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Non-ideal behaviour of silica-based stationary phases in trifluoroacetic acid–acetonitrile-based reversed-phase high-performance liquid chromatographic separations of insulins and proinsulins

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ABSTRACT

Several C_{18} stationary phases were found to behave non-ideally when insulins and proinsulins were eluted with shallow acetonitrile gradients in 0.1% trifluoroacetic acid, resulting in poor peak shapes or no elution at all. With triethylammonium phosphate or ammonium sulphate as buffer components, the insulins and proinsulins were eluted with excellent peak shapes, presumably owing to better masking of residual silanol groups on the stationary phases. Similar use of trifluoroacetic acid–acetonitrile gradients on the less hydrophobic C_4 or C_3 stationary phases resulted in excellent peak shapes. The difficult separation of rat proinsulin I and II, which are important for the study of rat insulin biosynthesis, was only achieved with two different stationary–mobile phase combinations.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become the most widely used separation mode for polypeptides owing to its high resolution capacity. For insulin and insulin-related compounds, the most popular mobile phases have been trifluoroacetic acid (TFA), alkylammonium phosphates or neutral salts in combination with acetonitrile (ACN) (see ref. 1 for a review).

Rats and mice are unusual among mammals in producing two different non-allelic proinsulins (I and II) in the islets of Langerhans, which subsequently are processed to insulin I and II and also C-peptide I and II. We recently described the successful RP-HPLC separation of rat proinsulin I and II (both containing 86 amino acids with four amino acid differences), a more difficult separation than that of the two insulins (51 amino acids, two differences) and the two C-peptides (31 amino acids, two differences). The separation was achieved using a LiChrosorb RP-18 column, eluted with a very shallow TFA–acetonitrile (ACN) gradient (0.1%/min) [2].

During this work we observed certain disturbing column-to-column variations, which could sometimes be overcome by "preconditioning" the columns with alkylammonium phosphate. However, from columns obtained during the last 12–

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18 months, rat insulins and proinsulins and also human insulin and proinsulin could not be eluted at all with TFA–ACN.

The aim of this study was to evaluate the usability of shallow TFA–ACN gradients on several silica-based C_{18}, C_8, C_4 and C_3 reversed-phase columns for the separation of rat and human insulins and proinsulins.

EXPERIMENTAL

Reagents

Trifluoroacetic acid (peptide synthesis grade) was obtained from Applied Biosystems, phosphoric acid (analytical-reagent grade) from Merck, ammonium sulphate (Aristar) from BDH, triethylamine (99%) from Janssen Chimica and acetonitrile (HPLC grade S) from Rathburn Chemicals. All other chemicals were of analytical-reagent grade. Distilled water was obtained from a Millipore Milli-Q plant, and all buffers were filtered (0.45 μm, Millipore) and vacuum/ultrasound degassed before use.

HPLC equipment

The HPLC system consisted of two Waters Assoc. Model M 6000A pumps, a WISP 710A autosampler, a Model 660 solvent programmer, a Model 730 data module and a Pye Unicam LC–UV detector.

Columns

LiChrosorb RP-18 (5 μm), LiChrospher 100 RP-18 (5 μm) and LiChrosorb RP-8 (5 μm) columns (250 $\times$ 4.0 mm I.D.) were obtained from Merck, Nucleosil 100-5C_{18}, 120-5C_{18}, 300-5C_{18} and 300-5C_4, all 5 μm, columns (250 $\times$ 4.0 mm I.D.) from Machery-Nagel & Co., an Ultrasphere-ODS (5 μm) column (250 $\times$ 4.6 mm I.D.) from Beckman, a Zorbax Bio Series Protein Plus (6 μm) column (250 $\times$ 4.6 mm I.D.) from DuPont and a Bakerbond Wide-Pore Butyl (5 μm) column (250 $\times$ 4.6 mm I.D.) from J. T. Baker.

Mobile phases and gradients

The columns were eluted with linear acetonitrile gradients (5 or 6% during 30 or 60 min) in 0.1% TFA, 0.125 *M* triethylammonium phosphate (TEAP) (pH 4.0) or 0.125 *M* ammonium sulphate (AS) (pH 3.0 or 4.0). The flow-rate was 1.0 ml/min and experiments were performed at room temperature (*ca.* 22°C) or 45°C. Detailed descriptions are given in the legends to the figures.

