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BINDING AFFINITY OF MONOIODINATED INSULIN TRACERS ISOLATED AFTER REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Insulin and insulin monoiodinated in tyrosine A14, A19, B16 and B26 can be separated using reversed-phase high-performance liquid chromatography on a number of C_{18} columns eluted with acetonitrile containing triethylammonium phosphate or acetate buffers. The monoiodoinsulins can be isolated using lyophilization, gel chromatography, or Sep-Pak purification. Compared with similar tracers purified and isolated by disc electrophoresis–ion-exchange chromatography, the resulting binding affinities to adipocytes of the purified tracers are more or less reduced dependent on the choice of column support, buffer, separation temperature, and isolation procedure.

INTRODUCTION

Iodination of polypeptide hormones generally yields a heterogenous mixture containing compounds that are non-labelled as well as those that are mono- and disubstituted in different tyrosine residues. In order to obtain a tracer in which the degree of iodination and the position of iodine substitution are accurately known it is necessary to fractionate the iodination mixture. Recent reports have described the use of reversed-phase high-performance liquid chromatography (RP-HPLC) for separating the iodination mixture of insulin[1–5], glucagon[1,6,7], cholecystokinin peptides[8], β-melanotropin[9] and several other peptide hormones[10,11]. In general RP-HPLC seems to be a very efficient method for separating the unlabelled peptide, labelled peptides with various degrees of substitution and position of the label, and eventually oxidized peptides. However, when the binding affinity to adipocytes of RP-HPLC purified monoiodinated insulin tracers was compared with that of similar tracers purified by disc electrophoresis–ion-exchange chromatography, the affinities of the RP-HPLC purified tracers was found to be reduced compared with the tracers purified by low-pressure methods[5]. The present report describes the RP-HPLC separa-

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tion of iodinated insulins and the binding affinity of the isolated individual monoiodinated insulin tracers obtained after changes of the chromatographic conditions (column support, buffer, organc modifier, temperature) and the isolation procedure.

MATERIALS AND METHODS

Insulin

Porcine insulin and iodinated porcine insulin were prepared as previously described[5].

Buffers

Phosphoric acid (0.25 *M*, Merck p.a.) and 1% trifluoroacetic acid (TFA) (Fluka, glass distilled before use) were titrated to the appropriate pH value with triethylamine (Janssen). Acetonitrile (Rathburn, grade S) was used as organic modifier. All other chemicals were of analytical grade. Water was drawn from a Millipore Milli-Q plant and the buffers were Millipore-filtered (0.45 μm) and vacuum/ultrasound degassed before use. The acetonitrile volumes were weighed out, assuming a density of 0.786 g/ml.

HPLC

Waters M45 or M6000 pump, U6K injector or WISP 910B sample processor, Waters Data Module and Pye Unicam UV-photometer were used. Fractions were collected at 1-min intervals in a Pharmacia FRAC 300 fraction collector, and the radioactivity was measured in a Hydrogamma 16-channel counter. Values of $E^{1\,cm}_{230}$ in the collected fractions were measured in a Uvikon 810 spectrophotometer (Kontron).

Columns

Lichrosorb RP-18, 5 μm, 250 × 4 mm I.D., Spherisorb ODS-2, 3 μm, 150 + 100 × 4 mm I.D. (precolumn 40 × 4 mm I.D. packed with the same material) and Vydac 218 TPB5, 5 μm, 250 × 4.6 mm I.D., were the columns used.

Methods

All samples were dissolved in 0.01 *M* hydrochloric acid to a final iodoinsulin concentration of 0.02–0.1 μg/ml. Some of the samples further contained unlabelled insulin (2 mg/ml). The injection volume was 50 μl.

Acetonitrile and salt were removed from the collected fractions by gel chromatography at 4°C using a 50 × 1 cm I.D. column packed with ToyoPearl HW 55 Superfine (Toyo Soda) in 3 *M* acetic acid–0.1% human serum albumin (HSA, Behringwerke), flow-rate 10 ml/h. Fractions were collected at 5-min intervals in a LKB UltroRac fraction collector, and the radioactivity was measured in a Searle gamma counter.

