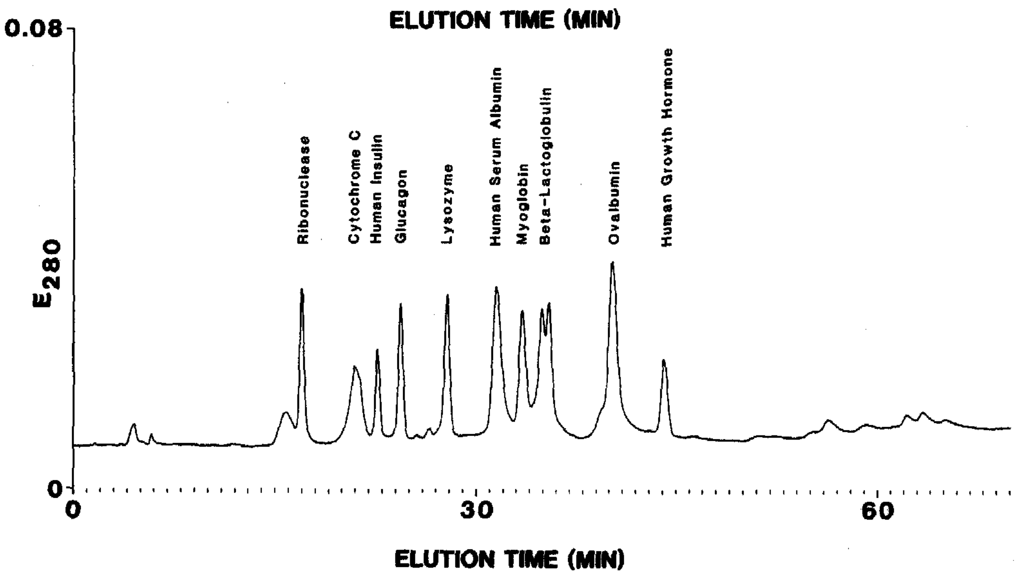
More than 200 RP-HPLC columns are available, and they all differ more or less with respect to plate numbers, peak shapes and selectivities. Further, a lack of column-to-column and batch-to-batch reproducibility is a common problem in many laboratories [8].

Polymer columns are primarily applied in gel permeation chromatographic, ion-exchange and reversed-phase analyses, and their use in RP-HPLC has recently been reported as "increasing" [1]. The main argument in favour of the polymeric RP columns is their increased chemical stability, which allows the use of alkaline mobile phases and also base washing in the case of contaminated columns. So far the potential differences between the various polymeric RP columns has not been analysed with respect to polypeptide separations.

Using our previously described alternative mobile phase of acetic acid gradients in water, we compared the chromatographic behaviour of "bare" polymer columns (Dynospheres, Chrompack) and derivatized columns (Asahipak C_4, C_8 and C_{18}, TSK Phenyl and Tosoh C_{18}) using acetic acid extracts of the normal and the diabetic pancreas as model samples (Figs. 1 and 2).

Although the two "bare" columns are polymerized from different monomers (DVB *versus* styrene) and contain different amounts of copolymerized non-aromatic compounds, their behaviours were surprisingly similar (Fig. 1, top). Almost identical


0.08
E280
0
Ribonuclease
Human Insulin
Glucagon
Lysosym
Human Serum Albumin
Beta-Lactoglobulin
Ovalbumin
Human Growth Hormone
0
30
60
ELUTION TIME (MIN)


0.08
E280
0
Ribonuclease
Cytochrome C
Human Insulin
Glucagon
Lysozyme
Human Serum Albumin
Myoglobin
Beta-Lactoglobulin
Ovalbumin
Human Growth Hormone
0
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60
ELUTION TIME (MIN)