Samples

Human insulin (HI) and proinsulin (HPI) were obtained from Novo Nordisk A/S. Medium from cultured newborn rat islet cells was used as a source of rat insulins. This medium contained 44 μg/ml insulin I and II (determined by radioimmunoassay [3]) and equimolar amounts of C-peptide I and II. Biosynthetically labelled rat islet polypeptides, including rat proinsulin I and II (10–20 ng per 50 islets) were prepared by incubation of rat islets with [^{3}H]leucine and [^{35}S]methionine as described previously [2].

Registration and counting

The column eluate was monitored at 210 nm. In experiments with labelled polypeptides, the eluate was collected in 0.5-min fractions (Pharmacia FRAC 300 fraction collector) and counted in a Packard Tri-Carb liquid scintillation counter (Model 460 C) after addition of 4 ml of Optiphase HiSafe (LKB).

RESULTS

The separation of rat insulin I and II, C-peptide I and II and proinsulin I and II (all present in rat islets) on a LiChrosorb RP-18 column is shown in Fig. 1. The UV profile in the upper panel represents the stored polypeptides and the lower panel shows the distribution of radioactivity in the newly synthesized polypeptides. It can be seen that the amounts of stored proinsulins compared with insulins are very small (1–2%), and only small amounts of newly synthesized proinsulins during this 60-min labelling period are converted to insulins and C-peptides. The fact that methionine is present only in proinsulin II and insulin II facilitates the peak identification.

Some batches of LiChrosorb RP-18 columns did not perform satisfactorily (non-ideal, tailing insulin peaks) when new columns were eluted initially with the TFA–ACN gradient (Fig. 2, top panel). We observed that repeated (4–6) chromato-

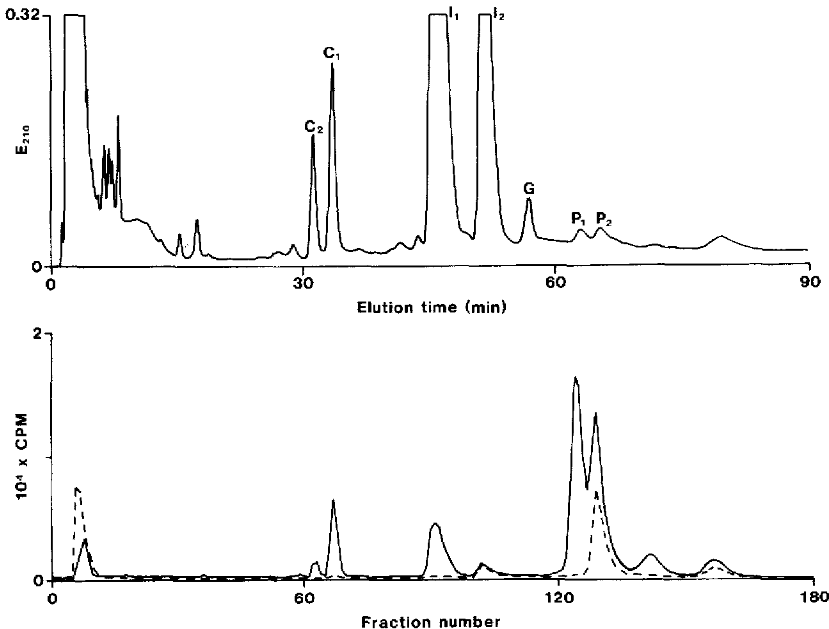

Fig. 1. RP-HPLC separation of a 3-*M* acetic acid extract of 9500 rat islets, labelled for 60 min with 0.5 mCi of [^{3}H]leucine and 0.5 mCi of [^{35}S]methionine (this amino acid being present only in insulin II and proinsulin II), using a LiChrosorb RP-18 column eluted at 1.0 ml/min with a linear ACN gradient (30–36%) in 0.1% TFA during 60 min. Peaks: C_1 = C-peptide I; C_2 = C-peptide II; I_1 = insulin I; I_2 = insulin II; G = glucagon; P_1 = proinsulin I; P_2 = proinsulin II. The absorbance at 210 nm (upper panel) represents the stored islet polypeptides and the radioactivity in the collected fractions (solid line, [^{3}H]leucine; dashed line, [^{35}S]methionine) (lower panel) represents the newly synthesized islet polypeptides.

