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Silica *versus* polymer-based stationary phases for reversed-phase high-performance liquid chromatographic analyses of rat insulin biosynthesis

A comparison of resolution and recovery

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ABSTRACT

Because of the problems caused by the irreversible binding of insulins and proinsulins to several silica-based reversed-phase columns, the use of polymeric reversed-phase columns was investigated for the analysis of rat islet polypeptides involved in insulin biosynthesis. No irreversible binding of insulins and proinsulins was observed for the polymeric reversed-phase columns, probably due to the absence of silanol groups. The six polypeptides involved in insulin biosynthesis in rat islets were equally well resolved in shallow trifluoroacetic acid–acetonitrile gradients on the silica-based Nucleosil 300-5C_4 column (45°C), the polymer-based Asahipak C4P-50 (25 and 45°C), and ODP-50 columns (45°C). In shallow triethylammonium phosphate–acetonitrile gradients (25°C) satisfactory resolution of the two rat proinsulins was only obtained on the polymer-based Asahipak C4P-50 and C8P-50 columns. Increasing the separation temperature to 45°C improved the separation of the two insulins and the two proinsulins in all cases. The shifts in retention times for the individual islet polypeptides observed in relation to the increased separation temperature were found to be different for the silica C_4 and the polymer C_4 columns. Recoveries of rat islet polypeptides were comparably high from the silica- and the polymer-based C_4 columns and linear load–response curves were obtained in the microgram to picogram mass range on both columns.

INTRODUCTION

Proinsulin is synthesized in the β-cells, one of the four cell types in the islets of Langerhans in the endocrine pancreas. The enzymatic cleavage of proinsulin at two positions with paired basic amino acid residues results in the formation of equimolar amounts of insulin and C-peptide. Most mammals produce a single insulin, but in the rat (and other rodents) two different non-allelic insulin genes are expressed, resulting in the formation of two sets of closely related proinsulins, insulins and C-peptides, differing in 4 out of 86 amino acids, 2 out of 51 amino acids, and 2 out of 31 amino acids, respectively.

We have recently described the successful separation of all these β-cell specific polypeptides by reversed-phase high-performance liquid chromatography (RP-HPLC) utilising carefully selected silica-based stationary phases eluted with very

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shallow trifluoroacetic acid (TFA)–acetonitrile gradients [1,2]. However, several of the stationary phases examined during this evaluation suffered from severely non-ideal behaviour (pronounced non-specific binding and lack of column-to-column reproducibility) [2]. Consequently, we have evaluated the use of polymeric RP columns for this type of analysis. Three different Asahipak columns with C_{18}, C_8 and C_4 anchored to the same polymer skeleton (polyvinylalcohol) were eluted with TFA or triethylammonium phosphate in acetonitrile, and the separation and recoveries of the rat C-peptides, insulins and proinsulins at ambient or elevated temperatures were optimized and compared to similar separations using selected silica-based stationary phases.

MATERIALS AND METHODS

HPLC equipment

The HPLC system consisted of two M6000A pumps, a WISP 710A, a 660 solvent programmer, a 730 data module (all from Waters), and a Pye Unicam LC-UV detector.

Silica-based columns

LiChrosorb RP-18 (5 μm) and LiChrosorb RP-8 (5 μm), both 250 × 4.0 mm I.D. were obtained from Merck, Nucleosil 120-5C_{18} (5 μm) and 300-5C_4 (5 μm), both 250 × 4.0 mm I.D. were obtained from Macherey-Nagel, Zorbax Protein Plus (6 μm), 250 × 4.6 mm I.D. was obtained from DuPont, and Bakerbond WP Butyl (5 μm), 250 × 4.6 mm I.D. was obtained from J. T. Baker.

Polymer-based columns

Asahipak ODP-50 (5 μm) 150 × 4.6 mm I.D. and Asahipak C8P-50 (5 μm) and C4P-50 (5 μm), both 250 × 4.6 mm I.D., were obtained from Asahi Chemical Industry Co.