Alternatively, salt and organic modifier were removed using Sep-Pak C_{18} (Waters). The cartridge was flushed with 10 ml of methanol–water (90:10) followed by 10 ml of water. The pooled fractions were diluted with 1 volume of water and loaded on the Sep-Pak. [^{125}I]Insulin was eluted with methanol–water (90:10), and 90% of the radioactivity was recovered in the first millilitre. The eluted tracer was diluted with 10 ml of water and lyophilized.

Disc electrophoresis and measurement of binding affinities to isolated adipocytes were performed as previously described[12–14]. All binding affinity values are given relative to the binding affinity of A14 monoiodoinsulin isolated by disc electrophoresis (A14 disc). Identification of the individual monoiodoinsulin derivatives is based on estimation of the iodine distribution after oxidative sulphitolysis followed by enzymatic cleavage of the isolated A- and B-chains as previously described[12,15]. The distribution of iodine as monoiodotyrosine (MIT) and diiodotyrosine (DIT) in diiodoinsulin peaks was determined after pronase digestion as previously described[15].

RESULTS

Fig. 1 shows the RP-HPLC separation of the four monoiodinated insulin tracers (A14, A19, B16 and B26) obtained using triethylammonium phosphate (TEAP) buffers at pH 3.00 and 4.00. At pH 4.00 the separation between B26 and B16 is more satisfactory than that obtained at pH 3.00. The separation between A19 and unsubstituted insulin at the two pH values is unchanged. If the pH of the TEAP solution was adjusted further towards more alkaline values, no immediate progress in the separation of the monoiodinated insulin derivatives was noticed but the separation between insulin and A19 at pH 5.00 and 6.00 was markedly improved (Fig. 2).

Fig. 3 shows the separation of the four monoiodoinsulin tracers using TEAP–acetonitrile at pH 4.00 on two different column supports (Spherisorb and LiChrosorb). The two chromatograms are almost identical. A very similar fractionation pattern can be obtained using Vydac 218 TPB5 with TEAP–acetonitrile at pH 4.00 (data not shown)[16].

Fig. 4 shows the separation of the insulin tracers using LiChrosorb RP-18 with TEAP–acetonitrile (pH 4.00) at 22°C (top) and 0°C (bottom). It should be noted that the two separations are performed at different flow-rates (1.0 ml/min at 22°C and 0.6 ml/min at 0°C) owing to pressure limitations in the pump. At 22°C the normal pressure at 1.0 ml/min is 3500 p.s.i. but at 0°C the pressure at 1.0 ml/min was greater than 4500 p.s.i. Two differences between the chromatograms are noteworthy: at 0°C B16 is eluted after A14 and the unsubstituted insulin is eluted in essentially the same position as A19.

Fig. 5 shows the separation of diiodoinsulins isolated from the iodinated mixture using disc electrophoresis[5], and the position of added insulin. The positions of the A19, B16 and A14 monoiodoinsulin peaks and the diiodoinsulin peaks 1 and 2 are shown in the insets.

The peaks 1–5 mainly contain the following iodine substitutions:

(1) DIT in Tyr A19 (8%)
(2) 2 MIT in the A-chain, Tyr A14 and Tyr A19 (25%)
(3) DIT in Tyr B26 (10%)
(4) MIT in Tyr B26 and MIT in Tyr A14 (29%)
(5) MIT in Tyr B16 and MIT in Tyr A14 (28%)

The recovery of [^{125}I]insulin from LiChrosorb RP-18 using TEAP–acetonitrile (pH 4.00) is *ca.* 70% for samples containing 1–5 ng of iodinated insulins. If a sample with low specific radioactivity was analysed (containing a mixture of 1–5 ng of iodinated insulin and 100 μg of unlabelled insulin) the recovery was *ca.* 100%.