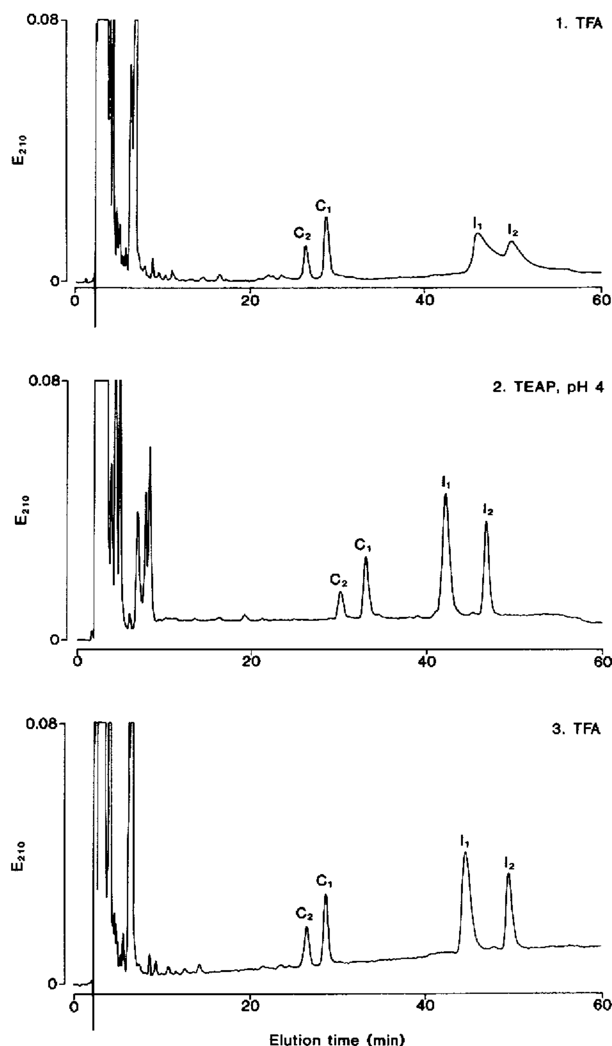

Fig. 2. RP-HPLC separation of 50 μl of rat islet cell culture medium containing 2.2 μg of insulin I + II plus equimolar amounts of C-peptides I and II. Peaks as in Fig. 1. The same LiChrosorb RP-18 column was eluted at 1.0 ml/min with a linear ACN gradient during 60 min in 0.1% TFA (30–36%, top panel), in 0.125 *M* TEAP (pH 4.0) (25–30%, middle panel) and again in 0.1% TFA (bottom panel).

grams of acidified culture medium containing rat insulins and C-peptides could precondition the columns to a better performance, *i.e.*, similar to the separation shown in Fig. 2, bottom panel. We further noticed that the LiChrosorb columns performed well (ideal peak shapes) when eluted with a TEAP–ACN gradient (Fig. 2, middle panel) and, thereafter, that elution with the TFA–ACN gradient resulted in excellent peak shapes (Fig. 2, bottom panel).

However, recent batches of LiChrosorb columns could not be "preconditioned" to elute the rat insulins, whereas the peak shapes of the C-peptides were excellent

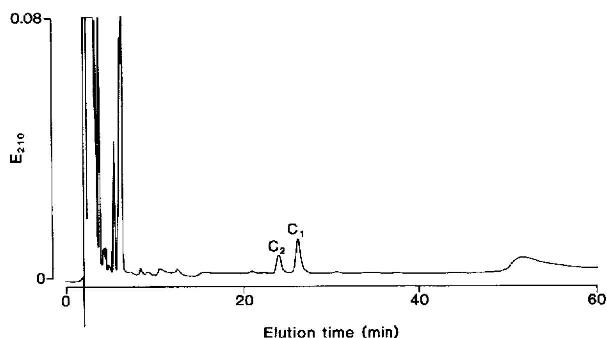

Fig. 3. RP-HPLC separation of 50 µl of rat islet cell culture medium in 0.1% TFA–ACN as in Fig. 2, but on a more recent batch of LiChrosorb RP-18. C_1 and C_2 are the C-peptides; the insulins were not satisfactorily eluted (irreversible binding).

