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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF THE FOUR MONOIODOINSULINS: EFFECT OF COLUMN SUPPORTS, BUFFERS AND ORGANIC MODIFIERS

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SUMMARY

The separation of mono- and diiodoinsulins has been performed using various C_{18} columns (LiChrosorb and Vydac), organic modifiers (acetonitrile, 2-propanol and ethanol) and trialkylammonium buffers at various pH values. One system (LiChrosorb–2-propanol–triethylammonium formate, pH 6.0) allows complete separation between unlabelled insulin, monoiodoinsulins and diiodoinsulins. The more-or-less reduced binding affinity of the reversed-phase high-performance liquid chromatographic purified tracers is most likely caused by column bleeding.

INTRODUCTION

Insulin monoiodinated in Tyr A14, A19, B16 or B26 shows different binding kinetics towards antibodies[1] and insulin receptors[2–5], and the use of specifically labelled monoiodoinsulin tracers is therefore desirable in these studies.

The separation of the four monoiodoinsulins can be performed using disc-electrophoresis–ion-exchange chromatography, but this process is rather time-consuming (two weeks)[2]. The same separation can be performed in *ca.* 2 h using reversed-phase high-performance liquid chromatography (RP-HPLC)[6–13], but a reduced binding affinity to isolated adipocytes of the HPLC-purified tracers compared with the binding affinity of similar tracers purified and isolated by disc-electrophoresis–ion-exchange chromatography necessitates further studies before the advantages of the RP-HPLC procedure can be fully exploited[10,11].

Besides the demand for a specifically labelled tracer, it is important that monoiodoinsulin used for binding assays is free of diiodoinsulins, because these derivatives degrade to iodide and polymers during storage, leading to reduced long-term stability and because some of the di-iodoinsulins containing di-iodotyrosine derivatives display very low binding affinity to insulin receptors[14].

This paper describes the RP-HPLC separation of insulin, monoiodoinsulins,

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and diiodoinsulins using different C_{18} supports, buffers, pH values and organic modifiers, and reports the binding affinities of the specifically labelled monoiodoinsulin tracers purified in this way.

MATERIALS AND METHODS

Insulin and iodinated insulin were prepared essentially as described previously[10].

The HPLC system consisted of two Waters M6000 pumps and a Waters 660 gradient controller or a Spectra-Physics SP8700 chromatograph. The injector was U6K (Waters) or WISP 910B (Waters), and a Pye Unicam UV detector was used. The columns were LiChrosorb RP-18, 5 μm, 250 × 4 mm I.D. (Merck), and Vydac 218 TPB, 5 μm, 250 × 8 mm I.D. (slurry-packed downwards in methanol in this laboratory). Buffers used were 0.25 *M* triethylammonium phosphate (TEAP), 0.25 *M* triethylammonium formate (TEAF), and 1% triethylammonium trifluoroacetate (TEATFA), and the pH was 3.0, 4.0, 5.0 or 6.0. The eluate was collected in 1-min fractions using a FRAC-300 (Pharmacia) fraction collector, and the radioactivity in the fractions was counted in a Hydrogamma 16-channel gammacounter.

Phosphoric acid and formic acid (Merck p.a.) and trifluoroacetic acid (Fluka p.a., glass-distilled before use) were titrated with triethylamine (Janssen) to the appropriate pH. Acetonitrile and 2-propanol were obtained from Rathburn (Grade S) and 99% ethanol from De Danske Spritfabrikker.

All buffers were Millipore filtered (0.45 μm) and vacuum/ultrasound degassed before use. The separations were performed at room temperature unless otherwise stated.

The identification of the HPLC-purified tracers was based either on co-chromatography of the individually labelled monoiodoinsulins prepared by disc-electrophoresis–ion-exchange chromatography or on estimation of the iodine distribution after oxidative sulphitolysis followed by enzymatic cleavage of the isolated A- and B-chains as previously described[4,14]. The distribution of iodine as monoiodotyrosine (MIT) and diiodotyrosine (DIT) in diiodoinsulin peaks was determined after pronase digestion as previously described[14].

The separated tracers were isolated as follows:

(1) Lyophilization.

(2) Removing salt and organic modifier using SEP-PAK C_{18} (Waters) as previously described[11].

(3) Gel chromatography on Toyo Pearl HW40 in 3 *M* acetic acid–0.1% human serum albumin followed by lyophilization as previously described[11].

The binding affinity to isolated rat adipocytes was measured as previously described[2–5].

RESULTS

Fig. 1 shows the isocratic elution of the four monoiodoinsulins using the LiChrosorb column, 28% acetonitrile as organic modifier and TEAF buffer at various pH values. At this fixed concentration of acetonitrile the four monoiodoinsulins are well separated only at pH 4.0. Figs. 2 and 3 shows the separation obtained using the

