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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INSULIN AND INSULIN DERIVATIVES

A COMPARATIVE STUDY*

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SUMMARY

The reversed-phase separation of crystalline insulin (I) and monoiodoinsulins (II) has been investigated, with respect to the effects of buffer, substitution group, pore-size and column support backbone. The separations were performed either isocratically (for II) or by gradient elution with very narrow gradients (for I).

Fourteen reversed-phase columns, the majority being silica-based, were investigated, and three main results emerged.

(1) Trifluoroacetic acid is unsuitable as a buffer for this type of analysis, whereas trialkylammonium phosphates are very suitable.

(2) The separation between the major components in crystalline insulin was comparable in the main for all the columns tested except one. However, the ability to distinguish between the numerous minor components (co-extracted with insulin peptide) varied a great deal between the columns.

(3) In an optimized buffer system only three columns were able to separate insulin peptide and the four monoiodoinsulin isomers; all three were 80–100-Å silica-based C_{18} columns.

INTRODUCTION

In recent years a large number of reports have been published describing the application of insulin to a variety of reversed-phase high-performance liquid chromatographic (RP-HPLC) systems. In the majority of these reports, insulin has been used as one among several polypeptides and proteins in order to characterize the separation capacity of the system. The separation of insulin, insulin-related and non-insulin-related substances has also been studied in more detail[1–14].

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The separation of crystalline insulin, containing one major constituent (insulin peptide) and a large number of insulin-like contaminants (desamidoinsulin, arginine insulin, ethylesterinsulin, des-alanine insulin, des-pentapeptide and des-octapeptide insulin, insulin dimers, proinsulin and intermediary insulins) as well as non-insulin-related substances (glucagon, pancreatic polypeptide) represents a typical challenge to the separation capacity of RP-HPLC.

Three types of buffer system have commonly been used: ion-pairing substances (*e.g.* alkylammonium phosphates, trifluoroacetic acid); salts (*e.g.* acetates, sulphates, phosphates); and chaotropic compounds (*e.g.* perchlorate). In almost all insulin separations acetonitrile was used as organic modifier. No matter which buffer, organic modifier and column support were used, insulin peptide and insulin-related substances with similar molecular weights (*ca.* 6000) were eluted before insulin-like substances with higher molecular weight [proinsulin and intermediary insulin (mol.wt. 9000) and insulin dimers (mol.wt. 12 000)]. The individual elution order of proinsulin, intermediary insulin and insulin dimers depended upon the actual buffer system[5].

However, with one exception[5], all analyses of the reversed-phase separation of insulin and insulin-like components have been performed with a single column and a single buffer–modifier system. In this paper we report the influence of a number of parameters (column, buffer, pore-size, column packing material, substitution group and batch-to-batch variation) on the RP-HPLC separation of crystalline porcine insulin.

MATERIALS AND METHODS

An M6000 A/M660 solvent programmer (Waters) or a Spectra-Physics SP 8700 chromatograph was used, with a WISP 710B or U6K injector (Waters). A Waters M440 with an extended-wavelength module (214 nm) or a Pye Unicam variable-wavelength UV detector was used. Fractions (1 min) were collected in a Pharmacia FRAC 300 fraction collector and assayed for radioactivity in a Hydrogamma sixteen-channel gamma counter.

The following fourteen columns were tested: LiChrosorb RP-18, 5 μm, 250 × 4.0 mm I.D. (Merck); Vydac 218 TPB 5, 5 μm, 250 × 8.0 mm I.D. (Separation Group); Spherisorb ODS2, 3 μm, 150 × 4.0 mm I.D. (Phase Separation); Spherisorb ODS2, 3 μm, 250 × 4.0 mm I.D. (Phase Separation); TSK ODS-120T, 5 μm, 250 × 4.6 mm I.D. (Toyo Soda); Nova-Pak C_{18}, 5 μm, 150 × 3.9 mm I.D. (Waters); Techogel C_4, 5 μm, 250 × 4.0 mm I.D. (HPLC Technology); Techogel C_{18}, 5 μm, 250 × 4.0 mm I.D. (HPLC Technology); Protesil diphenyl, 10 μm, 250 × 4.6 mm I.D. (Whatman); Protesil octyl, 10 μm, 250 × 4.6 mm I.D. (Whatman); Chrompack C_{18}, 8 μm, 100 × 3.0 mm I.D. (Chrompack); PEP-RPC, 10 μm, 50 × 5.0 mm I.D. (Pharmacia); PRP-1, 10 μm, 150 × 4.0 mm I.D. (Hamilton); PLRP-S, 5 μm, 150 × 4.0 mm I.D. (Polymer Laboratories).

Three buffers were used: 0.25 *M* triethylammoniumphosphate, pH 3.00 (TEAP); 0.25 *M* triethylammoniumformate, pH 6.00 (TEAF); 0.1% trifluoroacetic acid (TFA). Acetonitrile and isopropanol (HPLC quality) were used as organic modifiers.

The gradients were prepared by mixing two solutions. The first was one of the three buffers, and the second was a mixture of equal amounts of the buffer and the organic modifier.

Except for Protesil diphenyl and Protesil octyl, each type of column was tested with three or more individual specimens.

All solvents were Millipore-filtered (0.45 μm) and degassed (vacuum/ultrasound) before use. During chromatography helium is bubbled continuously through the mobile phases.

The samples were crystalline porcine sodium insulinate, batch G-63 (Nordisk Gentofte), containing *ca.* 75% insulin constituents and 25% sodium chloride and water. The dilute iodination mixture was prepared as described previously[15], 50 μl containing *ca.* 100 ng of insulin, 1–5 ng of monoiodoinsulins and 50 pg of diiodoinsulins.