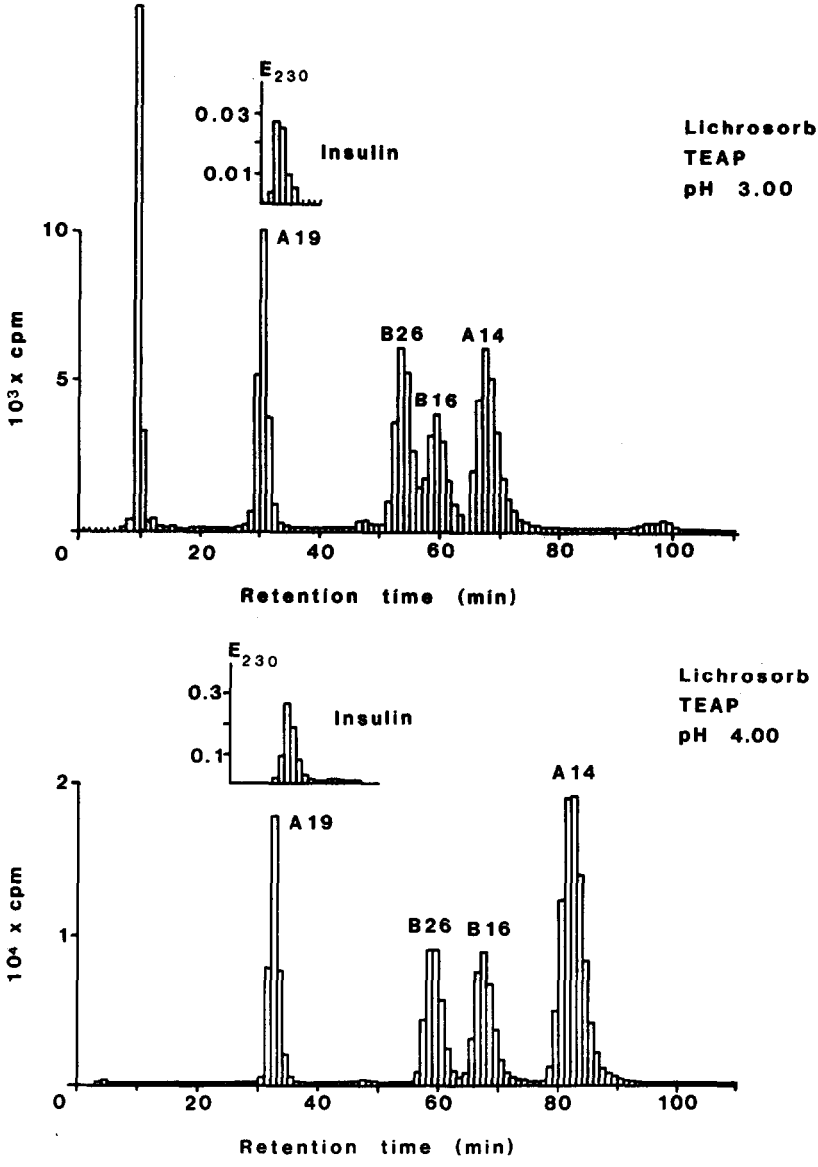

Fig. 1. Isocratic RP-HPLC separation of diluted iodination mixture (containing the four monoiodinated insulin isomers) plus added insulin at 22°C. Upper panel: LiChrosorb RP-18 (5 μm) eluted at 0.35 ml/min with 0.25 *M* TEAP, pH 3.00, containing 26.9% acetonitrile; samples, 20 μl of iodination mixture plus 10 μg of insulin. Lower panel: LiChrosorb RP-18 (5 μm) eluted at 1.0 ml/min with 0.25 *M* TEAP, pH 4.00, containing 25.5% acetonitrile; samples, 50 μl of iodination mixture plus 100 μg of insulin. The histograms represent the radioactivity in the collected fractions and the insets show the position of the unsubstituted insulin measured in the fractions at 230 nm. See Materials and methods for further details.