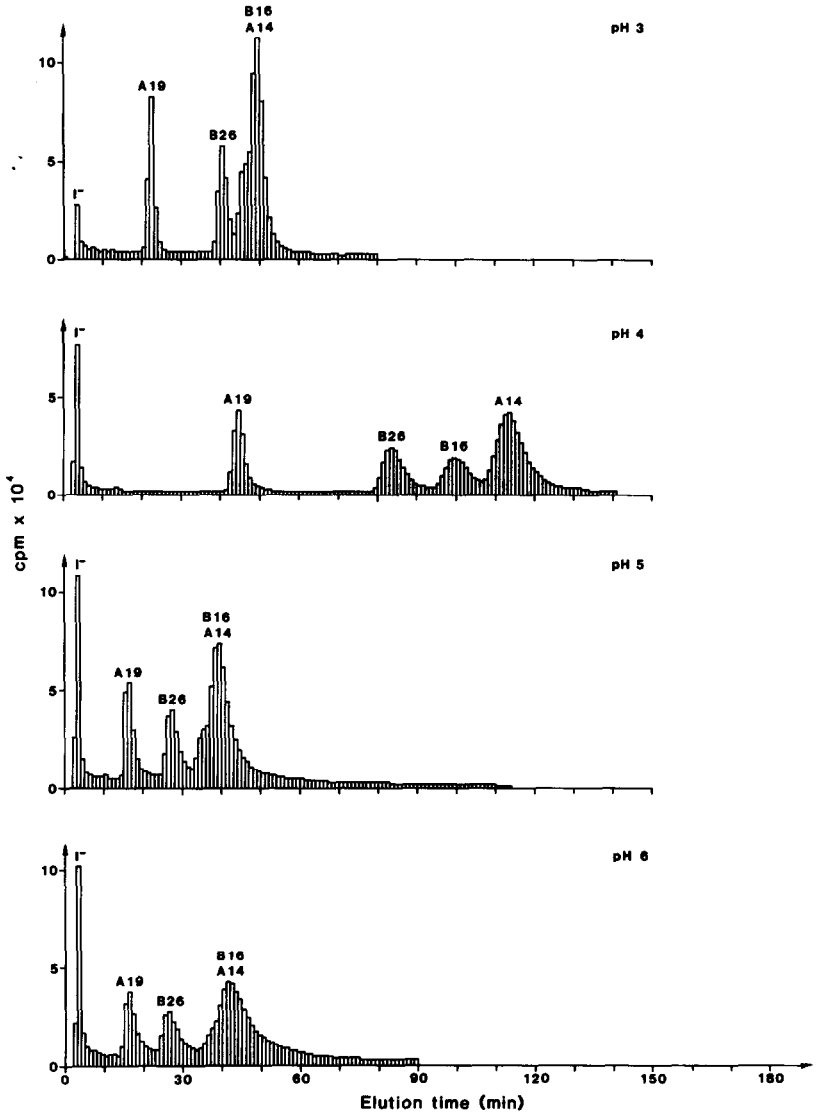

Fig. 1. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture (containing 1–5 ng of the four monoiodinated insulins and 5% diiodoinsulins in 3 *M* acetic acid) using a LiChrosorb RP-18 (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAF buffer containing 28% acetonitrile. The pH in the TEAF buffer was changed from 3 to 6 (from top to bottom) as indicated. The histograms represent the radioactivity in the collected fractions.

same column, buffer and pH values, but 2-propanol or ethanol as organic modifier.

The separation patterns in Figs. 1–3 are not the best possible separations for each organic modifier and pH. The fixed concentrations of acetonitrile, ethanol and 2-propanol are chosen such that at least one of the four pH values results in (more or less) satisfactory separation of the four monoiodoinsulins within a reasonable time.

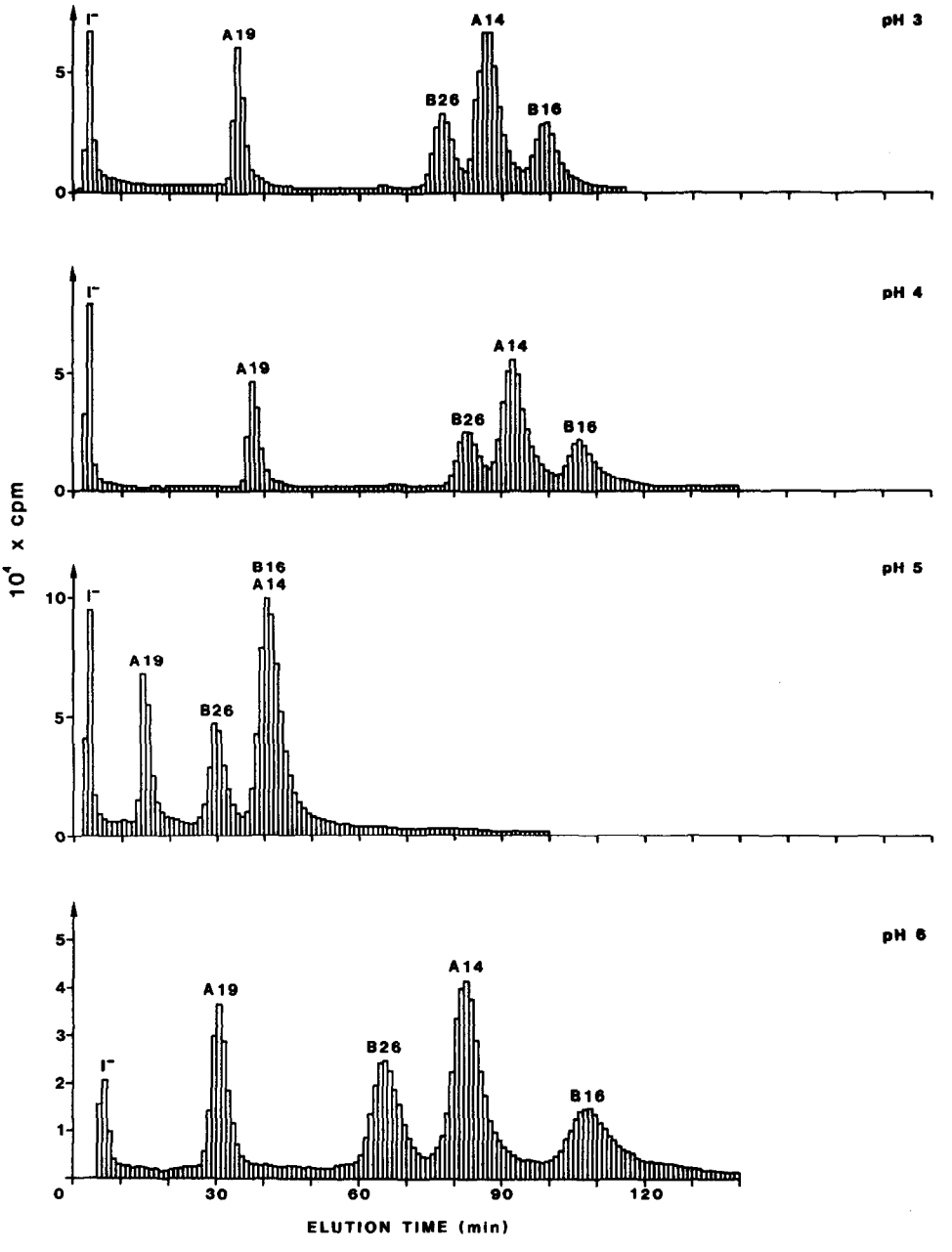

Fig. 2. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture using a LiChrosorb RP-18 (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAF buffer containing 21% 2-propanol. The pH in the TEAF buffer was changed from 3 to 6 (from top to bottom) as indicated; other details as in Fig. 1.