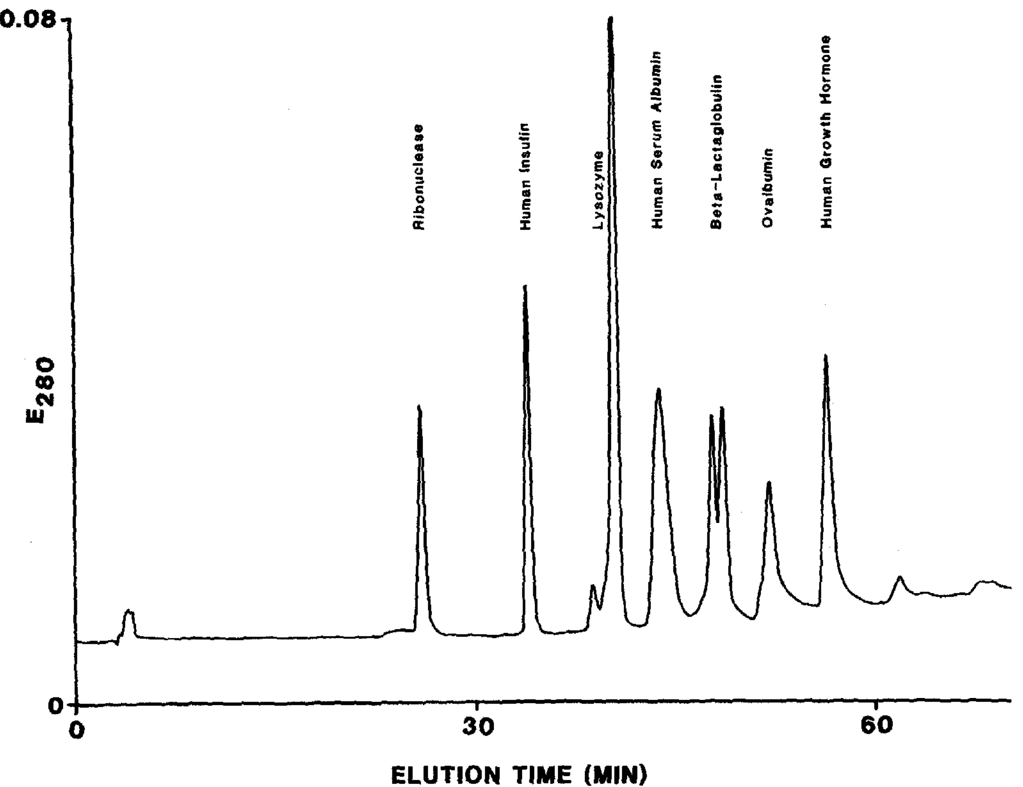


Fig. 6. RP-HPLC of a number of "standard" proteins using a 150 × 4.6 mm I.D. Asahipak ODP column eluted with a 2-propanol–acetic acid gradient (from 24% acetic acid–12% 2-propanol to 40% acetic acid–60% 2-propanol linearly for 60 min followed by 10 min isocratically at the final conditions; top), an acetonitrile–acetic acid gradient (from 24% acetic acid–12% acetonitrile to 40% acetic acid–60% acetonitrile linearly for 60 min followed by 10 min isocratically at the final conditions; middle) or an acetic acid gradient (from 34% to 90% acetic acid linearly for 60 min followed by 10 min isocratically at the final conditions; bottom). Flow-rate, 0.5 ml/min. Sample load, 50–100 μg of each "standard" protein.

TABLE I

RECOVERIES OF INSULIN (Ins), b-COMPONENT (b-comp), PROINSULIN (Proins), HUMAN GROWTH HORMONE (hGH), OVALBUMIN (Ovalb) AND HUMAN SERUM ALBUMIN (HSA) OBTAINED ON DIFFERENT SILICA- AND POLYMER-BASED RP-COLUMNS

The recoveries were calculated as the area under the UV curves (280 nm) obtained after gradient elution of the column relative to that obtained after bypassing the column with a PTFE capillary and injecting a similar sample.