Reagents

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

Samples

Human insulin (HI), human proinsulin (HPI) and mono-[^{125}I]-(TyrA14)-porcine insulin (200–300 mCi/mg) were obtained from Novo-Nordisk. Rat pancreatic polypeptide and porcine glucagon were from Sigma. Medium from cultured newborn rat islet cells was used as a source of rat insulins. This medium contained 63 μg/ml insulin I + II and equimolar amounts of C-peptide I and II. Biosynthetically labelled rat islet polypeptides, including rat proinsulin I and II (nanogram amounts), were prepared by the incubation of rat islets with [^{3}H]leucine and [^{35}S]methionine as described [1].

HPLC conditions

The columns were eluted with linear acetonitrile gradients in 0.1% TFA (6% during 60 min) or in 0.125 *M* triethylammonium phosphate (TEAP), pH 4.0 (5% during 60 min). Flow-rate: 1.0 ml/min for the silica-based columns and 0.5–1.0 ml/min for the polymer-based columns (pressure limit, 150 bar). Separation temperature: ambient or 45°C. The column eluate was monitored at 210 nm. In experiments with labelled polypeptides the eluate was collected in 0.5-min fractions and counted in a Packard Tri-Carb liquid scintillation counter (Model 460 C) after the addition of 4 ml Optiphase "HiSafe" (LKB).

Resolution

The resolution (R_s) was calculated as $2 \cdot (t_2 - t_1)/w_1 + w_2)$, where t_2 and t_1 are the retention times of two adjacent peaks, and w_1 and w_2 their base widths. A baseline separation results in $R_s = 1$, a 12.5%-overlap in $R_s = 0.5$ assuming Gaussian peaks.

Recovery

The recoveries of microgram amounts of polypeptides were calculated from UV areas (rat insulin I and II), nanogram amounts from [^{3}H]leucine counts (rat proinsulin I and II), and picogram amounts from [^{125}I]counts (A14-monoiodoinsulin) after the HPLC separations in comparison with identical samples either injected into a loop (10 m × 0.2 mm I.D.) or counted directly.

Dose–response curves were determined for all three mass ranges on a silica-based (Nucleosil 300-5C_4), and a polymer-based column (Asahipak C4P-50).

RESULTS

Resolution

The separation of rat insulins (I and II), C-peptides (I and II), and proinsulins (I and II) from islets biosynthetically labelled with [^{3}H]leucine and [^{35}S]methionine (this amino acid being present in insulin II and proinsulin II, only) for 60 min [1] was attempted on several silica- and polymer-based RP columns. As can be seen from Table I the resolution (R_s) obtained for the two C-peptides and the two insulins were satisfactory (> 0.5) for all columns eluted with TEAP–acetonitrile, whereas the two proinsulins in most cases would not be resolved. Only two polymeric columns, Asahipak C_8 and C_4, were able to separate the proinsulins satisfactorily.

When the same columns were eluted with shallow acetonitrile gradients in TFA at room temperature (Table II), all the columns were able to separate the C-peptides, the silica-based C_4 and C_3 columns as well as all the polymer-based columns separated the insulins satisfactorily, but none of the silica-based columns, and only one of the polymer-based columns (Asahipak C_4), were able to separate the two proinsulins.

Temperature effect

The RP-HPLC separation of rat islet polypeptides in TFA–acetonitrile was furthermore performed at 45°C (see Table II).

Chromatograms of two optimized separations on the silica-based Nucleosil C_4 and the polymer-based, Asahipak C_4 column performed at room temperature and at 45°C are shown in Fig. 1. The identity of the individual peaks was based upon amino

TABLE I

RESOLUTION (R_s) OF BIOSYNTHETICALLY LABELLED RAT ISLET POLYPEPTIDES

Mobile phase: 0.125 *M* TEAP, pH 4.0 in acetonitrile, linear gradient 0.08% acetonitrile/min, 25°C.

