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PREPARATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF IODINATED INSULIN RETAINING FULL BIOLOGICAL ACTIVITY*

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SUMMARY

Insulin monoiodinated in Tyr A14, A19, B16 and B26 can be separated from insulin and diiodoinsulins using reversed-phase high-performance liquid chromatography on LiChrosorb RP-18 columns. Monoiodoinsulins with high and low specific activities were isolated from a number of buffer systems without any reduction in binding affinity and biological activity in isolated rat fat cells. The reason for the previously observed reduction in the binding affinity was probably column bleeding, *i.e.*, chemical degradation of the column support.

INTRODUCTION

Iodination of insulin leads to a heterogeneous mixture of insulin, mono- and diiodinated insulins. Insulin monoiodinated in Tyr A14, A19, B16 or B26 can be isolated after iodination in urea-containing buffer using disc electrophoresis/ion-exchange chromatography[1,2]. It has been shown that A14 monoiodoinsulin exhibits the same binding affinity and biological activity in fat cells as native insulin, making it an ideal tracer for insulin[3].

Several groups have been studying the isolation of monoiodoinsulins, recently mostly using reversed-phase high-performance liquid chromatography (RP-HPLC)[4–12]. It is generally accepted that the A19 isomer shows a decreased binding affinity[1–4,13] and the B26 isomer an increased binding affinity in isolated rat adipocytes[2,3,7,8]. The receptor binding properties of these isomers also differ in other cell types, such as hepatocytes and cultured human IM-9 lymphocytes[1,2,5–8].

We have recently described several RP-HPLC systems for the separation of

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insulin and all four monoiodoinsulins using different C_{18} columns, organic modifiers and triethylammonium buffers at various pH values[9–12,14,15]. We observed a reduced binding affinity of these HPLC-purified monoiodoinsulin tracers to isolated rat adipocytes compared with the affinity of similar tracers isolated by disc electrophoresis/ion-exchange chromatography. Different observations of the deterioration of the biological properties of other peptides and proteins after RP-HPLC fractionation have been published in recent years[16–23], although some reports of retention of biological activity have also been published[24,25].

The present paper describes the RP-HPLC separation of all four monoiodoinsulins with maximum specific activity, as well as the preparative isolation in milligram-amounts of insulin and monoiodoinsulins with low specific activity using RP-HPLC on LiChrosorb RP-18 columns. By employing different isolation procedures it was possible to obtain monoiodoinsulins (and insulin) with retained biological properties in isolated rat adipocytes.

MATERIALS AND METHODS

Insulin

Highly purified porcine insulin was prepared as described previously[10].

Iodinated insulin with high specific activity

Insulin was iodinated in phosphate buffer containing 6 *M* urea using the lactoperoxidase method as described previously[1,26]. The average iodination degree was 0.14 I/mol of insulin. The iodination mixture (40 μl) was adjusted with 15 μl glacial acetic acid to pH about 3 prior to RP-HPLC fractionation as described below.

Iodinated insulin with low specific activity

A 150-mg amount of insulin was iodinated on a preparative scale (25 mg/ml) analogously to the method for preparing iodinated insulin with high specific activity, except that the added iodide was "cold" $Na^{127}I$ plus 1 mCi $^{125}I^-$ as tracer. The average iodination degree was 0.16 I/mol of insulin. To remove lactoperoxidase and iodide, the iodination mixture was applied to a 90 cm × 2.5 cm I.D. Sephadex G-50 column eluted with 0.1 *M* ammonium hydrogencarbonate, pH 8.0. The fractions containing the iodinated insulin were pooled and lyophilized.