RESULTS

Figs. 1 and 2 show the isocratic elution pattern of insulin peptide and monodesamidoinsulin on two different silica-based C_{18} column supports: LiChrosorb RP-18 (Fig. 1) and Chrompack C_{18} (Fig. 2). Both columns were eluted with acetonitrile as organic modifier, with TFA (upper panel) or TEAP (lower panel) as buffer substance. For comparable capacity factors (k') notable differences were observed (with respect to peak shape and resolution) by the change from one buffer substance to the other. The peak shape obtained with TFA was non-ideal and considerably inferior to that achieved with TEAP [also for higher and lower k' values (data not shown)], the latter was used throughout the insulin analyses.

Figs. 3–10 show the resolution obtained using an acetonitrile gradient in TEAP

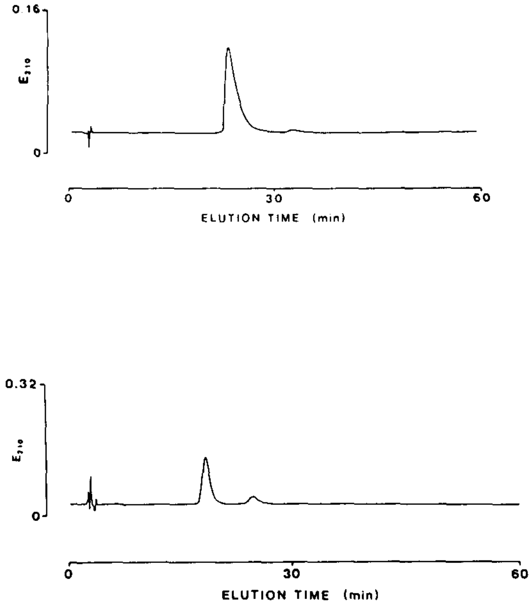

Fig. 1. Isocratic separation of 5 μg of crystalline porcine insulin on a 250 × 4.0 mm I.D. LiChrosorb RP-18 column eluted with 0.1% TFA–27.0% acetonitrile (upper curve) and 0.25 *M* TEAP–22.0% acetonitrile (lower curve), at 0.5 ml/min.

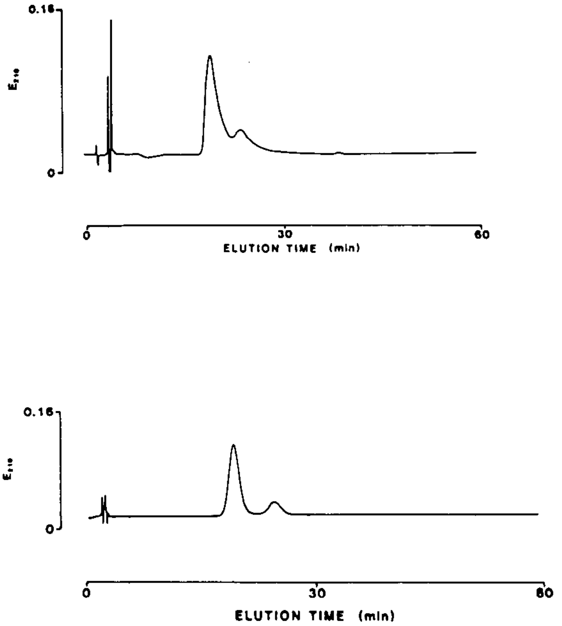

Fig. 2. Isocratic separation of 5 μg of crystalline porcine insulin on a 100 × 3.0 mm I.D. glass cartridge packed with Chrompack C_{18}. Elution was performed at 0.5 ml/min, with 0.1% TFA–23.5% acetonitrile (upper curve) and 0.25 *M* TEAP–22.5% acetonitrile (lower curve).

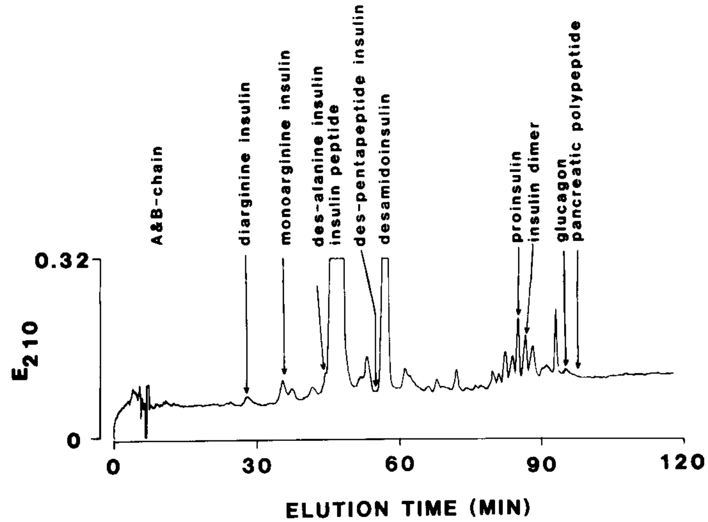

Fig. 3. Separation of 200 μg of crystalline porcine insulin on a 250 × 4.0 mm I.D. LiChrosorb RP-18 column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23–28% acetonitrile over 60 min (gradient No. 8), at 0.5 ml/min.