	Bonded phase	Stationary phase	R_s[a]			Comments
			C-peptides	Insulins	Proinsulins	
Silica-based	C_{18}	LiChrosorb RP-18	0.8	1.0	0	Incipient separation of the proinsulins at 45°C (R_s=0.4)
	C_{18}	Nucleosil 120-5C_{18}	1.6	1.2	0.4	
	C_8	LiChrosorb RP-8	1.1	1.3	0	
	C_4	Nucleosil 300-5C_4	1.2	0.5	0.4	Inverse elution of the proinsulins
	C_4	Bakerbond WP Butyl	1.1	1.1	0	
	C_3	Zorbax Protein Plus	1.3	1.0	0	
Polymer-based	C_{18}	Asahipak ODP-50	1.1	1.3	0	Non-ideal peak shapes for the proinsulins
	C_8	Asahipak C8P-50	0.8	1.2	0.5	
	C_4	Asahipak C4P-50	0.6	1.8	1.3	Baseline separation of the proinsulins

[a] An R_s value of 0.5 or higher is required for satisfactory separation.

TABLE II

RESOLUTION (R_s) OF BIOSYNTHETICALLY LABELLED RAT ISLET POLYPEPTIDES AT DIFFERENT TEMPERATURES

Mobile phase: 0.1% TFA–acetonitrile, linear gradient 0.1% acetonitrile/min.

	Bonded phase	Stationary phase	R_s						Comments
			C-peptides		Insulins		Proinsulins		
			25°C	45°C	25°C	45°C	25°C	45°C	
Silica-based	C_{18}	LiChrosorb RP-18	0.6		n.e.[a]		n.e.		Irreversible binding of insulins and proinsulins
	C_{18}	Nucleosil 120-5C_{18}	1.2		n.e.		n.e.		(see ref. 2)
	C_8	LiChrosorb RP-8	1.8		n.e.		n.e.		
	C_4	Nucleosil 300-5C_4	1.2	0.8	1.1	1.8	0.4	0.8	See Fig. 1, left panels
	C_4	Bakerbond WP Butyl	1.2	1.0	1.1	2.9	0	0.9	
	C_3	Zorbax Protein Plus	1.3	0.8	1.0	1.5	0	0.8	
Polymer-based	C_{18}	Asahipak ODP-50	0.7	0.8	1.4	2.2	0.4	0.8	
	C_8	Asahipak C8P-50	0.7	0.4	1.2	3.0	0	0.6	
	C_4	Asahipak C4P-50	0.6	0.6	1.9	2.9	0.7	1.3[b]	See Fig. 1, right panels.

[a] n.e. = Not eluted.

[b] Flow-rate 0.8 ml/min. (At 0.5 ml/min rat insulin II coeluted with rat proinsulin I, see Fig. 2).