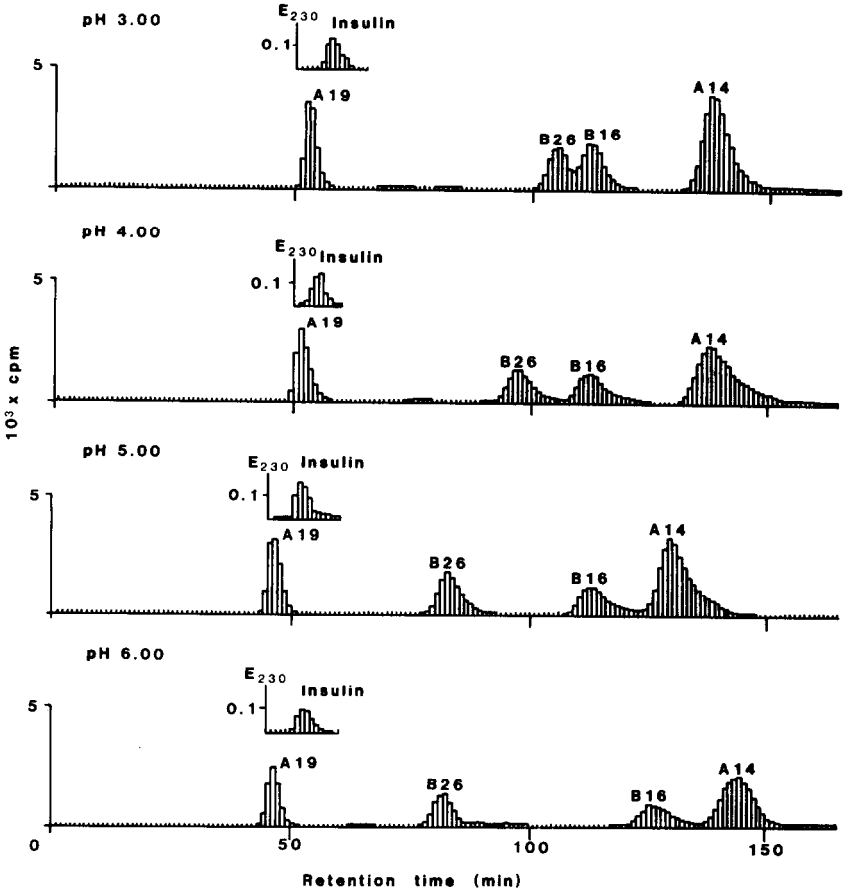

Fig. 2. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture (containing the four monoiodinated insulin isomers) plus 100 μg of added insulin using a LiChrosorb RP-18 (5 μm) column eluted at 1.0 ml/min with 0.25 *M* TEAP buffer containing 25.5% acetonitrile at 26°C. The pH in the TEAP buffer was changed from 3.00 to 6.00 from top to bottom as indicated; other details as in Fig. 1.

Table I shows the binding affinities to isolated adipocytes of the four monoiodoinsulin derivatives purified and isolated by disc electrophoresis–ion-exchange chromatography[14], and the binding affinities of A14 disc and A14 disc that has been subjected to repeated disc electrophoresis, lyophilization or Sep-Pak purification. These procedures causes no reduction in the binding affinity.

Table II shows the binding affinities of A14 disc isolated by Sep-Pak or lyophilization after incubation in various buffers and organic solvents. The binding affinity seems in most cases to be less reduced after incubation with 2-propanol than with acetonitrile.

Table III shows the measured relative binding affinities of the four monoiodoinsulins isolated by lyophilization, Sep-Pak, gel chromatography, or lyophilization–Sep-Pak after RP-HPLC separation using different buffers, column supports and temperatures. Elution on Vydac TPB5 with TEAP–acetonitrile, followed by gel