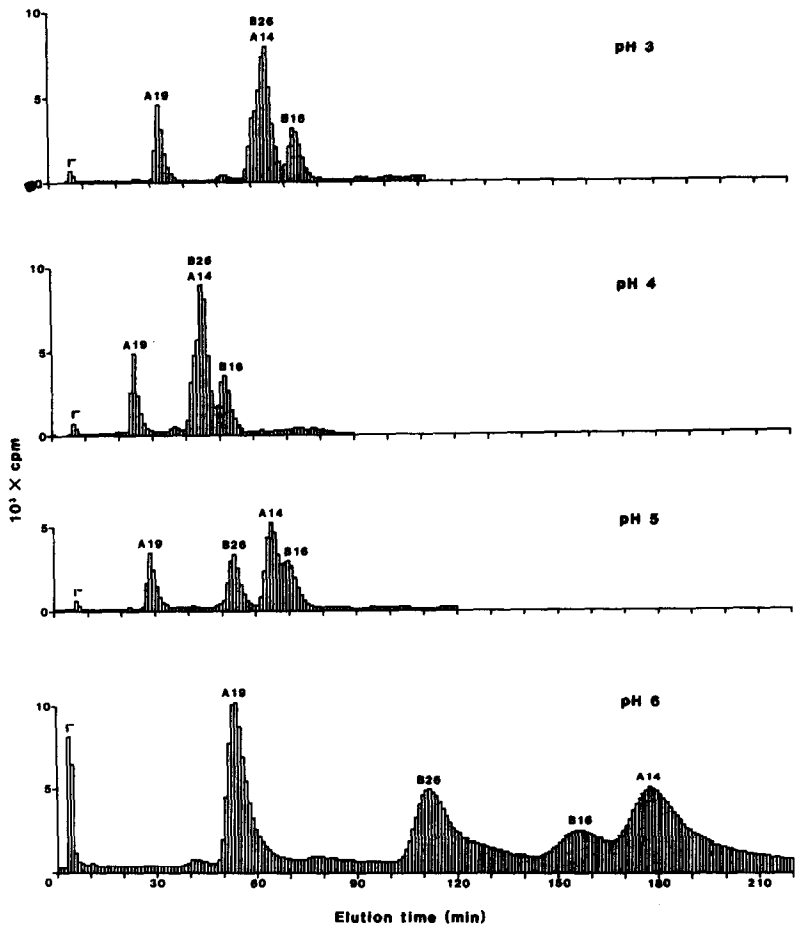

Fig. 3. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture using a LiChrosorb RP-18 (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAF buffer containing 37% ethanol. The pH in the TEAF buffer was changed from 3 to 6 (from top to bottom) as indicated; other details as in Fig. 1.

The order in which the four isomers are eluted depends on the choice of organic modifier. In acetonitrile-containing buffers the order is A19–B26–B16–A14 (Fig. 1) whereas in 2-propanol it is A19–B26–A14–B16 (Fig. 2). In these two solvents the order is the same at all four pH values, whereas in ethanol–TEAF the elution order changes when the pH value is increased from 5 to 6 (Fig. 3). Ethanol is the only organic solvent that does not lead to a satisfactory separation of the four isomers at any of the pH values used.

The best possible separations of the four monoiodoinsulins using a LiChrosorb column eluted with 0.25 *M* TEAF buffer (pH 6.0) containing acetonitrile, ethanol or 2-propanol as organic modifier are shown in Fig. 4. The same optimized separations using a Vydac reversed-phase column are shown in Fig. 5. From these two figures it can be seen that the selectivity towards the iodinated insulin derivatives differs as follows: Vydac–2-propanol will never lead to any reasonable separation between A14

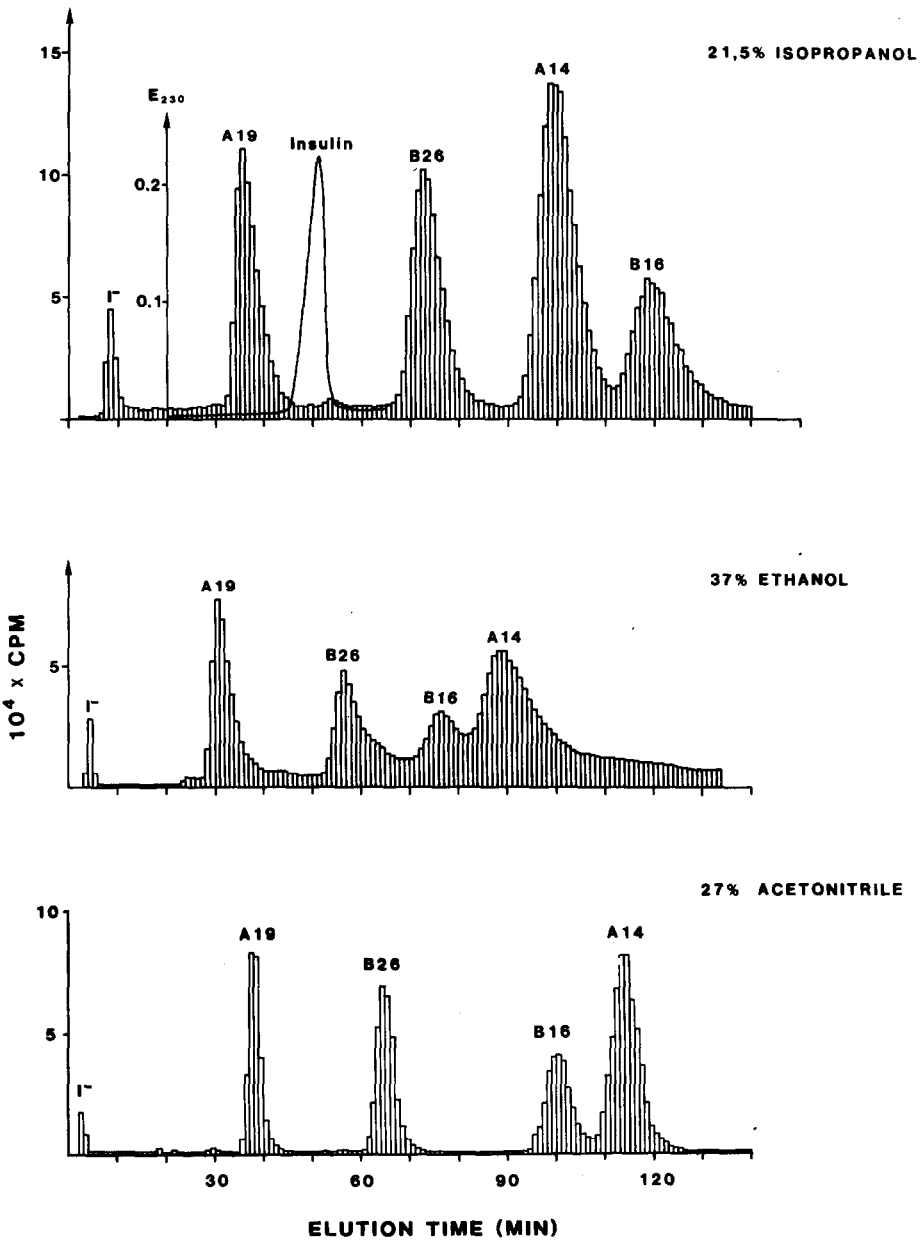

Fig. 4. Isocratic RP-HPLC separation of 50 μl diluted iodination mixture using a LiChrosorb RP-18 (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAF buffer, pH 6.0, containing (from top to bottom) 21.5% 2-propanol, 37% ethanol and 27% acetonitrile, respectively. In the top panel, 200 μg of insulin were added to the iodination mixture, and the solid line represents the continuously measured absorbance at 230 nm. Other details as in Fig. 1.