RP-HPLC

The HPLC system consisted of a Spectra-Physics SP 8700 chromatograph, a U6K (Waters) injector and a Pye Unicam UV detector. The columns were LiChrosorb RP-18, 5 μm, 250 mm × 4 mm I.D. (Merck) and LiChrosorb RP-18, 7 μm, 250 × 25 mm I.D. (Merck). The buffers were 0.25 *M* triethylammonium phosphate (TEAP), pH 4.0 and 0.25 *M* triethylammonium formate (TEAF), pH 6.0. Acetonitrile and isopropanol were used as organic modifiers. The columns were eluted isocratically, the eluate collected in 1-min fractions (Pharmacia, FRAC 300 fraction collector) and the radioactivity was measured in a 16-channel γ-counter (Hydrogamma 16) or in a manual gamma spectrometer (Mølsgaard Medical ApS, Denmark) equipped with a damping device (1000 times damping) made from stainless steel. All separations were performed at room temperature.

Reagents

Phosphoric acid and formic acid (p.a.) were from Merck, triethylamine (99%) from Janssen Chimica and acetonitrile and isopropanol (HPLC grade S) 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.

Silicon estimation

The silicon content in the column eluate (0.25 *M* TEAF, pH 6.0–21.5% isopropanol) was measured using inductively coupled plasma atomic emission spectrometry (ICP-AES). The effect of different organic modifiers on the silicon content was analyzed using graphite-furnace atomic absorption spectrometry (GFAAS).

Isolation procedures

(1) A Sep-pak C_{18} cartridge (Waters) was prewashed with 10 ml isopropanol–water (90:10, v/v) followed by 10 ml water. The pooled HPLC fractions were diluted in 1 volume of water and loaded on the Sep-Pak, followed by 10 ml water and 10 ml of 1 *M* acetic acid. The [^{125}I]insulin was eluted with 5 ml isopropanol–1 *M* acetic acid (90:10, v/v).

(2) Gel chromatography on a 70 cm × 1.6 cm I.D. Fractogel TSK HW-40 (s) (Merck) column eluted at 20 ml/h with 0.1 *M* ammonium hydrogencarbonate pH 8.0 containing 40% ethanol. The pooled fractions containing the [^{125}I]insulin were diluted in 2 volumes of water and lyophilized.

(3) Extraction with cyclohexane: 4 volumes of cyclohexane were shaken with the pooled fractions from HPLC or the concentrate from the Sep-Pak. The water phase was isolated, flushed with nitrogen and lyophilized.

The purity of the monoiodoinsulins

The purity was determined using analytical RP-HPLC (as described above).

The iodine distribution was determined using oxidative sulphitolysis and enzymatic cleavage of the separated A- and B-chains as previously described[13].

The diiodoinsulin content was estimated by disc electrophoresis, slicing the gel and measuring the radioactivity in the slices[13]. The content of diiodoinsulin with two iodine atoms in the same tyrosine group was determined by pronase digestion followed by gel chromatographic determination of diiodotyrosine (DIT) as described[27].

Biological determinations

The binding affinity to isolated rat adipocytes was measured as previously described[1–3,13].

The biological activities of mono[^{125}I]iodoinsulin and mono-[^{127,125}I]iodoinsulin were determined using the enhancement of the conversion of [U-^{14}C]glucose into lipid in isolated rat adipocytes as described previously[2,3]. In some of the determinations of mono[^{127,125}I]iodoinsulin the conversion of [3-^{3}H]glucose into lipid was measured without separation of the water and toluene phases.

The samples for biological determinations were dissolved in 0.01 *M* hydrochloric acid to give two different stock concentrations (≈0.3 and ≈0.1 mg/ml) and

the optical density was measured at 277 nm. The insulin concentration was calculated based on an optical density of 0.958 for an insulin solution containing 1 mg/ml in 0.01 *M* hydrochloric acid.