(Fig. 3). Neither human insulin nor proinsulin could be eluted at all from these columns (data not shown), indicating irreversible binding to the stationary phase.

In order to obtain a satisfactory elution of insulins and proinsulins in the preferred TFA–ACN mobile phase, several different stationary phases were examined. The characteristics of the individual stationary phases are summarized in Table I and the results are given in Table II. Although some of these columns initially separated the insulins and proinsulins with excellent peak shapes, insulins and proinsulins gradually become irreversibly bound to the C_{18} stationary phases. No irreversible binding of the C-peptides was ever observed.

An example of the increasing irreversible binding of the insulins and proinsulins is shown in Fig. 4. Human insulin (HI) and proinsulin (HPI) were initially eluted as

TABLE I

CHARACTERISTICS OF THE STATIONARY PHASES

Bonded phase	Stationary phase	Particle shape	Particle size (µm)	Pore size (Å)	Carbon load (%)	End-capped
C_{18}	LiChrosorb RP-18	Irregular	5	100	16.0	No
	LiChrospher 100 RP-18	Spherical	5	100	12.5	No
	Ultrasphere-ODS	Spherical	5	80	10–11	Yes
	Nucleosil 100-5C_{18}	Spherical	5	100	14	HMDS[a]
	Nucleosil 120-5C_{18}	Spherical	5	120	11	HMDS
	Nucleosil 300-5C_{18}	Spherical	5	300	6	HMDS
C_8	LiChrosorb RP-8	Irregular	5	100	9.5	No
C_4	Nucleosil 300-5C_4	Spherical	5	300	1	HMDS
	Bakerbond WP Butyl	Spherical	5	300	n.a.[b]	n.a.
C_3	Zorbax Protein Plus	Spherical	6	300	n.a.	n.a.

[a] HMDS = hexamethyldisilazane.
[b] n.a. = Not available.

TABLE II

BINDING PHENOMENA OF POLYPEPTIDES FROM PANCREATIC ISLETS TO REVERSED-PHASE STATIONARY PHASES

Mobile phase, 0.1% TFA in acetonitrile; gradient, 0.1%/min; room temperature (22°C).

Bonded phase	Stationary phase	C-peptides	Insulins and proinsulins	Comments	Fig. No.
C_{18}	LiChrosorb RP-18	No irreversible binding	Sometimes irreversible binding	In some instances preconditioning was possible	1, 2, 3
	LiChrospher 100 RP-18	No irreversible binding	Irreversible binding		
	Ultrasphere-ODS	No irreversible binding	Increasing to irreversible binding	Initially excellent peak shapes	
	Nucleosil 100-5C_{18}	No irreversible binding	Increasing to irreversible binding	Initially excellent peak shapes	
	Nucleosil 120-5C_{18}	No irreversible binding	Increasing to irreversible binding	Initially tailing peaks	4
	Nucleosil 300-5C_{18}	No irreversible binding	Increasing to irreversible binding	Initially tailing peaks	
C_8	LiChrosorb RP-8	No irreversible binding	Some non-specific binding	Non-ideal peak shapes	
C_4	Nucleosil 300-5C_4	No irreversible binding	No irreversible binding	Excellent peak shapes[a]	5
	Bakerbond WP Butyl	No irreversible binding	No irreversible binding	Excellent peak shapes[a]	
C_3	Zorbax Protein Plus	No irreversible binding	No irreversible binding	Excellent peak shapes[a]	5

[a] None of these stationary phases was able to separate rat proinsulin I and II.