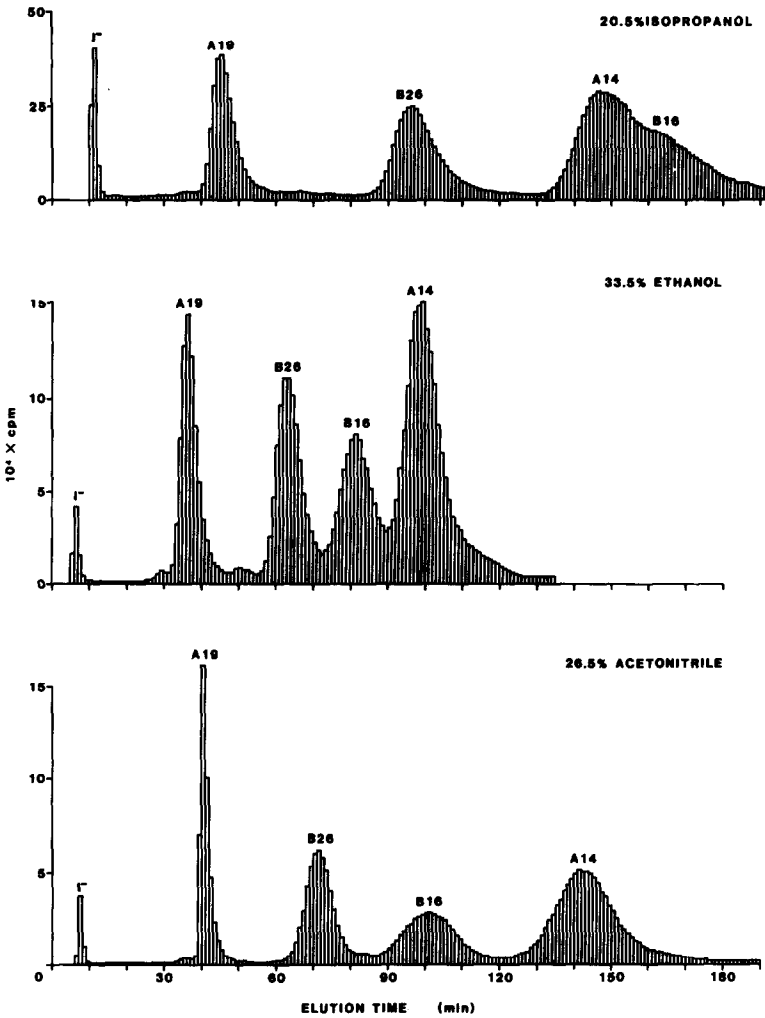

Fig. 5. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture using a Vydac 218 TPB (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAF buffer, pH 6.0, containing (from top to bottom) 20.5% 2-propanol, 33.5% ethanol and 26.5% acetonitrile, respectively; other details as in Fig. 1.

and B16 whereas the separation using LiChrosorb–ethanol, although better than that obtained using Vydac–2-propanol, is the least satisfactory of the three organic modifiers. In other contexts the two columns are comparable: the concentrations of organic modifier needed for a satisfactory separation using the two columns are remarkably similar, at least in the case of acetonitrile and 2-propanol, and the elution order (A19–B26–B16–A14 for acetonitrile and ethanol, A19–B26–A14–B16 for 2-propanol) is identical for the two columns.

It was also possible to obtain a satisfactory separation on the Vydac column eluted with TEAP or TEAF buffer and acetonitrile (pH 4.0, 5.0 and 6.0), as shown in Figs. 6 and 7.

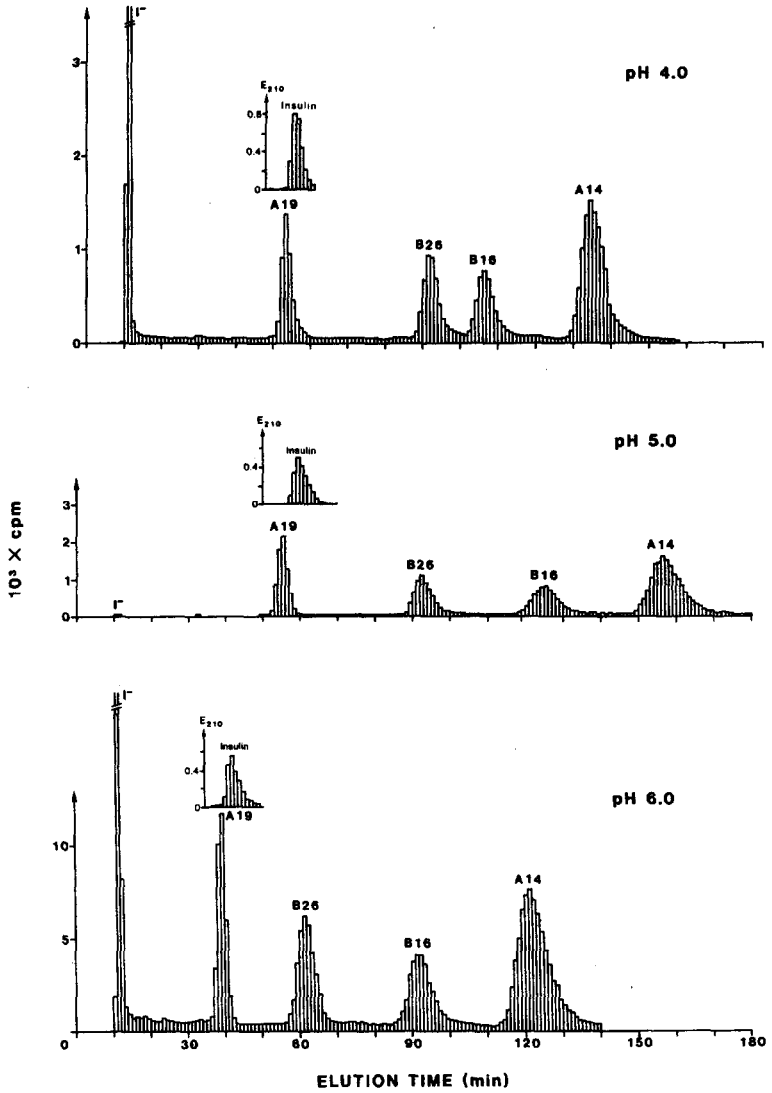

Fig. 6. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture plus 100 μg of insulin using a Vydac 218 TPB (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAP buffer containing (from top to bottom) 25.5%, 27% and 26.5% acetonitrile, respectively. The pH in the TEAP buffer was (from top to bottom) adjusted to 4.0, 5.0 and 6.0. The insets show the position of the unsubstituted insulin measured in the fractions at 210 nm; other details as in Fig. 1.

The elution position of insulin was always found between that of A19 and B26, normally close to A19. Under some conditions unlabelled insulin elutes together with A19[11]. However, with the combination LiChrosorb–TEAF–2-propanol at pH 6.0, insulin is well separated from A19 monoiodoinsulin (Fig. 4, top panel). If 200 μg of unlabelled insulin are added to the iodination mixture (containing *ca.* 5 ng of iodoinsulin), separation under the conditions described in Fig. 4 (top panel) allows