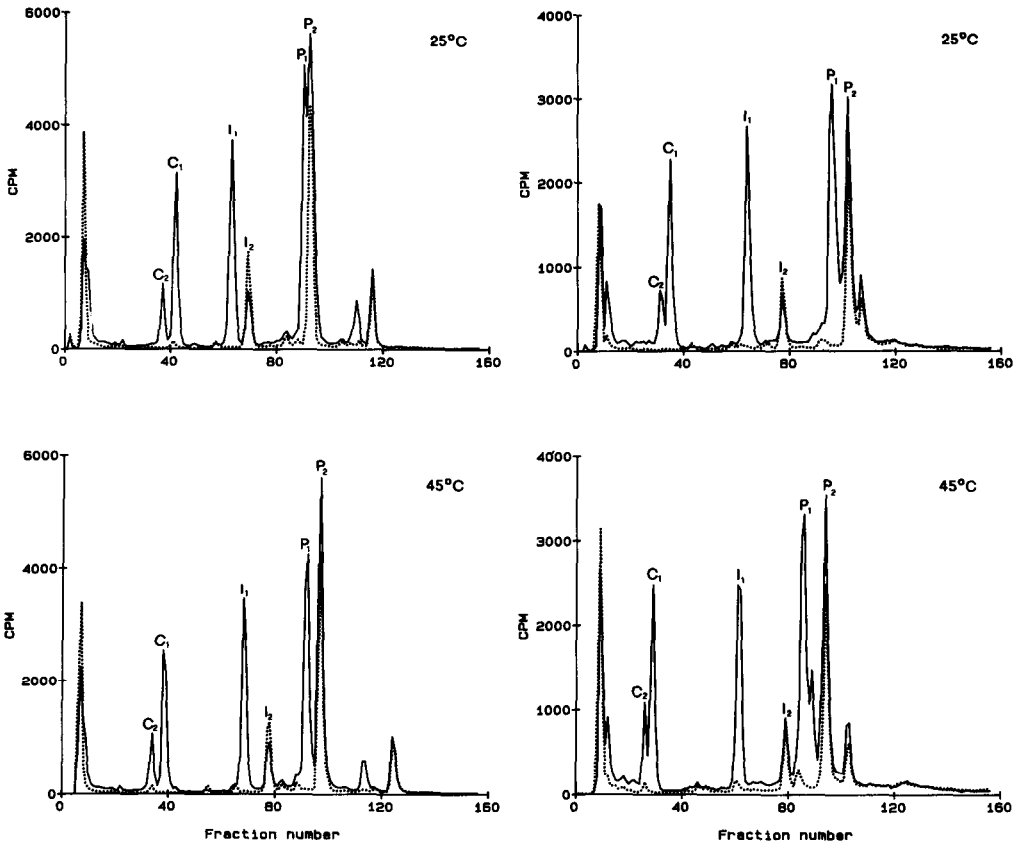

Fig. 1. RP-HPLC separation of a 3 *M* acetic acid extract of 50 rat islets labelled for 60 min with 2.5 μCi[^{3}H]leucine and 2.5 μCi[^{35}S]methionine, using a Nucleosil 300-5C_4column, eluted at 1.0 ml/min with a linear acetonitrile gradient (27–33%) in 0.1% TFA during 60 min (left panels); and an Asahipak C4P-50 column eluted at 0.8 ml/min with a linear acetonitrile gradient (26–32%) in 0.1% TFA during 60 min (right panels). Fractions at 0.5-min intervals were collected and counted for [^{3}H]- and [^{35}S]radioactivity. The separations shown in the upper panels were performed at ambient temperature (25°C), the separations in the lower panels at 45°C. The solid line represents [^{3}H]-radioactivity, the dotted line [^{35}S]radioactivity. The peaks are C_1 (C-peptide I), C_2 (C-peptide II), I_1 (insulin I), I_2 (insulin II), P_1 (proinsulin I) and P_2 (proinsulin II).

acid analysis and amino acid sequencing as described in ref. 1. The effect of temperature on the retention times of several pancreatic islet polypeptides was further examined on the same two columns and are depicted in Fig. 2.

Recovery

Load–response curves of pancreatic islet polypeptides were determined for three mass ranges using either the areas of the UV curves (microgram amounts of insulin) or radioactivity [^{3}H]leucine proinsulin for the nanogram range, [^{125}I]insulin for the picogram range), as shown in Fig. 3.