RP column type	Mobile phase		pH	Ligand	Modifier	Recovery (%)					
						Ins	b-comp	Proins	hGH	Ovalb	HSA
Silica-based	$(NH_4)_2SO_4$–NaH_2PO_4–$HClO_4$[a]		2.5	C_{18}	CH_3CN	93[13]			87[13]		2–21[13]
	0.1% TFA[a,b]		2.0	C_{18}	CH_3CN	Inapplicable for recovery studies of Ins and hGH[11,15]					
	0.1% TFA[b]		2.0	C_4	CH_3CN	100[16]		100[16]	80[12]	79	90
Polymer-based	0.25 *M* TEAP	(TSK Phenyl 5PW RP+)	3.0	Phenyl	CH_3CN				68[14]		
	0.25 *M* TEAP	(PLRP-S)	3.0	Phenyl	CH_3CN				37[14]		
	$(NH_4)_2HPO_4$	(TSK Phenyl 5PW RP+)	7.0	Phenyl	CH_3CN	95[14]			68[14]		91[14]
	CH_3COOH	(TSK Phenyl 5PW RP+)	2.2	Phenyl	Water				98	86	97
	CH_3COOH	(Dynospheres)	2.2	Phenyl	Water	85			90		
	CH_3COOH	(Dynospheres)	2.2	Phenyl	CH_3CN	86			76		
	CH_3COOH	(Dynospheres)	2.2	Phenyl	2-Propanol	76			85		
	CH_3COOH	(Asahipak)	2.2	C_8	Water					64	83
	0.075% TFA	(Asahipak)	2.0	C_8	CH_3CN					38	90
	0.025 *M* TEAP	(Asahipak)	3.0	C_8	CH_3CN					12	96
	0.1% TFA	(Asahipak)	2.0	C_4	CH_3CN	100[16]		100[16]	98	63	90

[a] LiChrosorb column.
[b] Nucleosil column.

chromatograms were obtained for both samples, and these chromatograms differed from those of a derivatized phenyl column (TSK Phenyl 5PW RP+; Fig. 1, bottom) with respect to chromatographic efficiency. At first glance, this column seems to be better qualified for the separation of proteins than the two other phenyl columns, and this observation is further supported by the good peak shapes obtained for several pure proteins (including high-molecular-weight samples such as transferrin and catalase) eluted from the TSK Phenyl column with an acetic acid gradient (Fig. 7, top).

These observations could indicate an influence of the stationary phase polymeric backbone in the TSK Phenyl 5PW RP+ column on the separation, in addition to that of the bonded phenyl phase, in accordance with the general opinion that differences between various alkylsilica columns is a function of the attached ligand as well as the silica [9].

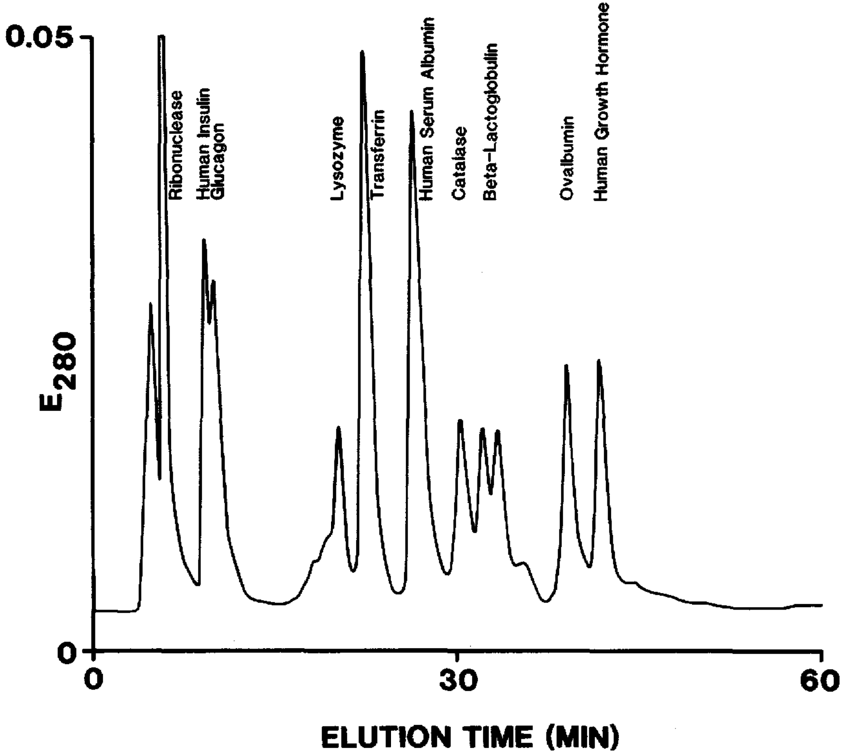
The three Asahipak alkyl columns clearly demonstrated the effect of the hydrophobicity of the attached ligand, in accordance with common experiences with alkylsilica columns. As this effect is equally distributed between sample components with high and low molecular weight, the effect of the stationary phase framework itself (polymerized vinyl alcohol) seems to be negligible.