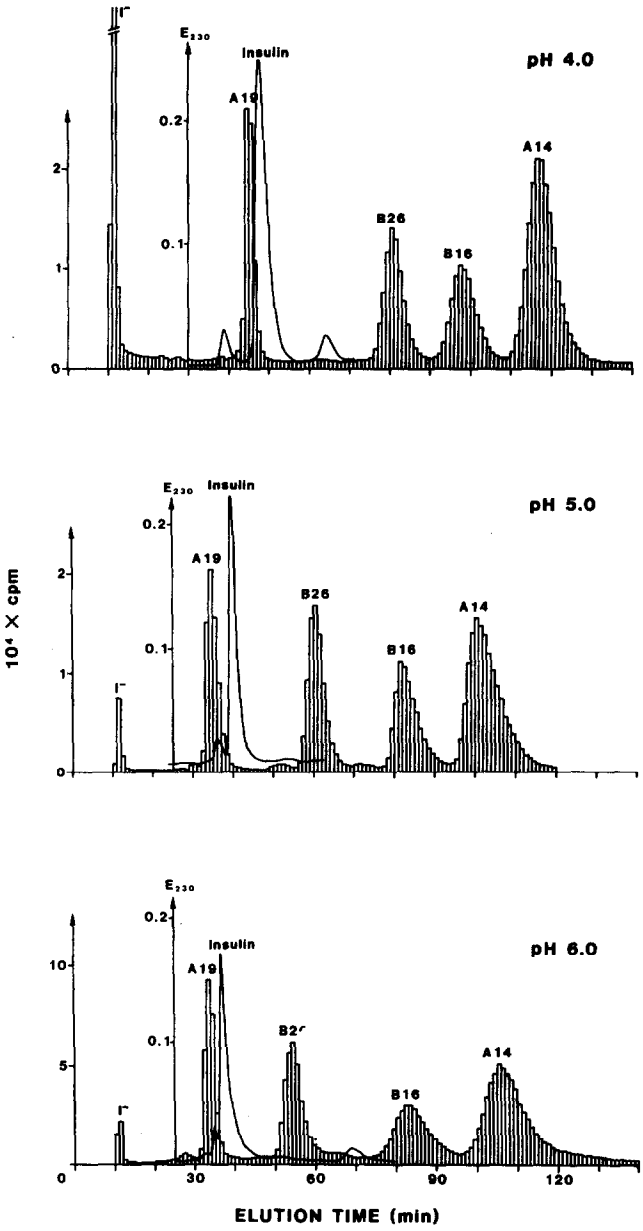

Fig. 7. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture plus 100 μg of insulin using a Vydac 218 TPB (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAF buffer containing (from top to bottom) 27.5%, 27% and 28.5% acetonitrile, respectively. The pH in the TEAF buffer was as indicated. The unbroken line represents the unsubstituted insulin measured continuously at 230 nm; other details as in Fig. 1.

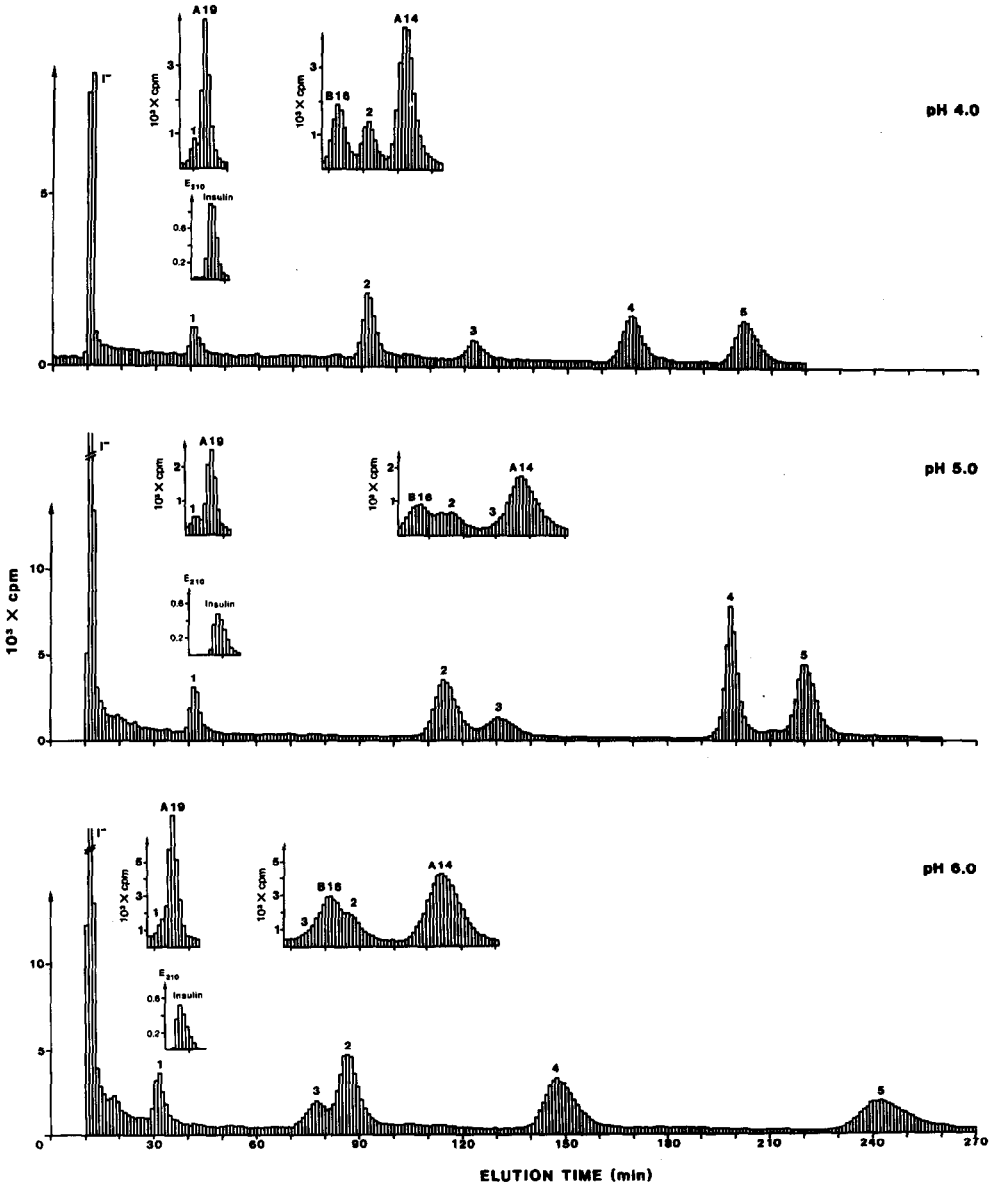

Fig. 8. Isocratic RP-HPLC separation of 50 μl of diiodoinsulin tracers plus insulin (100 μg) using a Vydac 218 TPB (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAP buffer and acetonitrile as organic modifier. The pH and acetonitrile concentration were (from top to bottom): pH 4.0, 26% acetonitrile; pH 5,0, 27% acetonitrile; pH 6.0, 27% acetonitrile. The insets show the separation between components 1, 2 and 3 in the diiodoinsulin mixture with elution volumes close to A19, B16 and A14-monoiodoinsulin. The amount of diiodoinsulins in the mixture applied is *ca.* 10 times larger than normally found in the iodination mixture. The relative amounts of each component is: 8% peak 1; 25% peak 2; 10% peak 3; 29% peak 4; 28% peak 5. Other details as in Fig. 1.