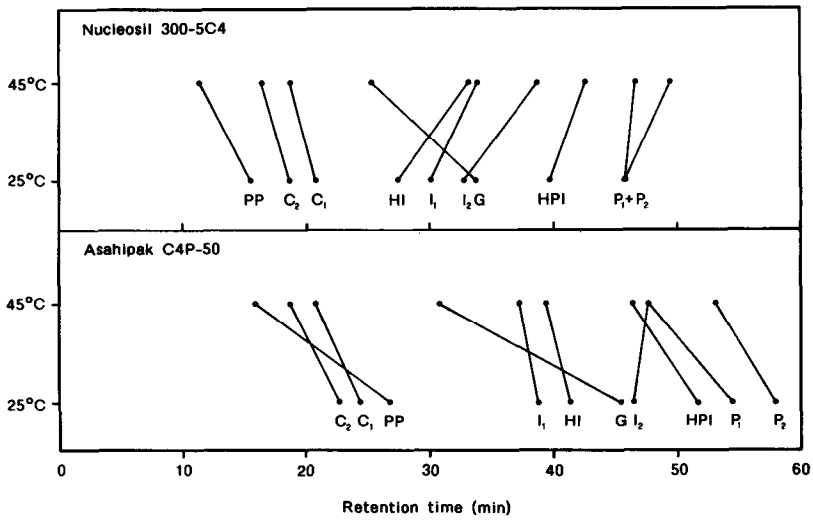

Fig. 2. The retention times of individual pancreatic polypeptides were measured after RP-HPLC of 1 μg of each polypeptide (except for the rat proinsulins, where only nanogram amounts were available) on a Nucleosil 300-5C_4 column (upper panel) and on an Asahipak C4P-50 (lower panel). The separations were performed at 25 and 45°C on both columns. The Nucleosil column was eluted at 1.0 ml/min, the Asahipak column at 0.5 ml/min. The gradients were the same as described in the legend to Fig. 1. The peak identities are, besides those described in the legend to Fig. 1, PP (rat pancreatic polypeptide), HI (human insulin), G (glucagon) and HPI (human proinsulin).

The ratios between individual rat islet polypeptides in identical samples (extracts of 50 rat islets) eluted from the silica-based Nucleosil C_4 column and the polymer-based Asahipak C_4 column are compared in Table III.

All major individual rat islet polypeptides were isolated after a preparative RP-HPLC separation of 500 biosynthetically labelled rat islets. After speed-vac concentration of the individual compounds, these samples were used for the measurement of the absolute recoveries (as described in Materials and Methods). The results are summarized in Table IV.

TABLE III

RATIOS BETWEEN INDIVIDUAL RAT ISLET POLYPEPTIDES AFTER RP-HPLC CALCULATED FROM [^{3}H]LEUCINE COUNTS

	Nucleosil 300-5C_4	Asahipak C4P-50
C-peptide I/C-peptide II	1.83 ± 0.06[a]	1.57 ± 0.20
Insulin I/insulin II	2.22 ± 0.07	2.20 ± 0.07
Proinsulin I/proinsulin II	0.88 ± 0.03	1.25 ± 0.11
C-peptides/insulins	0.75 ± 0.01	0.80 ± 0.03
C-peptides/proinsulins	1.05 ± 0.01	1.08 ± 0.03
Insulins/proinsulins	1.39 ± 0.02	1.34 ± 0.05

[a] Mean ± S.D., n = 5.

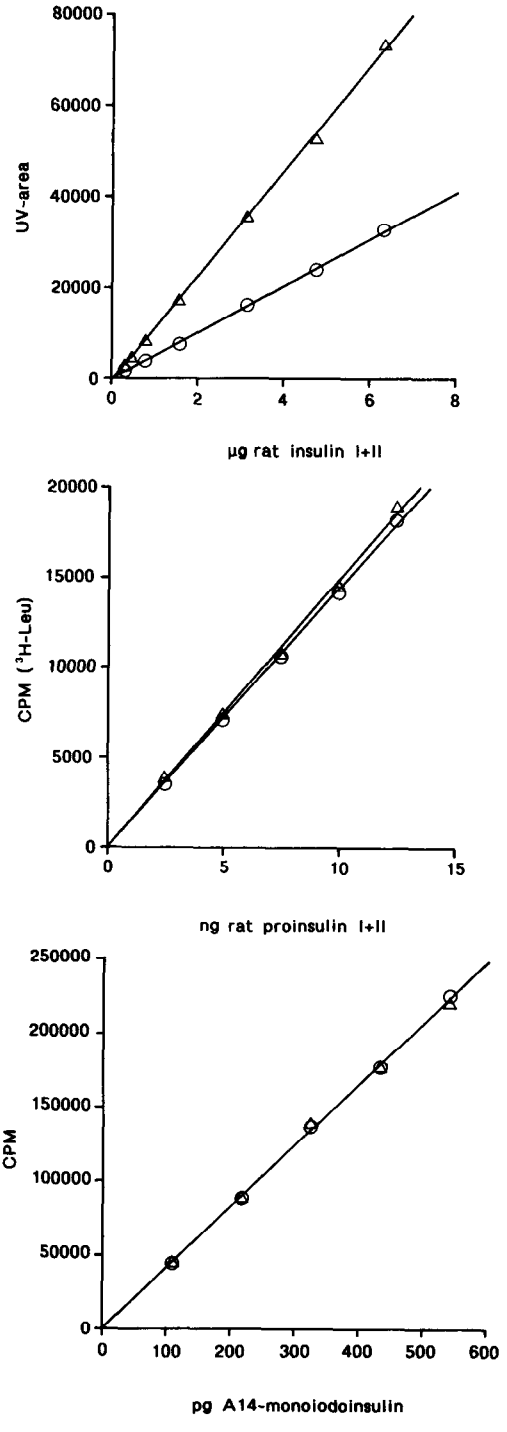
80000
60000
40000
20000
0
UV-area
0
2
4
6
8
µg rat insulin I+II
20000
15000
10000
5000
0
CPM (^{3}H-Leu)
0
5
10
15
ng rat proinsulin I+II
250000
200000
150000
100000
50000
0
CPM
0
100
200
300
400
500
600
pg A14-monoiodoinsulin