The concentration in the most concentrated stock solutions of insulin and the monoiodoinsulins with low specific activity was determined using amino acid analysis (Kontron Liquimat III). The determination was based on eight stable amino acids from the 24-h hydrolysis in 6 *M* hydrochloric acid (lys, his, arg, asp, glu, gly, ala, leu). The concentration in the more diluted solutions was determined using staining with Folin-Ciocalteau's reagent according to Lowry *et al.*[28] and with Coomassie Brilliant Blue in the microassay procedure (Bio-Rad). Linear calibration plots were obtained in the range 1–10 μg/ml. The validity was assessed by the identity of the calibration plots for insulin and iodoinsulin substituted to an average of 1 I/mol of insulin.

RESULTS

Fractionation of iodination mixtures

The fractionation of 20 μg insulin iodinated with 1 mCi $^{125}I^-$ using isocratic elution in TEAF–isopropanol, pH 6.0 is shown in Fig. 1. This iodination mixture contained about 17.5 μg of unsubstituted insulin plus 2.5 μg of monoiodoinsulins. The amount of unreacted $^{125}I^-$ was in agreement with the amount (about 5%) determined by TCA precipitation of the iodination mixture. Using this buffer, baseline separation of the four monoiodoinsulins and unlabelled insulin is obtained. Using TEAP–acetonitrile, pH 4.0 as eluent, the critical separation between A19 monoiodoinsulin and unsubstituted insulin was less satisfactory, as previously reported[11].

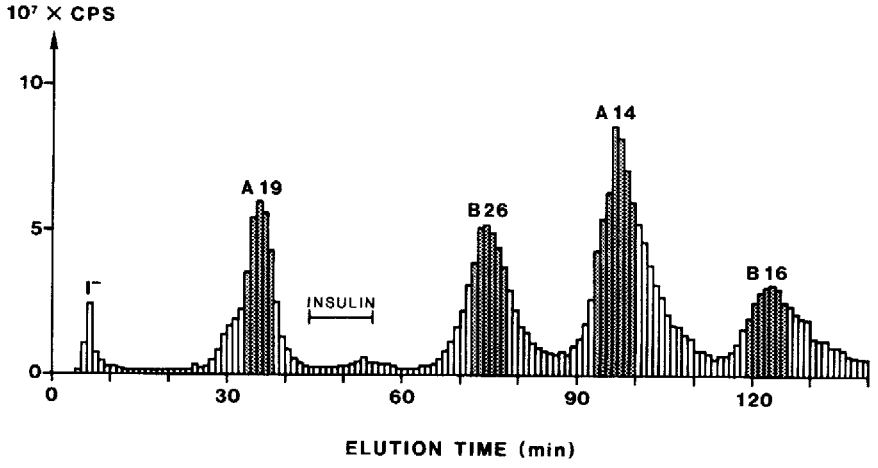

Fig. 1. Isocratic RP-HPLC separation of 50 μl iodination mixture (20 μg insulin iodinated with 1 mCi $^{125}I^-$) using a 250 mm × 4.0 mm I.D. LiChrosorb RP-18 column (5 μm) eluted at 0.5 ml/min with 0.25 *M* TEAF, pH 6.0–21.5% isopropanol. The histogram represents the radioactivity in the collected 1-min fractions. The dotted fractions in each peak were pooled and purified further (see Materials and Methods).

Fractionation of milligram amounts of iodinated insulin

Fractionation of [^{127,125}I]iodoinsulin in the TEAF–isopropanol, pH 6.0 system on a 250 mm × 4 mm column was satisfactory only with an amount up to 1 mg iodoinsulin containing about 170 μg of monoiodoinsulins (data not shown).

Scaling up to a 250 mm × 25 mm column and applying 25 mg of iodinated insulin was satisfactory with respect to the separation of A19 monoiodoinsulin and insulin, whereas A14 and B16 monoiodoinsulin were eluted as a single peak (data not shown). When the same column was eluted with TEAP–acetonitrile, pH 4.0 a satisfactory separation was obtained when 30 mg iodinated insulin were applied, as shown in Fig. 2.

Column bleeding

The contents of silicon in eluates from different batches of the LiChrosorb RP-18 column are given in Table I. The influence of different organic modifiers on the bleeding of silicon from a single LiChrosorb RP-18 column is shown in Table II.