In this series, the Tosoh 4PW C_{18} column is different from the other derivatized alkyl columns. Compared with these columns, and with the TSK Phenyl column (which has a similar polymeric backbone), the efficiency for peak I material (MW >6000 dalton) is reduced and, in accordance with this, the separation of "standard proteins" in acetic acid gradients (Fig. 5, bottom) is considerably less satisfactory than those of the TSK Phenyl column (Fig. 7, top) and the Asahipak C_{18} column (Fig. 6, bottom).

The Asahipak C_{18} column has an unusual selectivity for acetic acid gradients: the best separation of the two chains in β-lactoglobulin on this column was actually achieved with acetic acid in water, *i.e.*, it was better than that obtained with acetic acid–acetonitrile gradients (Fig. 6, middle), in contrast to the behaviour of the Dynospheres column [5], the Chrompack column (data not shown) and the Tosoh C_{18} column (Fig. 5).

Insulin was eluted with a good peak shape and with satisfactory recoveries from all polymer-based RP columns after acetic acid gradient elution, with the exception of the Tosoh C_{18} NPR column. Interestingly, serum albumin and thyroglobulin were eluted from this column with good peak shapes, and the separation of the two β-lactoglobulin chains was even better than that with the other two TSK columns (Fig. 7, bottom). The irreversible binding of insulin under these circumstances remains to be explained.

When the Asahipak C_8 column was eluted with a "classical" mobile phase for RP polypeptide analyses (TFA–acetonitrile), the major change observed is an increased plate number, especially in the first part of the chromatogram where peak II material (MW <6000 dalton) is eluted (Fig. 4, right). In the case of the Dynospheres column, the separation of peak I material is improved (contradictionary to the Asahipak column; compare with Fig. 2, top right). However, a dramatic increase in the chromatographic efficiency of the Asahipak C_8 column towards peak I material was noted when the separation temperature was increased from ambient to 45°C (Fig. 4, bottom left). A parallel increase in chromatographic efficiency for peak II material (MW <6000 dalton) was not observed, in accordance with earlier observations that



0.08
0
E280
Ribonuclease
Cytochrome C
Lysozyme
Human Serum Albumin
Beta-Lactoglobulin
Thyroglobulin
Human Growth Hormone
0
10
20
ELUTION TIME (MIN)

the use of elevated temperatures seemed to be of no advantage for peptide RP-HPLC [10]. Only very limited effects were observed when the elution with acetic acid gradients was performed at elevated temperature (data not shown).

The use of a high content of acetic acid in mobile phases for polymeric RP columns is an interesting alternative to "classical" polypeptide analyses with a C_{18} column and TFA–acetonitrile as mobile phase. From this work it is clear that different groups of polymer columns under these conditions behave as differently as alkylsilica columns eluted under comparable conditions, and that the potential value of a polymer-based column for acetic acid elution may be quickly judged by using "standard" proteins. Some of the columns seem to be virtually free from any influence from the stationary phase backbone, in contrast to the disturbing effects of free silanol groups in several silica-based RP columns. It is also noteworthy that the polypeptide selectivity of these columns can be changed with variations in the mobile phase, and that the columns with optimum performance with acetic acid maintain their superiority when they are eluted with mobile phases containing organic modifiers.

In the evaluation of the potential usability of polymeric RP columns for peptide and protein separations the recovery is an important factor. The pancreatic extracts are highly complex, containing peptides from a few amino acids to proteins with MW >300 000 dalton with equally large variations in hydrophobicity. Recoveries with these extracts would be extremely difficult to measure, and the information obtainable from such analyses would be of limited scientific value with respect to the general performance of the columns. Therefore, the recoveries from various polymer-based RP columns for a number of individual polypeptides and proteins with very different molecular weights and hydrophobicities were measured using various stationary–mobile phase combinations, and the results were compared with similar values obtained for various silica-based RP columns (Table I).