Fig. 3.

TABLE IV

ABSOLUTE RECOVERY (%) OF INDIVIDUAL PANCREATIC ISLET POLYPEPTIDES AFTER RP-HPLC

Polypeptides[a]	Mass range	Nucleosil 300-5C_4	Asahipak C4P-50
Rat C-peptide I	μg–ng	88.5 ± 1.8[b]	94.2 ± 8.2
Rat C-peptide II	μg–ng	99.2 ± 3.7	122.8 ± 6.2
Rat insulin I	μg–ng	97.8 ± 2.3	100.7 ± 2.5
Rat insulin II	μg–ng	88.7 ± 4.1	90.9 ± 7.6
Rat proinsulins	ng	105.3 ± 2.5	109.9 ± 4.6
Porcine insulin	pg	98.8 ± 2.3	99.2 ± 2.3
Mean recovery		96.4	102.9

[a] The polypeptide samples are described in the legend to Fig. 3.
[b] Mean ± S.D., $n = 5$.

DISCUSSION

RP-HPLC is the obvious method to use for the study of insulin biosynthesis due to its extremely high resolving power of closely related insulins and insulin-related polypeptides [3]. Optimization of the separation of the pairs of rat insulins I and II, C-peptides I and II, and proinsulins I and II resulted in the satisfactory separation of all these polypeptides which are involved in insulin biosynthesis in the rat [1,2].

The use of a silica-based C_{18} column (LiChrosorb RP-18) in combination with TFA–acetonitrile as mobile phase (room temperature) was the preferred system in our initial studies [1] until certain batch-to-batch variations (resulting in irreversible binding of insulins and proinsulins) necessitated further investigations. These binding phenomena were also observed for other C_{18} and C_8 columns in combination with TFA–acetonitrile, but were absent for C_4 and C_3 silica-based columns [2].

However, the selectivities for these columns were reduced to a level where no separation of rat proinsulin I and II was possible, in accordance with the finding that C_{18} and C_8 columns are more resolutive than C_4 columns [4].

Since no irreversible binding was ever observed when all these columns were

Fig. 3. Load-response curves for rat insulin I and II (upper curve), rat proinsulin I and II (middle curve) and A14 monoiodoinsulin (lower curve) after RP-HPLC on a Nucleosil 300-5C_4 column (○), and on an Asahipak C4P-50 column (△) eluted at 45 and 25°C, respectively, as described in the legend to Fig. 1. The samples were dilutions in 3 *M* acetic acid, 0.1% human serum albumin of medium from cultured newborn rat islet cells containig from 0.3 μg to 6.3 μg insulin I + II (upper curve), of rat islets biosynthetically labelled as described in the legend to Fig. 1, containing from 2.5 ng to 12.4 ng of proinsulin I + II (middle curve) and of carrier-free mono-[^{125}I]-(Tyr A14)-porcine insulin containing from 100–500 pg of monoiodoinsulin. The column eluates were registrated at 210 nm, and the peak areas integrated (Waters 730 data module) in the case of insulin or counted in the collected 0.5-min fractions for [^{3}H]leucine radioactivity (specific radioactivity approximately 1500 cpm/ng of proinsulin) or for [^{125}I]radioactivity (specific radioactivity approximately 400 cpm/pg of monoiodoinsulin).

eluted with TEAP–acetonitrile, the observations were ascribed to the presence of silanol groups, not being effectively masked by poor ion-pairing reagents (such as TFA) [2].