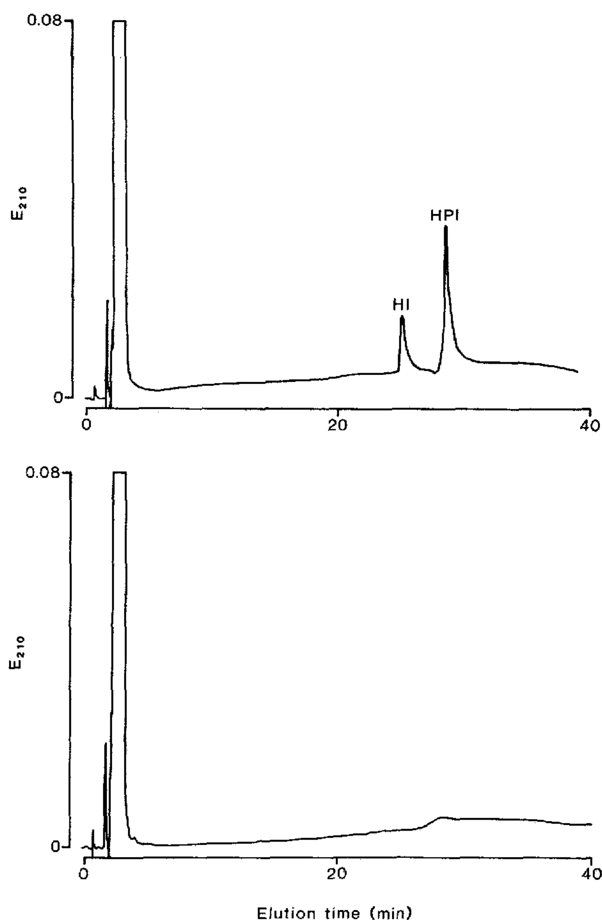

Fig. 4. RP-HPLC separation of *ca.* 1 μg of human insulin (HI) and *ca.* 3 μg of human proinsulin (HPI) on a Nucleosil 120-5C_{18} column eluted at 1.0 ml/min with a linear ACN gradient (30–36%) in 0.1% TFA during 30 min. Upper panel, first injection; lower panel, fourth injection.

tailing peaks (upper panel), but in later experiments these polypeptides were bound to the stationary phase, Nucleosil 120-5C_{18} (lower panel).

A C_8 stationary phase (LiChrosorb RP-8) also showed non-ideal insulin and proinsulin peak shapes and partial binding (data not shown), whereas insulins and proinsulins were eluted with excellent peak shapes from C_4 and C_3 stationary phases (Table II), exemplified for HI and HPI on Zorbax Protein Plus and Nucleosil 300-5C_4 (Fig. 5). However, none of these columns was able to separate rat proinsulin I and II.

It should be mentioned that excellent insulin and proinsulin peak shapes were obtained from all stationary phases after elution with TEAP (pH 4.0)–ACN (data not shown). Unfortunately, the rat proinsulins were not separated, except partly after chromatography on the Ultrasphere-ODS columns [2].