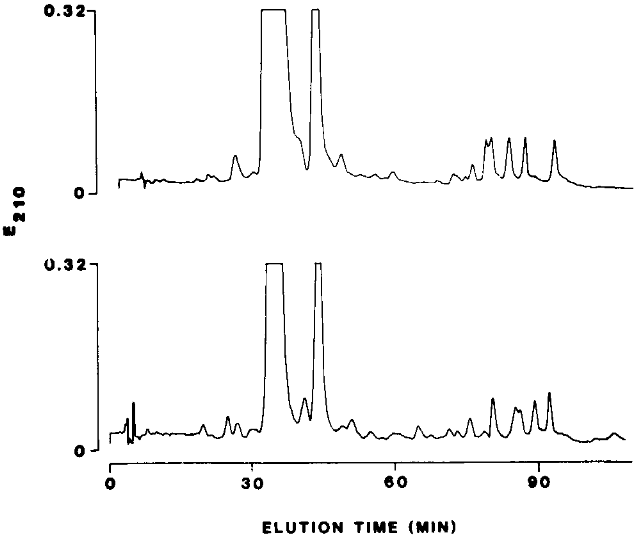

Fig. 4. Separation of 200 μg of crystalline porcine insulin on a 250 × 8.0 mm I.D. Vydac 218 TPB 5 column (top), or a 250 × 4.0 mm I.D. Spherisorb ODS2 column (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23–28% acetonitrile over 60 min (gradient No. 8). Flow-rate, 1.0 ml/min (top), 0.5 ml/min (bottom).

buffer for the elution of a number of reversed-phase column supports, silica-based (Figs. 3–9) as well as polymer-based (Fig. 10). Each column was evaluated in the following way. With a target *k'* value for insulin peptide between 15 and 25, the shape, duration and end-point of the gradient was varied in order to obtain the best possible resolution between insulin peptide and monodesamidoinsulin, as well as between proinsulin, insulin dimers and intermediary insulin in the last part of the chromatogram. A slightly concave acetonitrile gradient (gradient No. 8 in the Waters M660 solvent programmer) which increased the acetonitrile concentration 2.5–5.5%

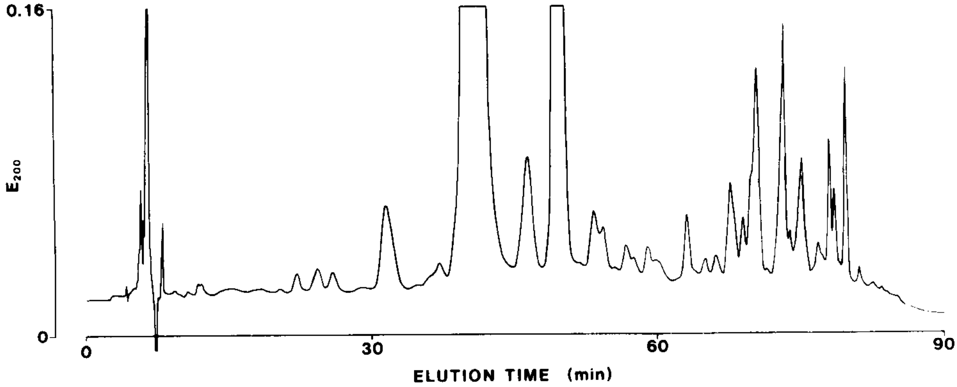

Fig. 5. Separation of 100 μg of crystalline porcine insulin on a 250 × 4.6 mm I.D. TSK ODS-120T column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 27.5–32.5% acetonitrile over 70 min (gradient No. 8) at 0.5 ml/min.

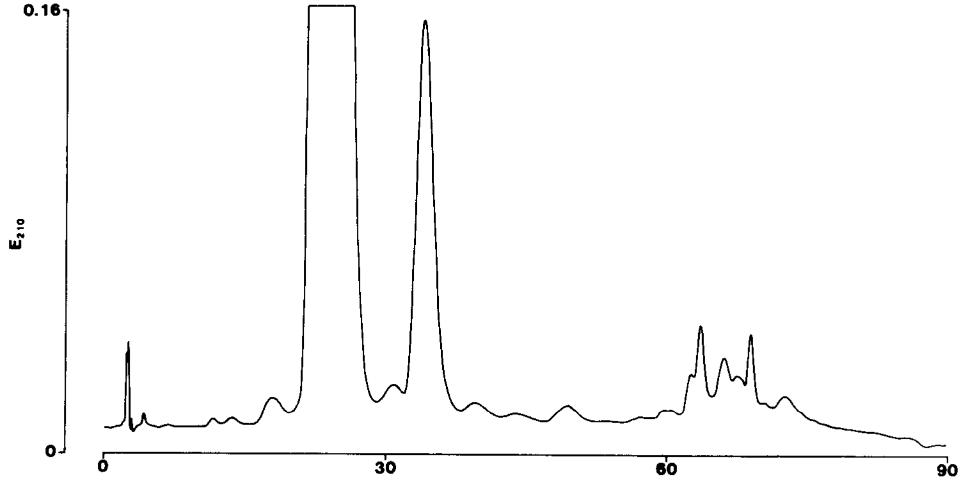

Fig. 6. Separation of 200 μg of crystalline porcine insulin on a 150 × 3.9 mm I.D. Nova-Pak C_{18} column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23.5–29% acetonitrile (gradient No. 8) for 60 min, at 0.5 ml/min.

during 50–70 min, was found to yield the best possible separation of crystalline insulin in all the columns tested except one. Except for the PEP-RPC column, these conditions allowed a more or less prominent separation between insulin peptide and desamidoinsulin, as well as between insulin-like components with a molecular weight higher than 6000. It was found impossible to separate insulin peptide and desamidoinsulin on the PEP-RPC column under these conditions.

Fig. 11 shows the isocratic elution of insulin peptide and the four monoiodoinsulins on three different silica-based C_{18} column supports with a pore-size of *ca.* 100 Å. The buffer system [0.25 *M* TEAF (pH 6.0)–isopropanol] has been optimized for this type of separation with respect to alkylammonium buffer, pH and organic modifier[16]. Although the three column supports separate the five constituents, it should be noted that the order of elution for the iodoinsulin isomers is different on the TSK ODS-120T from what it is on the LiChrosorb and the Spherisorb columns.