The logical conclusion of these observations was an evaluation of the use of polymer-based RP-columns and in contrast to the silica-based C_{18}- and C_8 columns, no irreversible binding was observed for the polymer-based C_{18}-, C_8- and C_4 columns eluted with TFA–acetonitrile, probably due to the absence of silanol groups in the polyvinylalcohol skeleton [5].

The two different types of stationary phases were further evaluated after elution with TEAP–acetonitrile, another popular mobile phase for the separation of insulin and insulin-like compounds [3,4]. None of the silica-based columns were able to resolve the two rat proinsulins (Table I), whereas this separation could be performed at room temperature on the polymer-based C_4 column in both mobile phases (Table I and II) and on the polymer C_8 column in TEAP–acetonitrile (Table I).

Increasing the separation temperature to 45°C, (previously noticed to improve the proinsulin separation under similar experimental conditions [2]) resulted in increased R_s values for the proinsulins on the silica-based C_3- and C_4- columns as well as on all polymer-based columns eluted with TFA–acetonitrile (Table II). The resolution of the two insulins was increased as well, whereas that of the two C-peptides was decreased. A similar temperature effect was also noticed for the LiChrosorb RP-18–TEAP–acetonitrile system (Table I), but with this stationary–mobile phase the improvement was not sufficient to obtain a satisfactory proinsulin separation.

In order to extend the examination of the influence of temperature on the retention times after RP-HPLC, several pancreatic islet polypeptides were analyzed at 25 and 45°C using the silica-based and polymer-based C_4 column, as shown in Fig. 2. Interestingly, the retention times of individual islet polypeptides were affected differently on the silica- and polymer-based columns. On the silica C_4 columns polypeptides with mol. wt. < 5000 (C-peptides, pancreatic polypeptide, glucagon) eluted earlier when the temperature was increased to 45°C, whereas polypeptides with mol. wt. > 5000 (insulins, proinsulins) eluted later. Especially, the retention time of glucagon was influenced by temperature. On the polymer C_4 column the retention times for both glucagon and pancreatic polypeptides were markedly lowered by increasing the temperature, in fact all the polypeptides, except insulin II, were eluted earlier at 45°C indicating that the selectivities of the two columns are different. These results suggest that the effect of the temperature can not apparently be described as a general effect, but depends upon several chromatographic parameters (the actual stationary and mobile phase) and the nature of the sample polypeptide. It is therefore not surprising that literature reports have described major [6–10] as well as minor improvements [11] and even negative effects [12] of the separation temperature upon the resolution of polypeptides after RP-HPLC.

Precise and quantitative calculations of the insulin biosynthesis (based on the measurement of the [^{3}H]leucine radioactivity of the individual polypeptides eluted from the RP-HPLC column) can only be performed if the recoveries of the individual components are known for all potential mass ranges.

Load–response curves for insulins and proinsulins (mass ranges from microgram to picogram amounts) are shown in Fig. 3. For microgram amounts of insulins the UV area is an accurate measure of the mass, but it should be noticed that due to

differences in the elution rate the areas were not comparable. In the nanogram and picogram range the UV areas could not be used for quantitation. [^{3}H]Leucine radioactivity was used for nanogram amounts and [^{125}I]radioactivity for picogram amounts, the measured radioactivity being unaffected by the elution rate. In all three mass ranges, a linear relation between the amount of polypeptide applied and that recovered after RP-HPLC was obtained (correlation coefficients from 0.9983 to 0.9996). Further, these relations were comparable for the two types of columns, suggesting identical recoveries over the whole mass range.