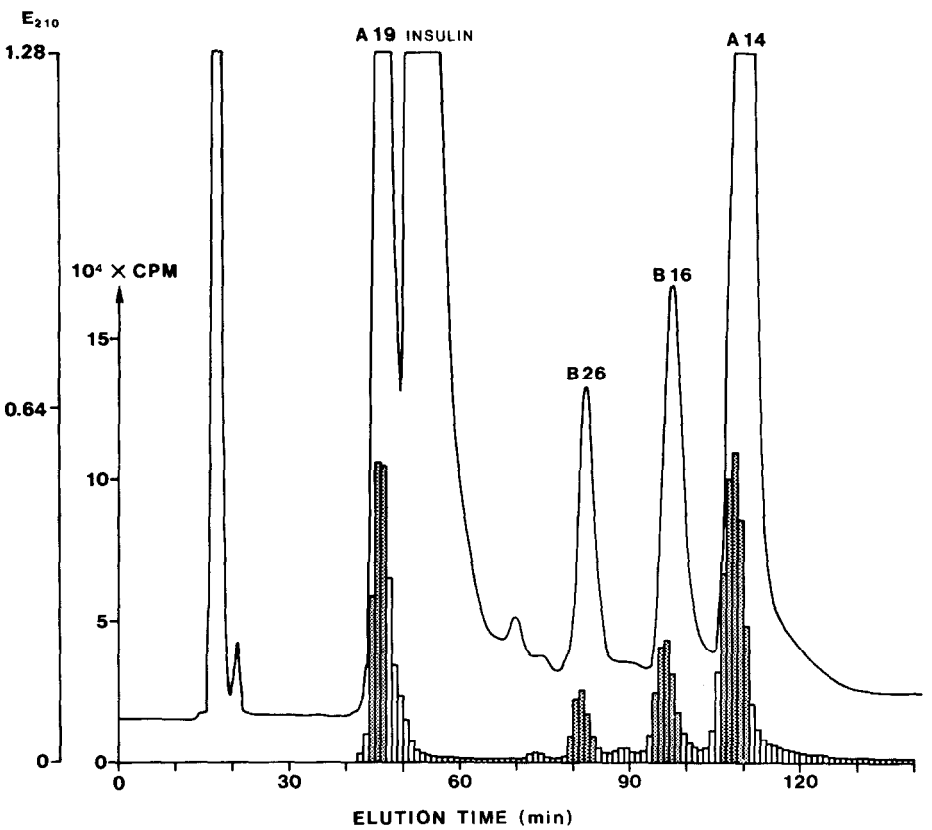

Fig. 2. Isocratic RP-HPLC separation of 300 μl iodinated insulin (30 mg insulin iodinated to 0.16 I/mol containing trace amounts of $^{125}I^-$) using a 250 mm × 25 mm I.D. LiChrosorb RP-18 column (7 μm) eluted at 5 ml/min with 0.25 *M* TEAP, pH 4.0–26% acetonitrile. The continuous curve represents the UV absorbance at 210 nm and the histogram represents the radioactivity in the collected 1-min fractions. The dotted fractions and fractions 53–56 containing insulin were pooled and purified further (see Materials and Methods).

TABLE I

THE CONTENTS OF SILICON (ng/ml) IN ELUATES FROM DIFFERENT BATCHES OF LICHROSORB RP-18, 5-μm COLUMNS ELUTED WITH 0.25 *M* TEAF, pH 6.0–21.5% ISOPROPANOL

Column batch No.	*Silicon (ng/ml)**
414999	188
415000	216
415016	30
Eluent	11

* By ICP-AES, see Materials and Methods.

Isolation of monoiodoinsulins

The recovery of monoiodoinsulins concentrated using Sep-Pak is shown in Table III. It was possible to obtain 90% of the monoiodoinsulin in 1 ml (loaded volume up to 50 ml). The recovery in the water phase after extraction with cyclohexane was *ca.* 90%.