The Techogel C_4, Techogel C_{18} and PLRP-S columns were all incapable of separating the four monoiodoinsulins (Figs. 12 and 13). Either A14 and B16 monoiodoinsulin are eluted in the same position (Techogel C_{18}, PLRP-S) or B26 and A14 monoiodoinsulin are unresolved (Techogel C_4).

Batch-to-batch variation for one column, LiChrosorb RP-18, is shown in Fig. 14, where three different columns obtained from the local manufacturer over a period of 6 months were tested under similar conditions. For each column the organic modifier concentration was adjusted in order to achieve a *k'* value of *ca.* 15 for A19 monoiodoinsulin. Under these conditions two of the columns were unable to resolve A14 and B16 monoiodoinsulin.

DISCUSSION

Reversed-phase separation of numerous compounds with small differences in

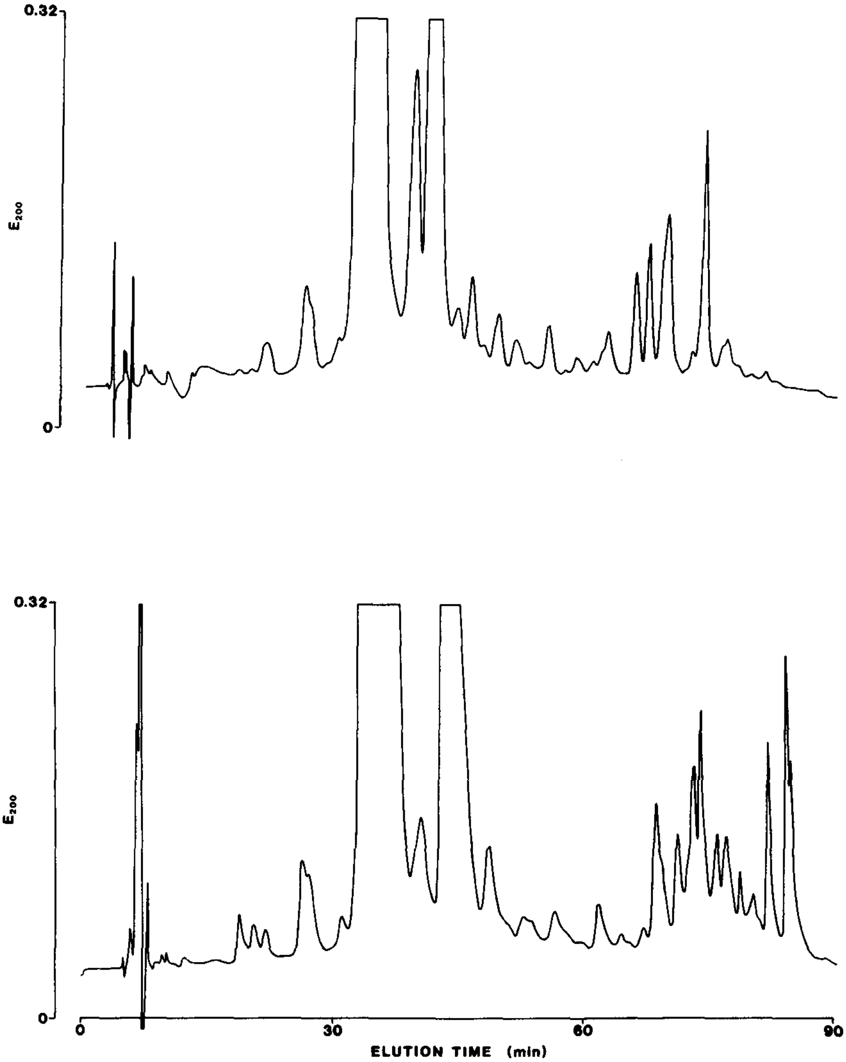

Fig. 7. Separation of 200 μg of crystalline porcine insulin on a 250 × 4.0 mm I.D. Techogel C_4 column (top) or a 250 × 4.0 mm I.D. Techogel C_{18} column (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 25–27.5% acetonitrile (gradient No. 8) for 60 min, at 0.5 ml/min.

hydrophobicity is generally performed using isocratic elution or a very narrow gradient of the organic modifier. In the case of crystalline insulin, the constituents can be divided in three groups (Table I). The main fraction contains insulin peptide (constituting 80–90% of the total amount of polypeptide) and a number of insulin derivatives with small structural differences from insulin peptide (loss of amide group(s), loss, addition or substitution of one or a few amino acid residues, etc.) and with a molecular weight similar to that of insulin peptide (5800). The second group