Fig. 6 shows the separations with TFA–ACN on Nucleosil 300-5C_4 eluted at

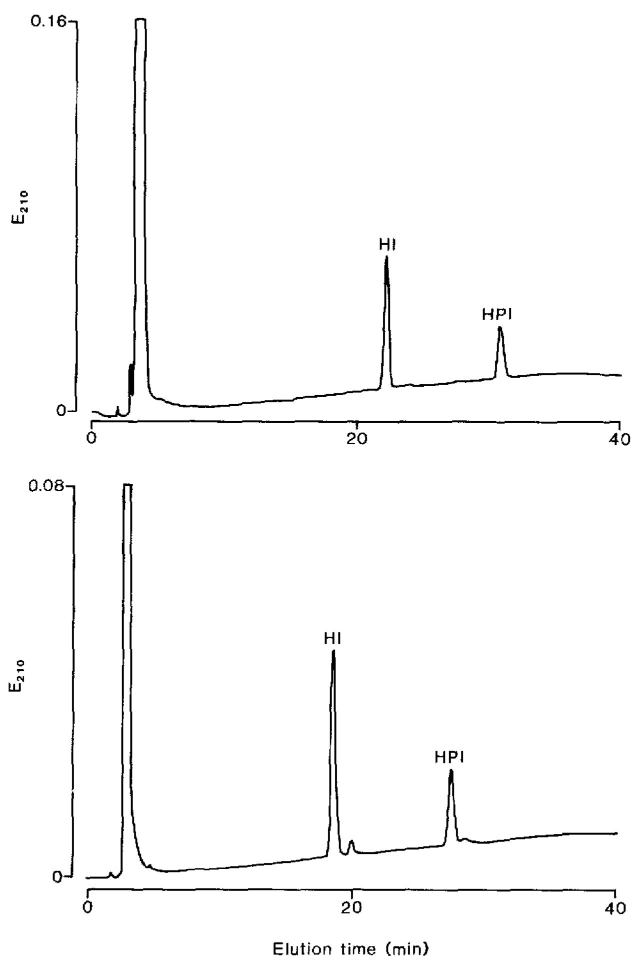

Fig. 5. RP-HPLC separation of *ca.* 1 μg of HI and *ca.* 1 μg of HPI on a Zorbax Protein Plus column (upper panel) and a Nucleosil 300-5C_4 column (lower panel), both eluted at 1.0 ml/min with a linear ACN gradient (26–32%) in 0.1% TFA during 30 min.

room temperature (upper panel) and at 45°C (lower panel); the higher separation temperature resulted in baseline separation of the two rat proinsulins.

The optimization of the separation of the labelled rat islet polypeptides on LiChrosorb RP-18 eluted with AS–ACN is shown in Fig. 7. Initial separation of the rat proinsulins was achieved by changing the pH from 4.0 to 3.0 (top and middle panels), whereas increasing the temperature to 45°C resulted in an almost baseline separation of the proinsulins (bottom panel). This beneficial effect of temperature was not observed when LiChrosorb with TFA–ACN was used (data not shown).

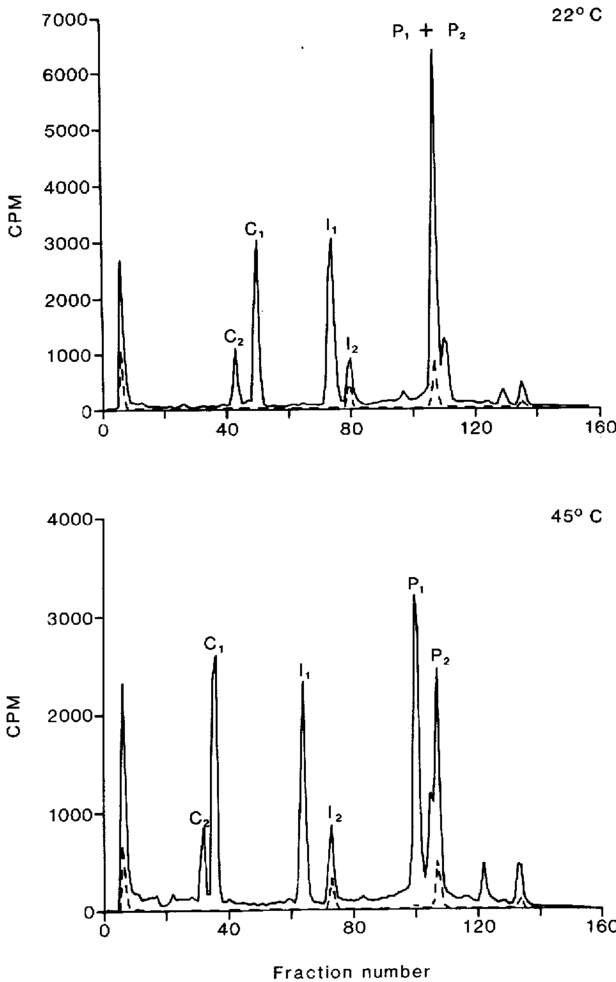

Fig. 6. RP-HPLC separation of a 3-*M* acetic acid extract of 50 rat islets (labelled as described in Fig. 1) using a Nucleosil 300-5C_4 column eluted at 1.0 ml/min with a linear ACN gradient (26–32%) in 0.1% TFA during 60 min. The separation shown in the upper panel was performed at room temperature (*ca.* 22°C) and that in the lower panel at 45°C. The solid lines represent ^{3}H radioactivity and the dashed lines ^{35}S radioactivity. Peaks as in Fig. 1.