Further elucidation of the recoveries may be gained from the ratios between the individual rat islet polypeptides after RP-HPLC (Table III). These ratios were comparable for the silica- and the polymer-based C_4 columns examined, except for that of the two proinsulins. An additional peak eluting between rat proinsulin I and II was observed after separation on the Asahipak C_4 column at 45°C (Fig. 1, lower panel right). This peak coeluted with proinsulin I at 25°C (Fig. 1, upper panel right). On the Nucleosil C_4 column we have observed a similar peak between the two proinsulins, when using a more shallow acetonitrile gradient (data not shown). In this case the peak coeluted with proinsulin II under the conditions shown in Fig. 1. This might explain the discrepancy of the ratios between the proinsulins after separation on the two column types. This component was not identified, but consists presumably of an intermediate form of proinsulin, as also is the case for the later eluting minor peaks seen in the chromatograms in Fig. 1. It has been shown that on the Nucleosil C_4 column the human proinsulin intermediates cleaved at the B–C junction elute earlier than human proinsulin, whereas the proinsulin intermediates cleaved at the A–C junction eluate later [13].

Furthermore, the ratios between the individual rat islet polypeptides after RP-HPLC on an Asahipak ODP-50 column (TFA–acetonitrile) were not changed when compared to a separation performed on two identical columns in series (data not shown) another demonstration of identical recoveries of the individual polypeptides.

In order to determine the absolute recoveries of the individual polypeptides, it was attempted to collect all the polypeptides from a separation of biosynthetically labelled rat islets on Nucleosil C_4 (60 min *ca.* 60 ml eluate), speed-vac concentrate and reinject the sample, but the resulting chromatogram was obscured by the occurrence of oxidized forms of the methionine containing insulin II and proinsulin II partly overlapping insulin I and proinsulin I, respectively (data not shown). This oxidation has been shown to occur during the sample concentration [14].

The absolute recoveries were therefore measured for the individually isolated polypeptides injected on the HPLC column and compared to injections bypassing the RP-HPLC column with a loop (or counting the sample before analysis), as shown in Table IV. The methionine sulphoxide forms of rat insulin II and proinsulin II (originated during speed-vac concentration) were added to the respective native forms. The recoveries were comparably high on both types of C_4 columns, with a slight tendency to higher recoveries from the polymer-based C_4 column (only significant for the C-peptide II).

From the literature it is well-known that the recoveries of polypeptides are not always quantitative, especially if small amounts are applied [15,16]. Reduced recoveries of larger polypeptides, *e.g.* greater than 20–30 amino acid residues have been described, even after injection of microgram amounts [17], but quantitative recoveries have been reported as well [12,18,19], even down to femtogram amounts [20].

It has been reported that insulin recovery from a silica-based C_{18} column eluted with shallow acetonitrile gradients in TEAP or ammonium sulphate is close to 100%, whereas the recovery of proinsulin (and related compounds) are substantially lower (60–80%) [3]. The reason for the higher recovery of proinsulin found in our study could be due to the use of the less hydrophobic C_4 columns.

In conclusion, we have shown that polymeric C_{18}, C_8 and C_4 columns eluted with shallow TFA–acetonitrile gradients were free of the irreversible binding of insulins and proinsulins previously noticed for certain silica C_{18}- and C_8 columns, probably due to the absence of silanol groups in the polymeric skeleton. At ambient temperature (25°C) only one polymeric RP column (C_4) was able to separate all polypeptides involved in the rat insulin biosynthesis whereas at 45°C all the polymeric RP columns (C_{18}, C_8 and C_4) and several silica RP columns (C_4 and C_3) were able to perform this separation satisfactorily. The recoveries were comparably high, and the ratios between the individual polypeptides recovered after RP-HPLC on the two C_4 columns were virtually identical, as was the case after separation on one compared to two columns connected in series (polymer C_{18} column). These results are a prerequisite for the quantitative calculations of the rat insulin biosynthesis.

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