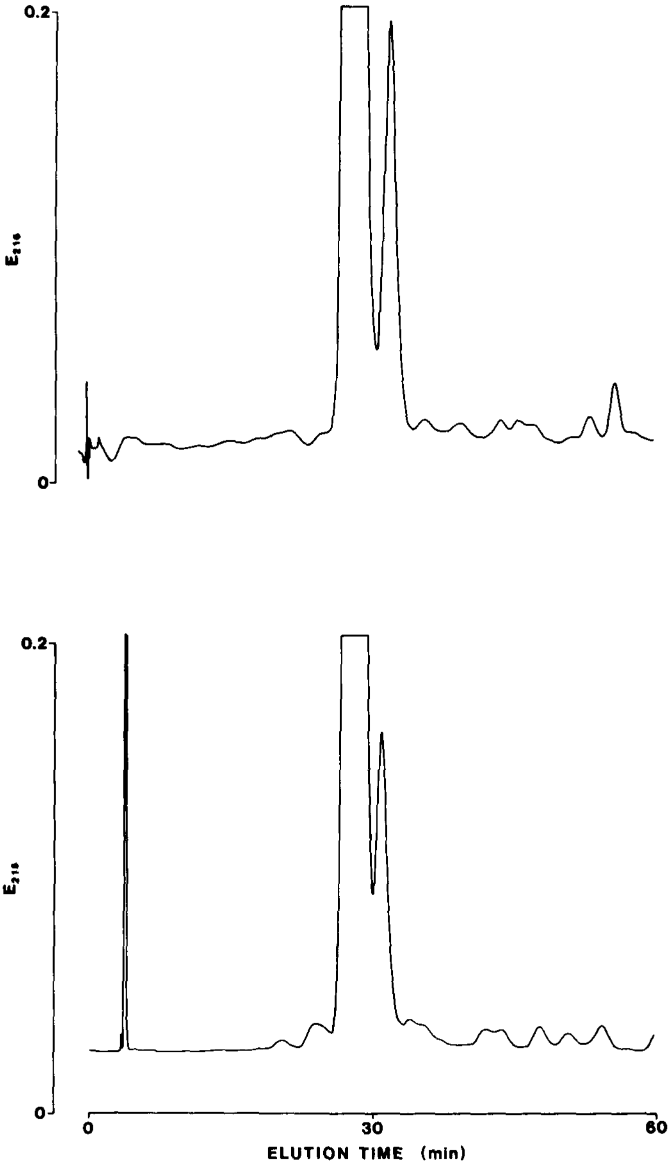

Fig. 8. Separation of 200 μg of crystalline porcine insulin on a 250 × 4.6 mm I.D. column packed with Protesil diphenyl (top) or Protesil octyl (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 23–28% acetonitrile (gradient No. 8) over 50 min, at 0.5 ml/min.

contains proinsulin, covalently bound insulin dimers and intermediary insulin, compounds with higher molecular weights than insulin peptide (9000–12 000) but containing the insulin peptide as part of the molecule. The third group contains non-insulin-related polypeptides co-extracted with insulin during the processing of the pancreatic glands (glucagon, pancreatic polypeptide, etc.).

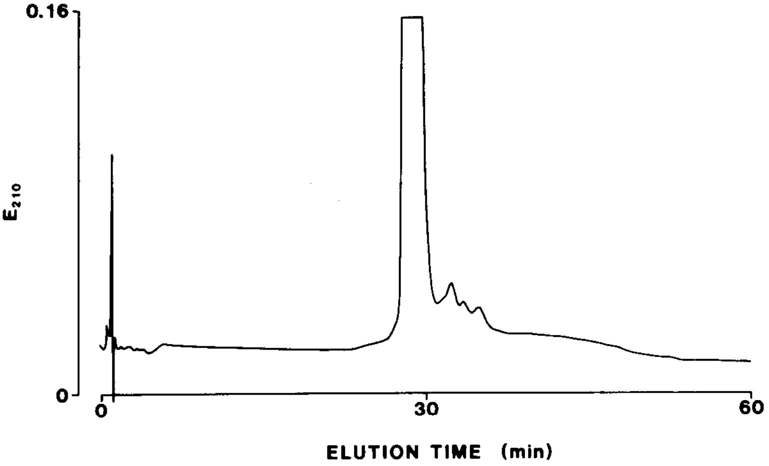

Fig. 9. Separation of 50 μg of crystalline porcine insulin on a 50 × 5.0 mm I.D. PEP-RPC column. Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 15–35% acetonitrile over 45 min (gradient No. 8), at 1.0 ml/min.

The members of the first group can be resolved by isocratic elution, but no published RP-HPLC separation of insulin peptide and proinsulin has been accomplished without the use of gradient elution.

In our studies we therefore used a very narrow, concave gradient, thereby operating close to isocratic conditions in the first part of the chromatogram where insulin and closely related substances are eluted, thereafter raising the organic modifier concentration to elute insulin-like components with higher molecular weights.

In isocratic elution —or close-to-isocratic elution— the choice of buffer is extremely important. As can be seen from Figs. 1 and 2, the peak shape of insulin peptide eluted isocratically is highly asymmetrical when TFA is used in combination with either C_{18} column support, whereas almost symmetrical peaks are obtained when the columns were eluted with TEAP buffer. This is probably because TEAP effectively masks residual silanol groups and thereby eliminates non-specific adsorption.

The isocratic elution pattern also reveals differences in selectivity, *i.e.* the ability to separate closely related substances. As can be seen from Figs. 1 and 2, there is a considerable difference in the abilities of the two C_{18} columns to distinguish between insulin peptide and monodesamidoinsulin (comparable k' values, identical mobile phase).

The separation pattern obtained for crystalline porcine insulin on a number of reversed-phase column supports, all eluted with an optimized TEAP–acetonitrile gradient, are shown in Figs. 3–10. The columns show considerable variation in their abilities to separate the numerous components in crystalline insulin, especially those with molecular weights higher than 6000. However, because many parameters are different (basic silica, substitution group, end-capping, pore size, particle size, etc.) from column to column, it seems too uncertain to correlate the fundamental characteristics —the hydrophobic interaction between the stationary phase and the sam-