DISCUSSION

For the study of the rat insulin biosynthesis, a method capable of separating and determining all the conversion products (*i.e.*, insulins and C-peptides), in addition to proinsulin I and II, is required. RP-HPLC methods giving partial separations of some of these polypeptides (coelution of proinsulins, no detection of C-peptides) have been published [4,5], whereas the successful separation of all the polypeptides involved (including the two rat proinsulins) could be achieved with the LiChrosorb RP-18 system with TFA–ACN [2]. A similar separation was obtained using an Ultrasphere

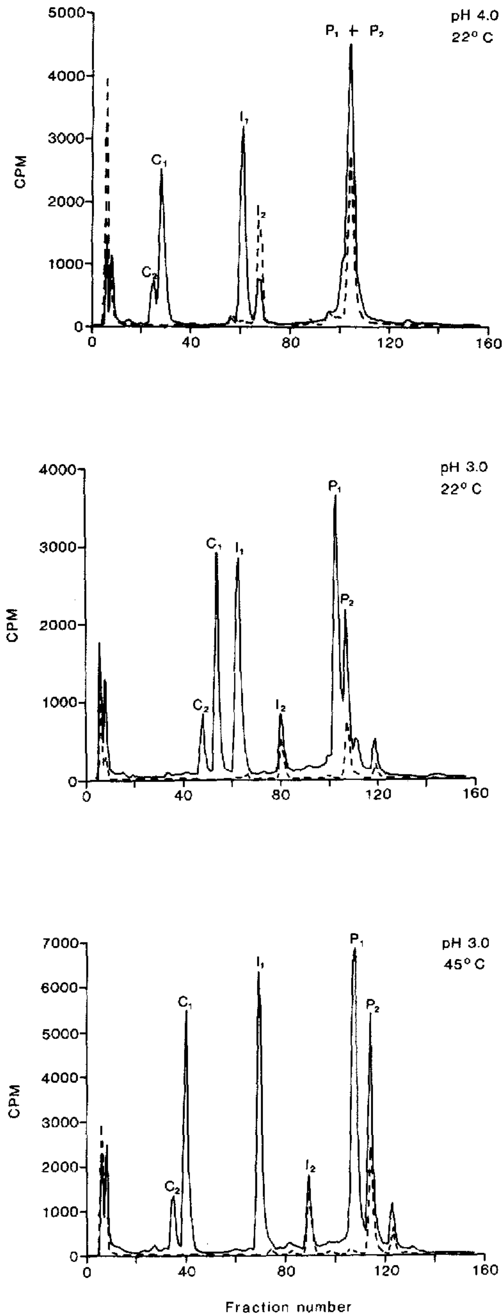

Fig. 7. RP-HPLC separation of a 3-*M* acetic acid extract of 50 rat islets (labelled as described in Fig. 1) using a LiChrosorb RP-18 column eluted at 1.0 ml/min with a linear ACN gradient in 0.125 *M* ammonium sulphate during 60 min. Top panel, 22°C, pH 4.0, 24–29% ACN; middle panel, 22°C, pH 3.0, 25–30% ACN; bottom panel, 45°C, pH 3.0, 26–31% ACN. The solid lines represent ^{3}H radioactivity and the dashed lines ^{35}S radioactivity. Peaks as in Fig. 1.

system with TEAP (pH 4.0)–ACN [2], but a volatile mobile phase would be preferable for these analyses.