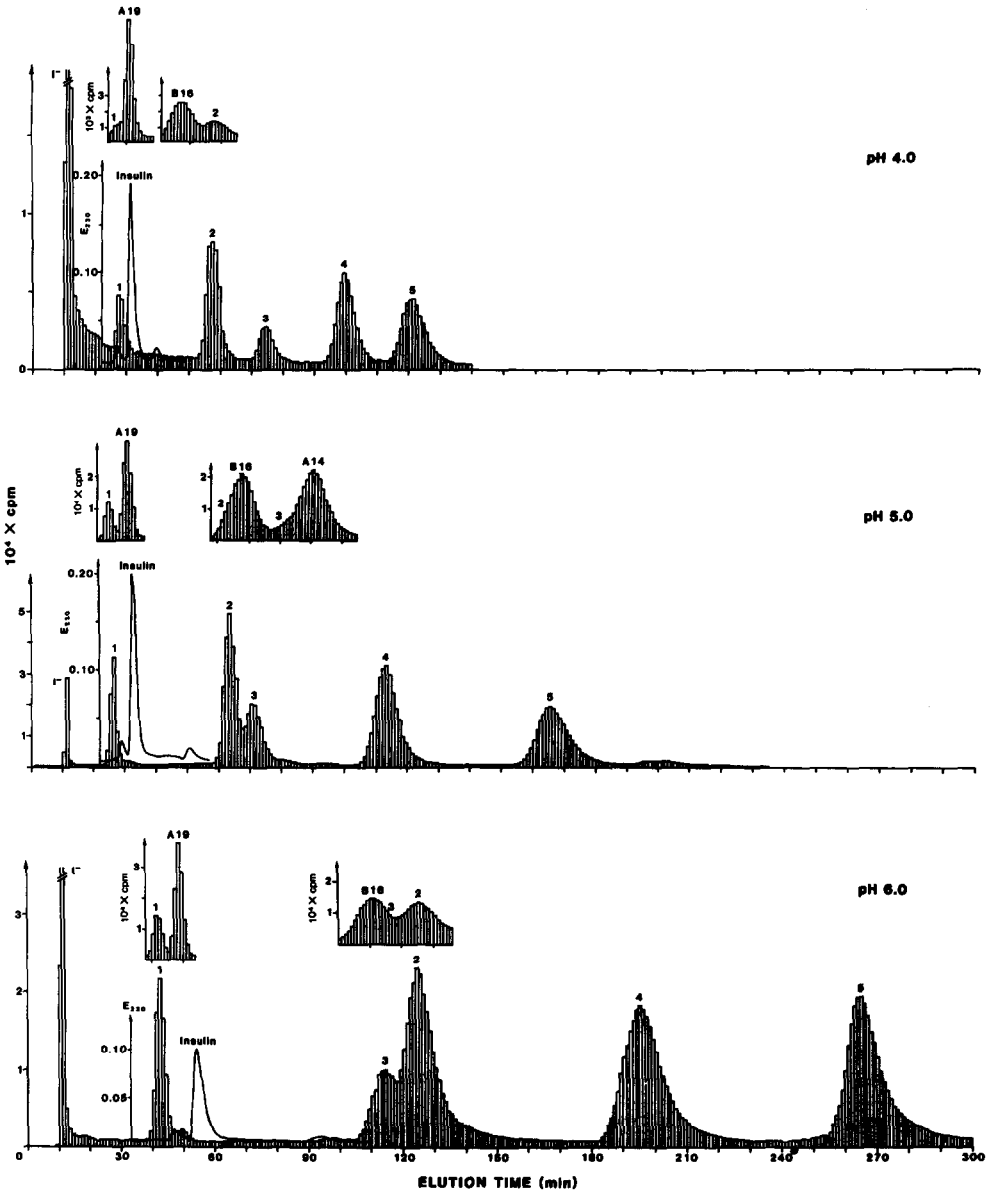

Fig. 9. Isocratic RP-HPLC separation of 50 μl of diiodoinsulin tracers plus insulin (100 μg) using a Vydac 218 TPB (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAF buffer and acetonitrile as organic modifier. The pH and acetonitrile concentration were (from top to bottom): pH 4.0, 27.5% acetonitrile; pH 5.0, 27% acetonitrile; pH 6.0, 28.5% acetonitrile. Other details as in Figs. 1 and 10.

direct isolation of A19 monoiodoinsulin without any detectable contamination by insulin.

The separation of the four monoiodoinsulins is very sensitive to temperature changes. With the LiChrosorb–TEAF–2-propanol system at pH 4.0, all four mono-

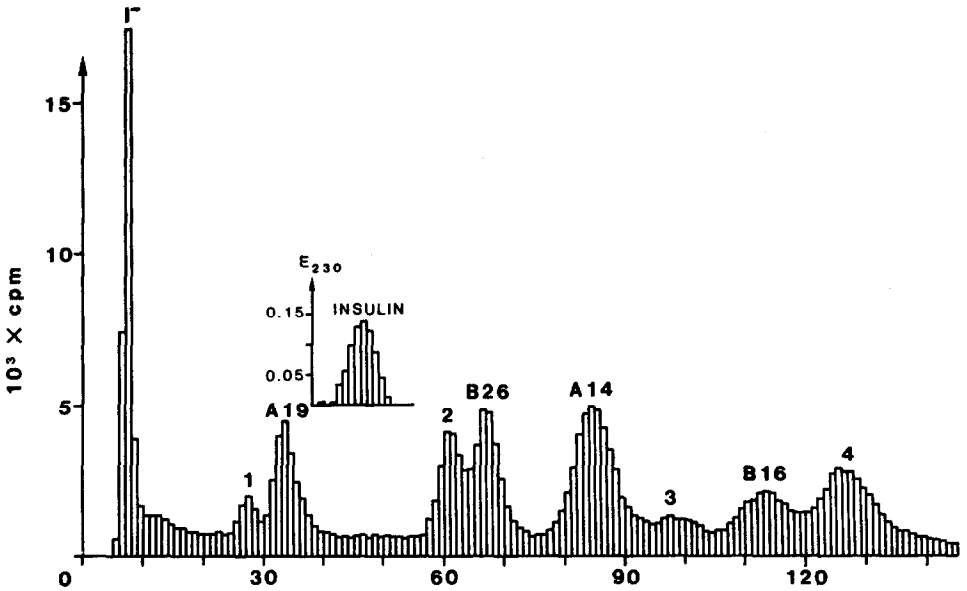

Fig. 10. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture plus insulin (100 μg) and diiodoinsulins (10 times the amount normally found in the iodination mixture) using a LiChrosorb RP-18 (5 μm) column eluted at 0.5 ml/min with 0.25 *M* TEAF, pH 6.0, containing 22.5% 2-propanol. Diiodoinsulin peak 5 (not shown) was eluted later than 200 min. Other details as in Fig. 1.

iodoinsulins were eluted within 120 min at 28°C, whereas only A19 was eluted within 140 min when the temperature was 19°C (data not shown).