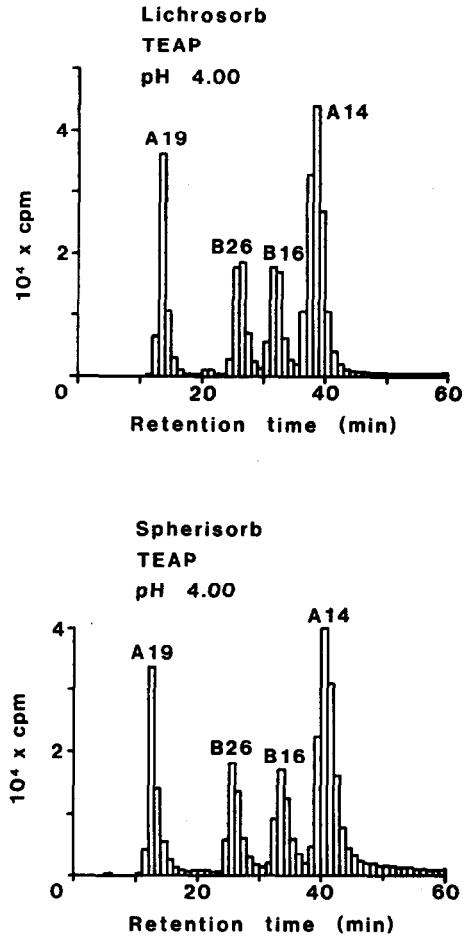

Fig. 3. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture using a LiChrosorb RP-18 (5 μm) column (upper panel) and a Spherisorb ODS-2 (3 μm) column (lower panel) eluted at 1.0 ml/min with 0.25 *M* TEAP, pH 4.00, containing 25.5% acetonitrile at 22°C; other details as in Fig. 1.

chromatographic isolation, resulted in monoiodoinsulin tracers with binding affinities almost identical with those of the reference tracers (Table I, left column).

Elution on Vydac with TEAP–acetonitrile, followed by Sep-Pak isolation, results in some reduction of the binding affinity. A further reduction results from the use of LiChrosorb–TEAP and Spherisorb–triethylammonium trifluoroacetate (TEATFA). It is noteworthy that the ratio between the binding affinities of the four HPLC-purified monoiodoinsulins is similar to the ratio between the reference tracers and is independent of the degree of reduction. The present results are only obtained when the HPLC-purified tracers are rapidly freed from acetonitrile and buffers (less than 3 h in the HPLC buffer).