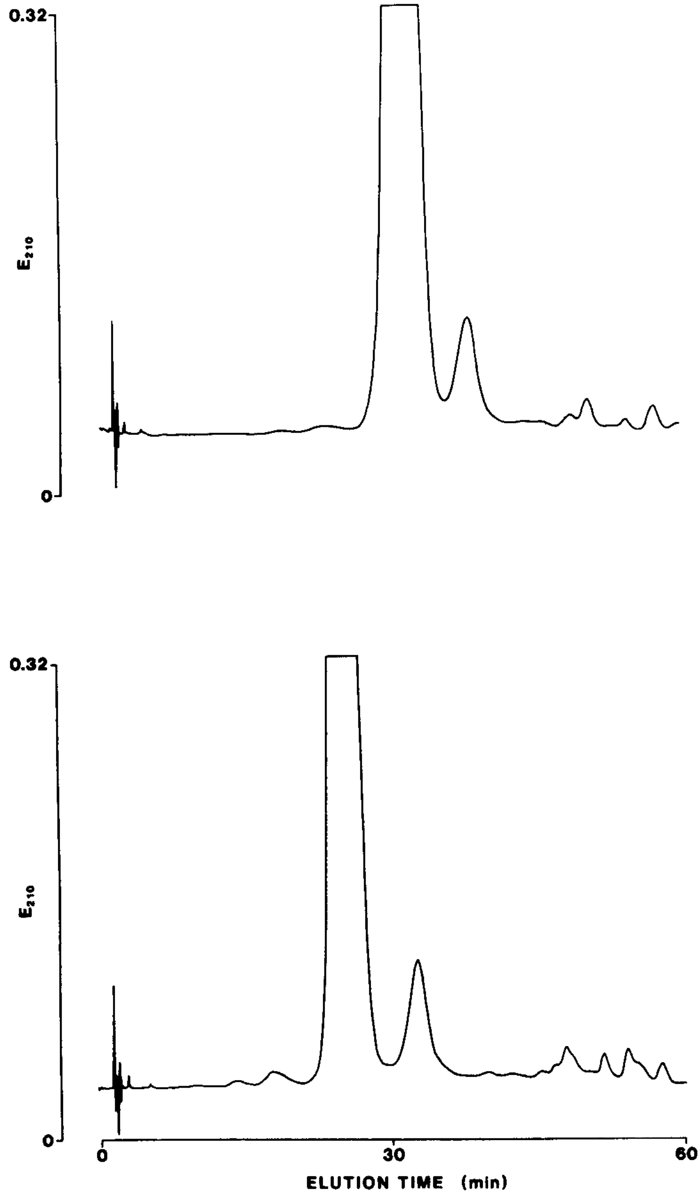

Fig. 10. Separation of 200 μg of crystalline porcine insulin on a 150 × 4.0 mm I.D. column packed with PRP-1 (top) or PLRP-S (bottom). Gradient elution, with 0.25 *M* TEAP (pH 3.0) and 22–25.5% acetonitrile (gradient No. 8), at 1.0 ml/min.

ple molecules— to one or more of the physicochemical parameters commonly used in column characterization.

The RP-HPLC separation of the four monoiodoinsulins (iodinated in Tyr A14, Tyr A19, Tyr B16 or B26) can be performed on LiChrosorb RP-18, TSK ODS-120T

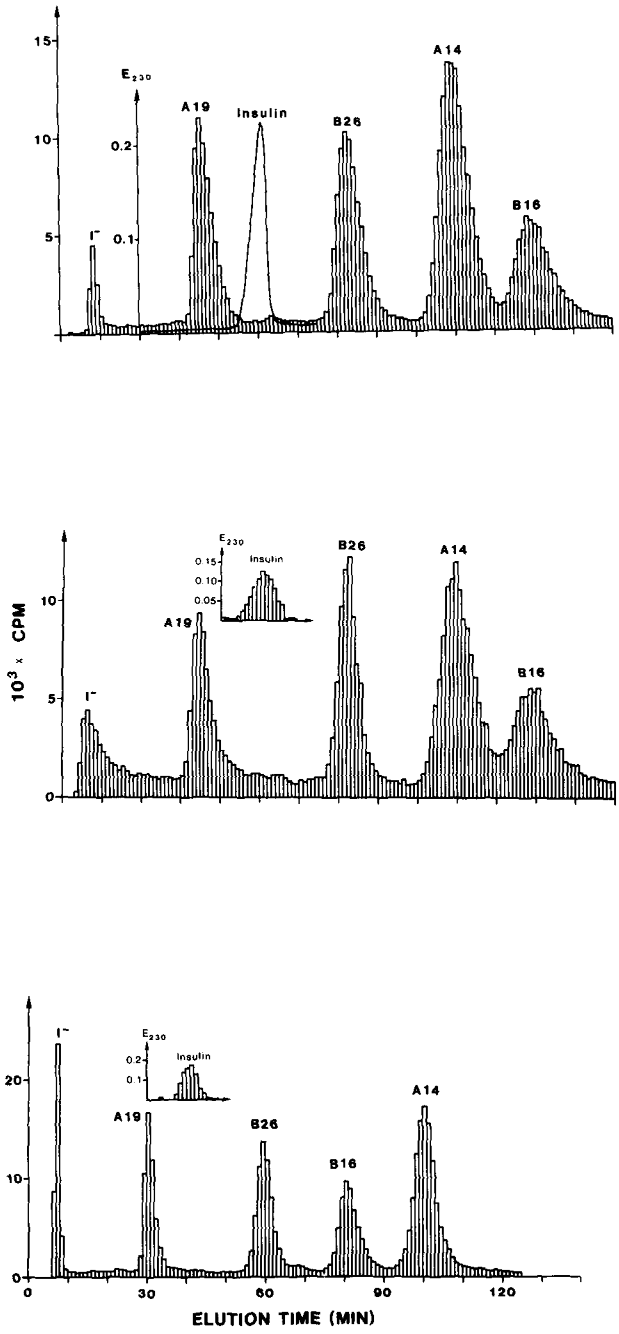

Fig. 11. Isocratic separation of 50 μl of diluted iodination mixture on a 250 × 4.6 mm I.D. TSK ODS-120T column (bottom), a 150 × 4.0 mm I.D. Spherisorb ODS2 column (middle) or a LiChrosorb RP-18 column (250 × 4.0 mm I.D.) (top), with 0.25 *M* TEAF (pH 6.0)–isopropanol concentrations of 21.5, 21.5 and 20.5% (from top to bottom). Flow-rate, 0.5 ml/min.