As our latest batches of LiChrosorb RP-18 either separated the insulins and proinsulins with unacceptable peak shapes, or the binding to the stationary phase was so strong that elution with TFA–ACN was impossible, we examined the behaviour of other RP columns in this mobile phase. The instantaneously or gradually increasing irreversible binding of insulins and proinsulins in shallow TFA–ACN gradients was found to be general for the all C_{18} stationary phases examined and also for a LiChrosorb C_8 column (Table II) independent of pore size (80–300 Å), carbon load (6–14%) or end-capping. In contrast, a 330-Å C_{18} Vydac column has been reported to separate several different insulin species with shallow TFA–ACN gradients without such problems [6]. In some instances, petreatment of new LiChrosorb RP-18 columns with TEAP–ACN allowed their use in TFA–ACN gradients, but even after this preconditioning recent batches of this column type could not be used with shallow gradients (Fig. 3).

After elution of RP columns with shorter alkyl chains (C_3 or C_4) with TFA–ACN, excellent peak shapes for insulins and proinsulins were obtained (Fig. 5), but the selectivity in these systems was too low to separate the two rat proinsulins (three different C_3/C_4 columns, Table II). Interestingly, the selectivity could be improved if the separation temperature was increased from 22 to 45°C (Fig. 6, lower panel), resulting in the separation of both proinsulins and insulins and C-peptides, although the effect of temperature on polypeptide retention times has been reported to be minimal [7].

A similar improvement in the efficiency following an increase in separation temperature was observed with a LiChrosorb RP-18 column eluted with AS–ACN (Fig. 7), whereas the results obtained on the same column eluted with TFA–ACN were comparable (*i.e.*, irreversible binding of insulins and proinsulins) at 22 and 45°C (data not shown).

Although RP-HPLC of peptides and proteins has been performed for more than a decade, the underlying mechanisms are far from well understood. Most experience was gained after the introduction of TFA [8], phosphoric acid [9] and alkylammonium salts [10] as mobile phase additives. The primary function of these substances is the (hopefully total) elimination of non-specific binding of peptides and proteins to the stationary phase in general and to free silanol groups in particular by ion pairing to charged groups in the sample molecules and to the free silanol groups [7,11].

The deleterious influence of these groups on the chromatographic process may vary from reduced recovery and non-ideal peak shapes to total loss of the injected peptide or protein sample [11–18]. This sample loss, which is directly related to an increasing molecular weight of the sample [19], is most pronounced for nanogram amounts [20,21], but losses also occur on the microgram scale [19]. It is most common after the use of C_{18} columns [7], which are more hydrophobic, but also better resolving, than C_4 or C_8 columns [11].

Free silanol groups are present in various amounts in all silica-based RP-HPLC columns (including the so-called "end-capped" columns), and additional groups are constantly created during chromatography owing to chemical instability of silica-based stationary phases in the commonly used highly acidic mobile phases [7,11,12,22]. Shielding of these groups is therefore of the greatest importance. Alkylammonium

salts (and especially TEAP) are very effective agents, whereas TFA is less suitable for this protection [7,11,18,23].

Blocking these very active sites on the stationary phase with smaller or larger amounts of the sample before the actual analysis has been reported [17,24], but the possibly beneficial effect seems to depend on the actual protein sample.

Our findings in this study that TEAP in some instances could "mask" the irreversible binding sites on the LiChrosorb columns (thereby allowing their successive use in TFA–ACN), and that the use of TEAP invariably resulted in excellent peak shapes, support some of the above-mentioned literature reports.

Further, a rapidly occurring stripping of ligands from C_{18} columns (perhaps a result of a poor binding procedure) has been reported to occur very rapidly with new columns [25]. The introduction of several sites with strong non-specific polypeptide binding combined with the poor ion-pairing capacity of dilute TFA and the very hydrophobic C_{18} ligand might explain most of our observations, but also the type of silica gel used may be important [15,26]. Since the irreversible binding to the C_{18} columns was only observed for the insulins and proinsulins (MW 6 and 9 kilodaltons, respectively) and not for the less hydrophobic C-peptides (MW 3 kilodaltons) it can be concluded that most C_{18} columns eluted with shallow TFA–ACN gradients are not suitable for the separation of polypeptides with molecular weights and hydrophobicities equal to or higher than those of insulins and proinsulins.

Finally, it should be emphasized that column reproducibility, recently reported to be a problem in many laboratories in the U.S.A. and Europe [27], is a phenomenon which must be taken very serious.

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