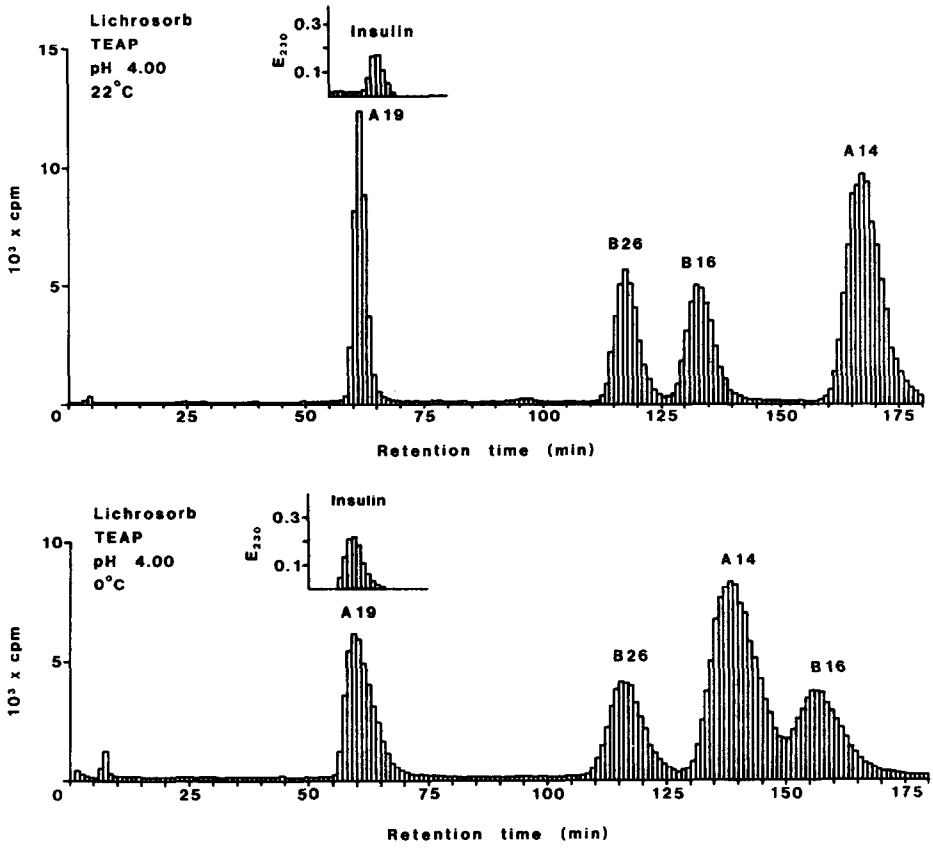

Fig. 4. Isocratic RP-HPLC separation of 50 μl of diluted iodination mixture plus added insulin (100 μg) using a LiChrosorb RP-18 (5 μm) column eluted with 0.25 *M* TEAP, pH 4.00, and acetonitrile. Upper panel: acetonitrile, 25.0%; elution rate, 1.0 ml/min; temperature, 22°C. Lower panel: acetonitrile, 24.0%; elution rate, 0.6 ml/min; temperature, 0°C. Other details as in Fig. 1.

DISCUSSION

We have recently shown that baseline separation of the four monoiodinated insulin derivatives can be obtained using Spherisorb ODS-2 eluted with TEATFA–acetonitrile, and that the binding affinity to adipocytes of the monoiodoinsulins separated and isolated by HPLC was reduced compared with similar tracers purified by low pressure methods[5]. The present study was undertaken in order to elucidate the significance of the HPLC separation parameters on the binding affinity obtained after isolation of the HPLC-separated insulin tracers.

The separation obtained between B16 and B26 monoiodoinsulin using TEAP–acetonitrile at pH 3.00[4] was markedly improved by adjusting the pH of the TEAP solution to 4.00 (Fig. 1). At higher pH values B26 and B16 move so much apart that B16 and A14 converge (Fig. 2), and a pH value of 4.00 was therefore chosen for further experiments.

These conditions result in baseline separation of the monoiodoinsulins not

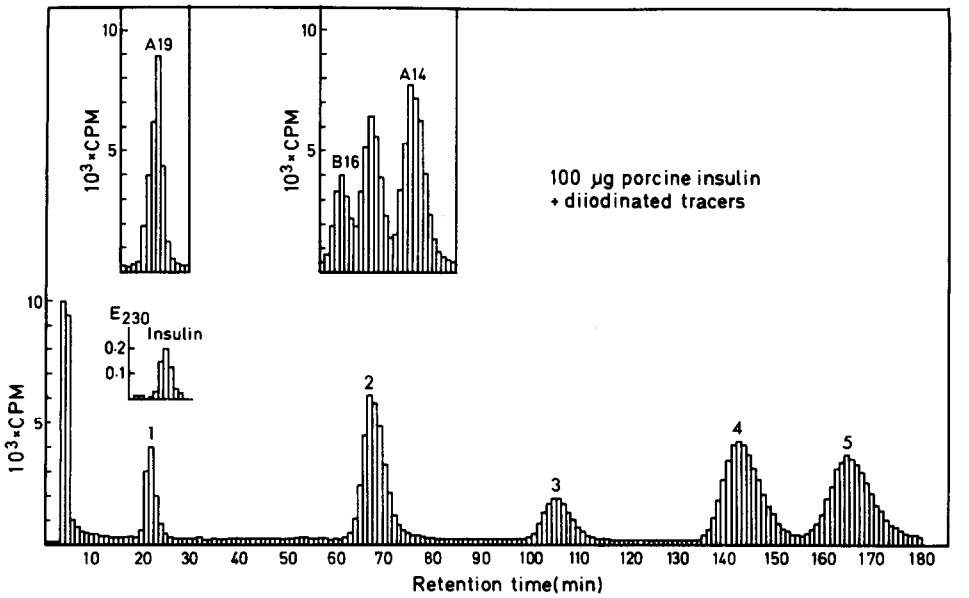

Fig. 5. Isocratic RP-HPLC separation of 50 µl of diiodoinsulin tracers plus added insulin (100 µg) using a LiChrosorb RP-18 (5 µm) column eluted at 1.0 ml/min with 0.25 *M* TEAP, pH 4.00, containing 25.5% acetonitrile at 22°C. The insets show the separation between components 1 and 2 in the diiodoinsulin mixture with retention times similar to A19, B16, and A14 monoiodoinsulin. The amount of diiodoinsulins in the mixture applied is *ca.* 15 times larger than that normally found in the iodination mixture.