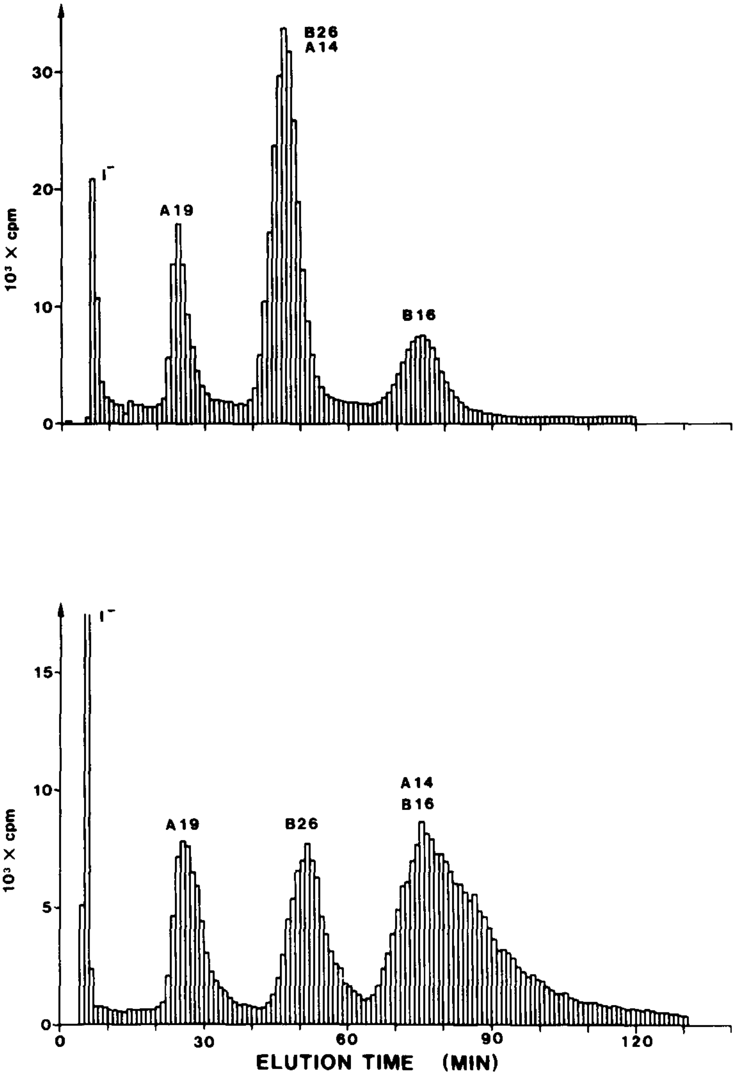

Fig. 12. Isocratic separation of 50 μl of diluted iodination mixture on a 250 × 4.0 mm I.D. column packed with Techogel C_4 (top) or Techogel C_{18} (bottom). The columns were eluted with 0.25 *M* TEAF (pH 6.0)–21.0% isopropanol, at 0.5 ml/min.

or Spherisorb ODS2 columns eluted isocratically with 0.25 *M* TEAF (pH 6.0)–isopropanol. This highly sensitive separation, which has been optimized with respect to alkylammonium buffer, pH, column and organic modifier[16], cannot be performed on the Techogel C_4 and C_{18} columns (Fig. 12), the resin-based PLRP-S column (Fig. 13), nor the PRP-1 column (data not shown). Furthermore, the Vydac 218 TPB 5 column was unable to separate the monoiodoinsulins[16].

Even though the separation can be performed on the LiChrosorb RP-18 col-

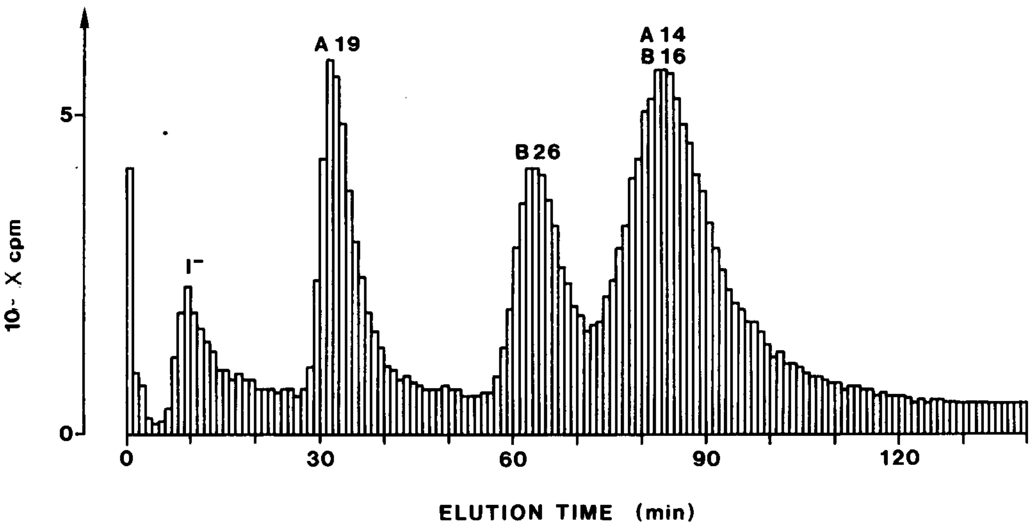

Fig. 13. Isocratic separation of 50 μl of diluted iodination mixture on a 150 × 4.0 mm I.D. PLRP-S column eluted with 0.25 *M* TEAF (pH 6.0)–22.5% isopropanol, at 0.5 ml/min.

umn, the task is only just within the capacity of this column. This is illustrated in Fig. 14, which shows the separation pattern obtained from three randomly selected LiChrosorb RP-18 columns. One column behaved well, but separation was not achieved on the two other columns, which suffered from lack of selectivity and theoretical plates and from too high an asymmetrical factor.