Figs. 8–10 show the elution of diiodinated insulin derivatives. Five components (peaks 1–5) are generally obtained, and peaks 1–3 may interfere with A19, B26 and/or B16. The system described in Fig. 10 is the only system without interference between mono- and diiodoinsulins. A reversed elution of the di-iodoinsulin peaks 2 and 3 was

TABLE I

BINDING AFFINITY OF THE HPLC-PURIFIED TRACERS (RELATIVE TO A14 DISC)

Column	*Buffer*	*pH*	*Organic modifier*	*Isolation procedure*	*A14*	*A19*	*B16*	*B26*
Spherisorb	TEATFA	3.0	Acetonitrile	Lyophilization	57	43	53	130
Spherisorb	TEATFA•	3.0	Acetonitrile	Sep-Pak	52	34	56	100
LiChrosorb	TEAP	4.0	Acetonitrile	Sep-Pak	91	46	103	177
LiChrosorb	TEAP	4.0	Acetonitrile	Gel chromatography	96	34	119	188
LiChrosorb	TEAP	3.0	Acetonitrile	Sep-Pak	61	33	92	145
Vydac	TEAP	3.0	Acetonitrile	Sep-Pak	70	34	95	144
Vydac	TEAP	3.0	Acetonitrile	Gel chromatography	96	44	92	192
LiChrosorb	TEAF	6.0	2-Propanol	Lyophilization	90	57	93	178
LiChrosorb	TEAF	6.0	Ethanol	Lyophilization	57	28	64	94
Vydac	TEAF	6.0	2-Propanol	Lyophilization	58	43	74	120
Vydac	TEAF	6.0	Ethanol	Lyophilization	41	24	61	77
Reference tracers purified by disc-electrophoresis–ion-exchange chromatography					100	57	114	183

TABLE II

RELATIVE BINDING AFFINITIES (%) OF THE REFERENCE TRACERS IN PRESENCE OF THE REMAINS LEFT AFTER LYOPHILIZATION OF 2.5 ml OF RP-HPLC COLUMN ELUATE

	A14	*A19*	*B16*	*B26*
Reference tracers	100	100	100	100
Reference tracers plus lyophilization residue	67	74	85	75*
	58	71	78	79**

* 0.25 *M* TEAF, pH 5.0, 27% acetonitrile, eluted from a Vydac 218 TPB column.
** 0.25 *M* TEAF, pH 3.0, 36% ethanol, eluted from a LiChrosorb RP-18 column.

found when the pH was changed from 5.0 to 6.0 in TEAP as well as in TEAF (Figs. 8 and 9), but not in TEATFA buffers (data not shown).

The relative binding affinities of the four monoiodoinsulins after various chromatographic conditions and isolation procedures are given in Table I. Most of the systems revealed tracers with reduced binding affinity.

To investigate the possible effect on the binding affinity assay of the RP-HPLC column eluate, 2.5 ml of eluate from each of the two columns investigated were lyophilized and added to each of the four monoiodoinsulin derivatives (prepared by disc-electrophoresis–ion-exchange chromatography) before the binding assay. This reduced the relative binding affinities of all the isomers by 20–40% (Table II).

DISCUSSION

We have recently described a number of RP-HPLC separations of insulin and the four monoiodinated insulin derivatives[9–11]. The binding affinity to isolated adipocytes of the HPLC-purified tracers was found to be more-or-less reduced compared with similar tracers purified by disc-electrophoresis–ion-exchange chromatography[10,11]. In order to perform the RP-HPLC separations using milder conditions, the separation has been performed at pH values near neutral and acetonitrile has been exchanged with ethanol or 2-propanol. The triethylammonium phosphate buffer and the triethylammonium trifluoroacetate buffer have been replaced by the lyophilizable triethylammonium formate buffer in order to avoid further purification steps after the HPLC separation.

As can be seen from Figs. 1–3, the separation in each of the three organic solvents is influenced by the pH of the buffer, each pH value requiring a particular concentration of organic modifier for the best separation. The reasons for these very different separations are probably the gradual neutralization of the triethylammonium ions as well as the silanol groups as the pH is increased, together with the different organic modifiers (varying degree of polarity, different hydrophobic parts).

The data in Figs. 1–3 indicate that the best separations at each pH value would be quite different, and Figs. 4 and 5 show that this is so at pH 6.0, not only for the LiChrosorb RP-18 column, but also for the Vydac C_{18} column. The LiChrosorb column separates satisfactorily with acetonitrile and 2-propanol, less so with ethanol. In contrast, it is not possible to perform a reasonable separation on the Vydac column using 2-propanol as organic modifier, whereas ethanol and acetonitrile perform well.

The fact that the separation can be performed perfectly on both columns using acetonitrile as organic modifier indicates why this solvent has found such widespread use in RP-HPLC.

In other respects the two C_{18} columns behave very similar. They both show a reversed elution order of A14 and B16 when 2-propanol is substituted for acetonitrile or ethanol, and the concentrations of organic modifier needed for the optimal separations are very alike for the individual columns: acetonitrile, 27.0% and 26.5%; 2-propanol, 21.5% and 20.5%; ethanol, 33.5% and 37.0% (for LiChrosorb and Vydac, respectively).

From these figures it is also seen that the often-described differences in ability to break the binding between the C_{18} phase and the hydrophobic parts of the protein/peptide molecule (2-propanol > acetonitrile > ethanol) is valid in the reversed-phase separation of insulin. The two 5-μm column supports differ in pore size (Vydac 330 Å; LiChrosorb 60–80 Å), and it has been claimed that large-pore silica should be advantageous for reversed-phase separations of proteins[15]. In the separations described here no advantages were noticed for the large-pore material, which even behaved less well than the 60–80 Å support when 2-propanol was used as organic modifier. It can therefore be concluded that conditions other than pore size will govern the separation of a polypeptide of MW 6000, primarily the chemistry behind the bonding of the C_{18} phase and the end-capping.

The separation between unlabelled insulin and insulin monoiodinated in A19 has caused some troubles in other published RP-HPLC separations of the iodinated insulins[6,12,13]. In order to prepare an A19 tracer free of unlabelled insulin, ion-exchange chromatography of the A19-insulin mixture obtained after RP-HPLC has been used[12]. We have previously shown that this separation is improved when the pH is raised from 3 to 6 in the LiChrosorb–TEAP–acetonitrile system[11]. The same improvement was *not* found in the Vydac acetonitrile–TEAP (or TEAF) system (Figs. 6 and 7).

The system described in Fig. 4 (top panel) allows complete separation between insulin and A19 monoiodoinsulin even when the column is loaded with a sample containing 40,000 times more unlabelled than labelled insulin. It is essential to avoid contamination of the A19 tracer with unlabelled insulin in order to obtain reliable results in bioassays that attempt to detect possible insulin contamination. Another detection method is analytical RP-HPLC, which in our laboratory (LiChrosorb, 0.25 *M* TEAP, pH 3.0, acetonitrile) has a detection limit (dependent on the choice of UV detector) of between 1 and 10 ng[16].

The principal disadvantages of using 2-propanol as organic modifier are the sensitivity to temperature changes and the high back-pressure over the column, which, when the temperature is lowered, often exceeds the limit for most HPLC equipment. However, we have performed satisfactory separations with flow-rates down to 0.2 ml/min.