TABLE I

BINDING AFFINITIES TO ISOLATED ADIPOCYTES OF REFERENCE TRACERS PURIFIED BY DISC ELECTROPHORESIS–ION-EXCHANGE CHROMATOGRAPHY (LEFT COLUMN) AND OF A14 DISC AFTER DIFFERENT PURIFICATION PROCEDURES (RIGHT COLUMN)

A14	100%	A14 Disc	100%
A19	57%	A14 Disc redisc	96%
B16	114%	A14 Disc lyophilized	100%
B26	183%	A14 Disc Sep-Pak purified	100%

only when LiChrosorb RP-18 is used as column support but also with Spherisorb ODS-2 (Fig. 3) and Vydac 218 TPB5[16]. This illustrates the efficiency with which TEAP masks the residual silanol groups in the LiChrosorb material.

Lowering the separation temperature resulted in a markedly decreased separation, primarily owing to the reversed elution order of B16 and A14 (Fig. 4). In all other experiments in which buffers, column supports or organic modifiers were varied, the order of the eluted monoiodoinsulins was always found to be A19–B26–B16–A14, and unsubstituted insulin eluted between A19 and B26. In addition to the reversed elution order of A14 and B16, the separation between unlabelled insulin and A19 monoiodoinsulin is lost at 0°C. The thermodynamic effect of the lowered tem-

TABLE II

BINDING AFFINITIES OF A14 DISC LYOPHILIZED AFTER INCUBATION IN VARIOUS BUFFERS AND ORGANIC MODIFIERS

The binding affinity of A14 disc with no further treatment is 100% (*cf.* Table I).

Buffer–modifier	*Incubation time (h)*	*Affinity (%)*
TEATFA–acetonitrile (68:32)	4	87
TEATFA–acetonitrile (68:32)	20	84
TEATFA–2-propanol (68:32)	4	87
TEAP–acetonitrile (68:32) pH 3.00	4	83
TEAP–2-propanol (68:32) pH 3.00	4	85
Ammonium bicarbonate–acetonitrile (68:32) pH 8.00	4	83
Ammonium bicarbonate–2-propanol (68:32) pH 8.00	4	89
Ammonium acetate–acetonitrile (68:32) pH 3.00	4	62
Ammonium acetate–2-propanol (68:32) pH 3.00	4	86
Ammonium acetate–acetonitrile (68:32) pH 4.00	4	82
Ammonium acetate–2-propanol (68:32) pH 4.00	4	86

TABLE III

BINDING AFFINITIES OF THE FOUR MONOIODOINSULINS PURIFIED BY RP-HPLC UNDER VARIOUS CHROMATOGRAPHIC CONDITIONS

The binding affinities presented should be compared with those obtained from the corresponding reference tracers isolated by low pressure methods (*cf.* Table I, left column).

Conditions	*Affinity (%)*			
	A14	*A19*	*B16*	*B26*
Spherisorb, TEATFA, lyophilization	50	37	54	90
Spherisorb, TEATFA, lyophilization, Sep-Pak	56	42	49	118
LiChrosorb, TEAP, 22°C, Sep-Pak	61	32	92	145
LiChrosorb, TEAP, 0°C, Sep-Pak	67	37	89	116
Vydac, TEAP, Sep-Pak	79	33	95	144
Vydac, TEAP, ToyoPearl	88	41	90	178

perature should be a shift in equilibrium between sample in the liquid phase and sample absorbed to the stationary phase towards more liquid sample, and this is reflected in the lower acetonitrile concentration needed at 0°C (even with the reduced flow-rate) to produce the retention times for the monoiodoinsulins similar to those obtained at 22°C. The change in elution order of B16 and A14 demonstrates the complex nature of the binding forces between a polypeptide and the C_{18} phase. The initial phase in a similar shift is demonstrated when the pH in the TEAP solution is raised to 5 or 6 (Fig. 2) where B16 starts to approach A14.