For the silica C_{18} columns eluted with acetonitrile-containing mobile phases, the recovery was directly related to the molecular weight of the sample: with the exception of TFA [11,12], use of all all other mobile phases resulted in *ca.* 100% recoveries for insulin (6000 dalton). However, already for the b-component {composed primarily of proinsulin (9000 dalton) and insulin dimer (12 000 dalton) [6]} the recoveries with two of these mobile phases were reduced and for the hydrophobic growth hormone (22 000 dalton) only the use of a single mobile phase (with perchloric acid) resulted in a satisfactory recovery. The use of TEAP-acetonitrile resulted in a minimal recovery and with TFA–acetonitrile the growth hormone was irreversibly bound [12].

The effect of the silica-bound ligand on the recovery was clearly seen when a C_4 column was compared with the C_{18} columns: insulin, proinsulin, growth hormone, ovalbumin and serum albumin were now recovered in excellent yields under mobile phase conditions where these samples were irreversibly bound to C_{18} columns.

Fig. 7. RP-HPLC of a number of "standard" proteins using two 75 × 4.6 mm TSK Phenyl 5PW RP+ columns in series eluted with an acetic acid gradient (from 34% to 90% acetic acid linearly for 60 min, top), or a 35 × 4.6 mm I.D. Tosoh octadecyl NPR column eluted with an acetic acid gradient (from 34% to 90% acetic acid linearly for 10 min followed by 10 min isocratically at 90% acetic acid; bottom). Flow-rate, 0.5 ml/min. Sample load, 50–100 μg (top) or 2–10 μg (bottom) of each "standard" protein.

In contrast to the results from the silica C_{18} columns, excellent recoveries for insulin, growth hormone and serum albumin were obtained on a single polymer-based stationary phase (TSK Phenyl) eluted with ammonium phosphate–acetonitrile. However, the recoveries of growth hormone in TEAP–acetonitrile from two different polymeric phenyl columns clearly demonstrated the impact of the individual stationary phase on the recovery (compare TSK Phenyl with PLRP-S).

For the Asahipak C_8 column, the results of elution with acetic acid in water and with TFA and TEAP in acetonitrile clearly depended on the actual sample: for serum albumin the use of acetonitrile resulted in higher recovery than after elution with acetic acid, whereas the use of acetonitrile resulted in extremely low recoveries for (the notoriously difficult) ovalbumin, which was recovered in the highest yield after elution with acetic acid. With this mobile phase, the effect of the individual polymeric stationary phase again was elucidated when the recoveries of serum albumin and ovalbumin on the Asahipak C_8 column were compared with the higher values obtained on the TSK Phenyl column. Further, the very high recovery of growth hormone demonstrated the general excellence of the TSK Phenyl column for elution with acetic acid in water.

For the Dynospheres column, the effects of the individual samples on the recoveries obtained with different mobile phases were found to parallel those on the Asahipak C_8 column, although smaller.

Comparison of a silica-based C_4 and a polymeric C_4 column eluted with TFA–acetonitrile demonstrated the superiority of the polymer-based C_4 column for growth hormone, whereas ovalbumin was recovered in higher yields on the silica-based column. However, in contrast to the results obtained after elution of ovalbumin from polymer-based columns with acetic acid gradients in water (where it was eluted as a single component), elution of ovalbumin with TFA–acetonitrile resulted in two peaks from both the silica-based and the polymer-based C_4 columns and from the Asahipak C_8 column (probably owing to the formation of a native and a denatured form of ovalbumin). As the ratio between the two forms was found to differ for the two C_4 columns, and as one of these main components was eluted with severe tailing, the two recovery figures may not be directly comparable.

In general, the recoveries from the polymeric RP columns were not only excellent for insulin and proinsulin, but were equally high for growth hormone, ovalbumin and serum albumin. Similar recoveries from silica C_{18} columns could either only be obtained with a single mobile phase (growth hormone) or not at all (serum albumin). In addition, the finding that the recoveries of all samples after elution with acetic acid gradients in water were either at the same level as or higher than those obtained after elution with acetonitrile gradients remains an outstanding characteristic of the polymer-based RP columns.

For environmental, economical and toxicity reasons, replacement of acetonitrile with a less offensive solvent would be an advantage. The efficiency of polymeric columns eluted with acetic acid is at present not comparable to that obtained with acetonitrile, but future improvements in the design of the stationary phase, backbone and ligands may well change this situation.

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