Purity of monoiodoinsulins

The purity of monoiodoinsulin tracers with high specific activity was evaluated using the different methods summarized in Table IV. These results are representatives for several batches.

The purity of monoiodoinsulins with low specific activity is shown in Table V. Although the purity was *ca.* 95%, the B-chain labelled monoiodoinsulins were less pure than the A-chain labelled ones, probably because the amount of B-chain incorporation is lower than that of the A-chain incorporation.

Binding affinity of monoiodoinsulin tracers

Full binding affinity relative to that of the same isomer prepared by disc electrophoresis/ion-exchange chromatography was retained following isolation by gel chromatography in 40% ethanol (A), extraction with cyclohexane (B) and dilution in large volumes of albumin-containing buffer (C) (Table VI).

TABLE II

THE CONTENT OF SILICON (ng/ml) IN ELUATES FROM LICHROSORB RP-18, 5-μm COLUMN NO. 208810.

Eluent	*Silicon (ng/ml)**
0.25 *M* TEAF, pH 6.0–21.5% isopropanol	504
0.25 *M* TEAF, pH 6.0–27% acetonitrile	244
0.25 *M* TEAF, pH 6.0	88
Eluent	56

* By GFAAS, see Materials and Methods.

TABLE III

RECOVERY OF MONOIODOINSULIN MEASURED AS THE RADIOACTIVITY AFTER SEP-PAK CONCENTRATION

	% of radioactivity ± S.D. (n = 7)
Loaded Sep-Pak	100
First ml eluted	91 ± 2
Second ml eluted	1 ± 0.5
Empty Sep-Pak	7 ± 2

TABLE IV

THE PURITY OF MONOIODOINSULIN TRACERS PREPARED BY RP-HPLC AS PER CENT OF RADIOACTIVITY IN CONTAMINATING MONOIODOINSULIN

Methods: A = analytical RP-HPLC; B = iodine distribution analysis; C = pronase digestion. The content of DIT (diiodotyrosine) represents the molar (per cent) amount of diiodoinsulin with two iodine substitutions in the same tyrosine group.

Monoiodo-insulin	*Method*	*Iodide*	*A14*	*A19*	*B16*	*B26*	*DIT*
A14	A	2.1	96.8		1.1		
	B		95.6	2.4		2.0	
	C	1.3					0.2
A19	A	2.6		97.4			
	B			99.7		0.3	
	C	1.7					0.3
B16	A	4.8			93.8	1.4	
	B			2.7	93.3	4.0	
	C	3.6					1.9
B26	A	2.9				97.1	
	B			4.3		95.7	
	C	2.0					0.2

TABLE V

THE PURITY (%) OF MONOIODOINSULINS (MILLIGRAM AMOUNTS) WITH LOW SPECIFIC ACTIVITY PREPARED BY RP-HPLC AND EVALUATED BY ANALYTICAL RP-HPLC OF 25 μg

	Iodide	*Insulin*	*A14*	*A19*	*B16*	*B26*
A19 monoiodoinsulin*				100		
Insulin*		100				
A19 monoiodoinsulin**				99		
Insulin**		100				
B26 monoiodoinsulin**		4.2		1.6		94.4
B16 monoiodoinsulin**	0.5		1.7	0.8	95.1	1.8
A14 monoiodoinsulin**			98.0		2.0	

* The preparative RP-HPLC was performed with TEAF, pH 6.0–isopropanol.
** The preparative RP-HPLC was performed with TEAP, pH 4.0–acetonitrile.

TABLE VI

BINDING AFFINITY (%)* OF RP-HPLC PURIFIED MONOIODOINSULIN TRACERS RELATIVE TO THE CORRESPONDING REFERENCE TRACER (= 100%)

Methods: A = gel chromatography in 40% ethanol; B = extraction with cyclohexane; C = dilution with albumin-containing buffer.