The separation between diiodoinsulin and monoiodinated insulins using TEAP at pH 4.00 (Fig. 5) is less satisfying with respect to the A19 tracer than the separation obtained using Spherisorb ODS-2 with TEATFA–acetonitrile[5], but it is possible to prepare A19 monoiodoinsulin with less than 1% diiodoinsulin. It is important to

avoid contamination with this diiodoinsulin derivative (A19 diiodotyrosine-insulin) because it was previously shown to be nearly devoid of binding affinity[15].

The reduced recovery obtained when 1–5 ng of iodinated insulin were applied could be raised to 100% by adding 100 μg of unlabelled insulin to the same amount of [^{125}I]insulin. It is well known that insulin in dilute solution strongly adsorbs to surfaces (glass, plastic etc.)[19] and that this adsorption can be decreased if serum albumin is added to the insulin solution. The reduced recovery of the injected 1–5 ng of iodination mixture propably reflects a non-specific adsorption to stainless steel and PTFE in the chromatographic set-up. A similar reduction in recovery has been described for ranatensin, an eleven-residue peptide. When 0.1–5 μg of peptide was loaded on a 300 × 4 mm I.D. MicroPak MCH-10 (Varian) octadecyl column the recovery dropped from 108% (5 μg) to 64% (0.1 μg)[20].

From Tables I and II it can be seen that some of the most common procedures involved in the purification process (disc electrophoresis, Sep-Pak purification, lyophilization) do not affect the binding affinity whereas incubation in TEAP, TEATFA, bicarbonate and acetate buffers containing organic solvents will cause some damage. As can be seen from Table III, the combination of buffer, organic solvent and RP-HPLC in most cases further intensifies the reduction of the binding affinity.

It should be borne in mind that estimation of binding affinity is a biological assay and that the standard deviation (S.D.) for the percentages given in Tables I–III are of the order of 10%. It could therefore be concluded that 90% binding does not necessarily reflect any reduction of the binding affinity, but values for the binding affinities of HPLC-purified tracers have always been less than 100% of the affinity of similar tracers purified by low pressure methods, therefore the postulated reduction is probably real.

The best results were obtained using the Vydac column support. This material differs from the LiChrosorb and Spherisorb materials in pore size (330 Å compared with 60–80 Å for LiChrosorb and Spherisorb) and Vydac is also known to be essentially monolayered and effectively end-capped. Which of these factors is responsible for the good performance of this material in the separation of polypeptides and proteins is unknown. It has been claimed that the principal factor governing the usefulness of any reversed-phase material for protein separations is the basic material: silica[17].

Recently the same problems were reported in RP-HPLC purification of hypothalamic brain somatostatin releasing factor (CRF), a 41-residue peptide[18]. When purified on μBondapak C_{18} or μBondapak CN eluted with ammonium acetate–acetonitrile, the peptide lost all biological activity. The reversed-phase purification with retained biological activity could be achieved in two ways using either μBondapak C_{18} (or CN) eluted with acetonitrile–trialkylammonium buffers at 0°C or by using effectively end-capped, large-pore C_{18} columns (Vydac or Perkin-Elmer) eluted with TEAP or TFA–acetonitrile. In the present study the separation at 0°C still produced some reduction of the binding affinity, whereas the use of the Vydac 330 Å column support eluted with TEAP at room temperature allowed the isolation of undamaged iodinated insulin tracers (with respect to binding affinity).

The reason for the loss of binding affinity of iodinated insulin which probably (but not necessarily) is an indicator for the biological activity is not clear. A (reversible or irreversible) conformational change in the polypeptide molecules induced by

the HPLC solvents could be the principal effect, or this conformational change could lead to altered binding of the sample molecules to the column support resulting in reduced biological activity.

Because a reduced binding affinity was observed after incubation of A14 monoiodoinsulin in HPLC buffer, and because a further reduction was found after the reversed-phase separation, it may be suggested that iodinated insulin is sentitive to HPLC buffers as well as to the actual separation.

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