A reversed elution order of B16 and A14 monoiodoinsulin has been described for the LiChrosorb–triethylammonium phosphate–acetonitrile system at pH 4.0 when the separation temperature was lowered from 22°C to 0°C[11]. No reversed elution order has been observed in the LiChrosorb–TEAP–2-propanol system when the temperature was lowered.

It is important to avoid contamination of the monoiodinated insulin tracers

with diiodoinsulin derivatives, because some of these derivatives show low binding affinity (especially the A19 diiodotyrosine derivative) and degrade to iodide and high molecular weight compounds[14]. The diiodoinsulins can be isolated from the iodination mixture using disc-electrophoresis[14], and RP-HPLC dissolves the isolated diiodoinsulins into five fractions, which have been characterized with respect to position of the iodines in the tyrosyl group in the insulin molecule[11]. As for the monoiodoinsulins, the elution order is influenced by choice of pH and buffer, and separation between all monoiodoinsulins and diiodoinsulin derivatives can be obtained only using LiChrosorb–TEAF–2-isopropanol (pH 6.0) (Fig. 10). However, concerning the total purification scheme, the formation of diiodoinsulins should be minimized in the iodination process, and the lactoperoxidase iodination method allows monoiodination with *ca.* 5% diiodoinsulins (0.4% peak 1, 1.3% peak 2, 0.5% peak 3, 1.4% peak 4 and 1.4% peak 5 material; see the legend to Fig. 10). Diiodoinsulin is not detectable in any of the monoiodoinsulin tracers prepared as described in Fig. 4 (top panel). We have previously described an RP-HPLC system allowing satisfactory separation between mono- and diiodoinsulins[10] (Spherisorb–TEATFA–acetonitrile, pH 3.0), but the separation between insulin and A19 monoiodoinsulin was less satisfactory than that described in Fig. 4 (top panel).

With LiChrosorb–TEATFA–acetonitrile the separation between B16 and A14 was not satisfactory within a reasonable time at pH 4.0, 5.0 or 6.0, nor was the separation between A19 and insulin (data not shown). Furthermore, at pH 5.0 and 6.0, insulin (100 μg applied) tends to precipitate at the top of the column, leading to non-ideal peak-shape.

Table I shows that the binding affinities of the RP-HPLC purified tracers are more-or-less reduced compared with similar tracers purified and isolated using disc-electrophoresis–ion-exchange chromatography. However, the ratio of the binding affinities of the HPLC-purified tracers is the same as that of similar tracers purified by disc-electrophoresis–ion-exchange chromatography. The reason for this reduced binding affinity can be related to several steps in the HPLC procedure: the buffer, the organic solvent, the HPLC column and the isolation procedure. Because no general effect was obtained on the binding affinity when acetonitrile at acidic pH was exchanged with 2-propanol or ethanol at neutral pH, and because insulin is known to be stable to prolonged exposure to a number of organic solvents, including ethanol, solvent-induced denaturation is unlikely to be responsible for the reduced binding affinities. Because addition of lyophilized column eluate reduced the binding affinity of monoiodoinsulins purified by disc-electrophoresis–ion-exchange chromatography (Table II), column bleeding, *i.e.* dissolution of the silica C_{18} matrix, could be responsible for the reduced binding affinities.

RP-HPLC purified tracers with reduced binding affinities have been subjected to gel chromatography (Sephadex G-50 SF, 1 *M* acetic acid–0.1% human serum albumin or 0.1 *M* ammonium hydrogen carbonate–0.1% human serum albumin, pH 8.0), but the effect on the binding affinity was neither convincing nor reproducible (data not shown). Column bleeding from HPLC columns has not been reported in the HPLC literature, probably because the majority of HPLC separations are performed from an analytical point of view. However, it seems reasonable to relate column bleeding to the basic material (silica) and the binding/end-capping chemistry, *i.e.* the column manufacturer, perhaps even batch-to-batch variations, age and state

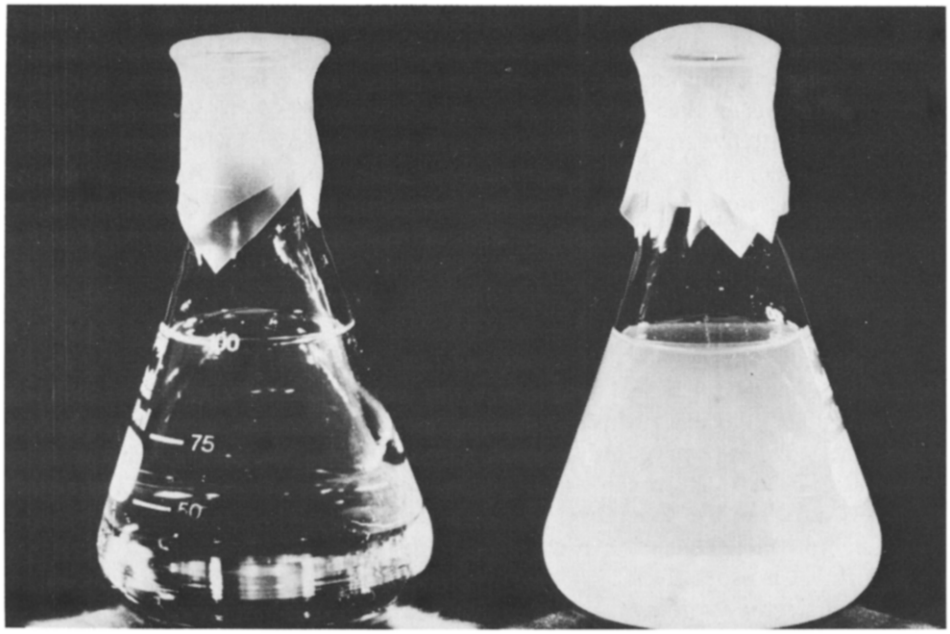

Fig. 11. A Waters Prep-Pak-C_{18} was equilibrated with 100% ethanol. After 48 h the storage ethanol was pumped out of the cartridge and mixed with one volume of distilled water (right). The conical flask to the left contains absolute ethanol–water (50:50).

of the column, the buffer, pH, ion strength and organic modifier. In the case of the LiChrosorb RP-18 column, the buffer (pH, ion strength) and the choice of modifier are known to determine the degree of column bleeding as well as the breakdown points in the chromatographic support[17].

This unpredictable effect could explain the fluctuating binding affinities of the RP-HPLC purified and isolated monoiodinated insulin derivatives.

Column bleeding is a phenomenon which must be considered in preparative HPLC separations. Fig. 11 shows the eluate resulting from a 48 h storage of a Waters preparative C_{18} cartridge in ethanol after dilution with one volume of water. The detection of bleeding products can at present probably only be performed using mass spectrometry or nuclear magnetic resonance spectroscopy.

In order to overcome the effects of column bleeding, a study of the subversion of the HPLC support in relation to the solvents used, as well as a characterization of the binding between the sample and the breakdown products, is necessary. Such studies are in progress in our laboratory, in relation to insulin and the RP-HPLC systems described here.

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