It has been claimed in the literature that reversed-phase columns with different chain lengths perform more or less identically. However, when the Techogel C_4 and C_{18} columns are compared (Fig. 12) it can be seen that they separate the four monoiodoinsulins in different ways: the C_4 column is unable to resolve the B26 and A14 monoiodoinsulin, whereas A14 and B16 are eluted in the same position from the C_{18} column.

It is worth emphasizing that the three columns that were able to separate the

TABLE I

INSULIN-RELATED AND NON-INSULIN-RELATED IMPURITIES COMMONLY FOUND IN CRYSTALLINE INSULIN

Insulin-related compounds, mol.wt. ca. 5800	*Insulin-related compounds, mol.wt. 9000–12 000*	*Non-insulin related compounds*
Insulin peptide	Proinsulin	Glucagon
Monoarginine insulin	Insulin dimer	Pancreatic polypeptide
Diarginine insulin	Intermediary insulin	
Desamidoinsulins		
Ethylesterinsulins		
Des-alanine insulin		
Des-pentapeptide insulin		
Des-octapeptide insulin		

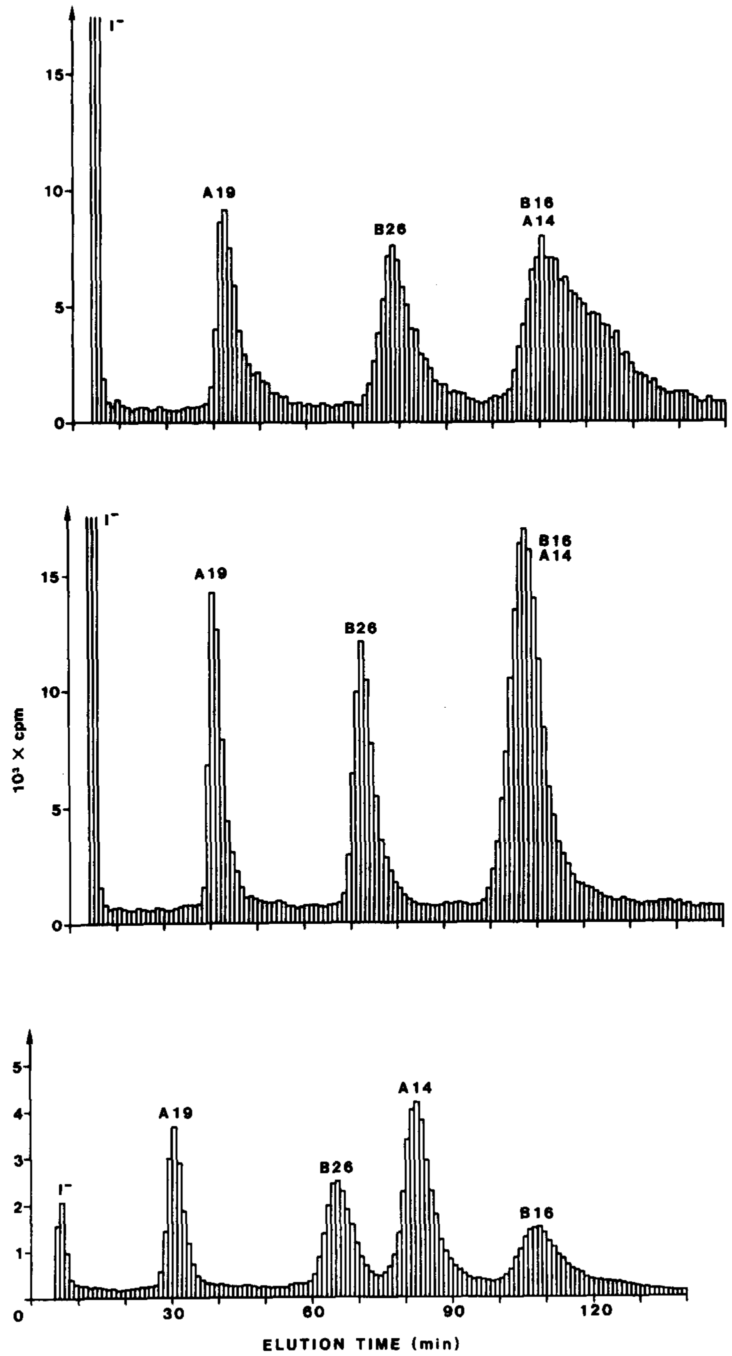

Fig. 14. Isocratic separation of 50 μl of diluted iodination mixture on three different LiChrosorb RP-18 columns (250 × 4.0 mm I.D.) eluted with 0.25 *M* TEAF (pH 6.0) containing (from top to bottom) 22.0, 21.0 and 21.0% isopropanol. Flow-rate 0.5 ml/min.

iodoinsulins were all silica-based C_{18} columns with 80–100 Å pore size. None of the wide-pore columns tested in this work could separate all four iodoinsulins, and, to the best of our knowledge, the successful use of wide-pore reversed-phase columns for complete separation of iodoinsulins has not been published. However, the three C_{18} columns found to be useful for this important separation differ with respect to other parameters, *i.e.* carbon load and end-capping, and it therefore seems premature to connect polypeptide selectivity with a single column parameter —or combination of parameters.

Although this investigation deals with only one type of buffer and one group of compounds (insulin and insulin derivatives), it may be concluded that, before a reversed-phase polypeptide separation is undertaken, some column research (bringing the actual sample into play) can be a very profitable investment.

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