Isolation procedure	*A14*	*A19*	*B16*	*B26*
A	97 ± 1	85 ± 17	93 ± 24	89 ± 3
B	93 ± 18	105 ± 5	107 ± 6	104 ± 10
C	112 ± 11	106 ± 5	104 ± 11	111 ± 14
Reference** tracer	100	100 (57)	100 (114)	100 (183)

* The binding affinity is mean ± S.D. of 2–6 independent experiments each consisting of four replicates.

** Purified by disc electrophoresis/ion-exchange chromatography. The values in parentheses are the binding affinities relative to A14 monoiodoinsulin.

Biological activity of monoiodoinsulins with low specific activity

The biological activity was calculated relatively based on the assumption that the specific activity of the four monoiodoinsulins is identical when prepared from the same iodination, *i.e.*, the amount of radioactivity is a measurement of the amount of monoiodoinsulin and is shown in Table VII. The absolute biological activity was based on the determination of the insulin/monoiodoinsulin concentrations primarily using amino acid analysis, since these cannot be determined based on weighing (less than 1 mg yields) or spectrophotometry (the molar extinction coefficients are not known). The concentrations found by amino acid analysis of the insulin isolated after RP-HPLC were in agreement with those estimated by spectrophotometry at 277 nm.

The absolute biological activities are shown in Table VIII.

TABLE VII

RELATIVE BIOLOGICAL ACTIVITY OF MONOIODOINSULINS ISOLATED AFTER PREPARATIVE RP-HPLC USING TEAP–ACETONITRILE AS ELUENT

Iodine substitution	*Per cent biological activity** *(mean ± S.D.)*
Tyr A19	51 ± 1
Tyr B26	164 ± 9
Tyr B16	104 ± 0
Tyr A14	100

* Determined per 10 000 cpm, calculated relative to A14 monoiodoinsulin (= 100%). The mean biological activity was determined from two different stock solutions, as described in Materials and Methods. The reproducibility varied from 3 to 13% within the same stock solution.

TABLE VIII

ABSOLUTE BIOLOGICAL ACTIVITY OF INSULIN AND MONOIODOINSULINS ISOLATED AFTER PREPARATIVE RP-HPLC AS IN TABLE VI

The absolute biological activity is calculated relative to an insulin standard prepared by low-pressure methods. The biological determinations were performed as described in Table VII.

Insulin/monoiodoinsulin	*Per cent biological activity (mean ± S.D.)*
A19*	61 ± 7
Insulin*	113 ± 4
A19**	49 ± 4
Insulin**	91 ± 4
B26**	157 ± 5
B16**	127 ± 0
A14**	95 ± 1

* The preparative RP-HPLC was performed with TEAF, pH 6.0–isopropanol.
** The preparative RP-HPLC was performed with TEAP, pH 4.0–acetonitrile.

DISCUSSION

Since it is well established that monoiodoinsulins substituted in different tyrosine groups behave differently in biological systems, the need for homogeneous well characterized monoiodoinsulin tracers is evident. The A14 monoiodoinsulin is widely accepted as the ideal tracer for insulin whereas the A19, B16 and B26 monoiodoinsulins are substituted in tyrosyl residues considered to be part of the putative binding site[30,31] and thus valuable in various biological examinations.

Insulin was iodinated in 6 *M* urea buffer by the lactoperoxidase method to increase the iodine substitution in the B-chain tyrosine groups to about 40% of the iodine in the B-chain. Without urea, the iodine substitution in the B-chain was about 10%[13]. These results are representative for carrier-free iodination with ^{125}I to an iodination degree of 0.14 I/mol of insulin.

Iodination of 150 mg insulin with $^{127}I^-$ containing trace amounts of $^{125}I^-$ resulted in a lower iodine incorporation in the B-chain (about 25% calculated from Fig. 2). This is probably a consequence of the higher insulin concentration during the preparative iodination. An increase in the average degree of iodination did not result in a higher incorporation in the B-chain and at the same time the iodine incorporation was less reproducible (data not shown).

Since diiodoinsulins may have different binding affinities and decay to iodide and polymers with high non-specific binding[27], the amount of diiodosubstitution must be minimized. With iodination degrees lower than 0.2 I/mol of insulin, the diiodoinsulin content was maximally 5% on a molar basis[13].

We have recently described a one-step RP-HPLC fractionation of insulin, all four monoiodoinsulin isomers and five different types of diiodoinsulins from diluted iodination mixtures[12]. A LiChrosorb RP-18, 5-μm column is eluted isocratically with 0.25 *M* TEAF, pH 6.0 containing 21.5% isopropanol and the separation between A19 monoiodoinsulin and insulin is superior to that with other RP-HPLC systems[8]. Several silica-C_{18} columns could be used, but batch-to-batch variations in individual

columns might be a problem[32]. In contrast to other reported RP-HPLC systems[8], A19 monoiodoinsulin is obtained free from unlabelled insulin in a single step.

When this system was applied to a 250 mm × 25 mm LiChrosorb RP-18 (7-μm) column the separation was not satisfactory, perhaps due to the greater particle size and a lower linear flow-rate. The linear flow-rate was not increased in accordance with the analytical system due to practical limitations (the elution was performed at 5 instead of 20 ml/min).

Fig. 2 shows that the preparative column performs very well using the TEAP, pH 4.0–acetonitrile system, yielding all four monoiodoinsulins plus insulin in high purity (Table V).

The use of acetonitrile instead of isopropanol as organic modifier resulted in sharper peaks and the individual batch properties tend to disappear. The critical separation between A19 monoiodoinsulin and insulin was even better in the preparative column than in previous separations using analytical columns[11].

Separations on analytical columns in TEAP–acetonitrile, pH 4.0 resulted in an A19 monoiodoinsulin tracer contaminated with insulin, as determined by comparison of the biological activity of the four monoiodoinsulins (data not shown). A reason for the better results on the preparative column could be the four-fold decrease in flow-rate.

Lyophilization of an RP-HPLC column eluate containing the four monoiodoinsulins always resulted in reduced binding affinity[12]. The biological activity in isolated adipocytes was also reduced when applying insulin on the analytical column in amounts increasing from 0.5 to 10 mg (data not shown). The addition of the lyophilization residue from an RP-HPLC column eluate to monoiodoinsulin reference tracers (disc electrophoresis/ion-exchange chromatography) resulted in a decrease in binding affinity, which provides supports to the assumption that column bleeding (chemical degradation of the column support) could be the responsible factors[12]. Other evidence is provided by the direct precipitations in the eluate from a preparative C_{18} cartridge[12].

The quantitative analyses of the products of bleeding obtained by elution of several LiChrosorb RP-18 stationary phases are given in Tables I and II. In previous RP-HPLC separations of diluted iodination mixtures, the amounts of monoiodoinsulins were about 1 ng of each isomer in 2–3 ml of column eluate. From the tables, the amount of silicon is several times higher than the amount of monoiodoinsulin.

If TEAF–isopropanol extracts of LiChrosorb RP-18 stationary phases were hydrolyzed under alkaline conditions, octadecane and octadecanol could be demonstrated using gas chromatography[29].

The variation with respect to different batches of LiChrosorb (Table I) and organic modifiers (Table II) may explain the observed fluctuations in binding affinity found previously[11,12]. Increasing the amount of monoiodoinsulins fractionated (about 2.5 μg in Fig. 1) did not *per se* preserve the binding affinity of the isolated monoiodoinsulins.

The addition of serum albumin to RP-HPLC column eluates containing minute amounts of the monoiodoinsulin isomers has been reported[5–8], but the resulting binding affinity has never been compared to that of the corresponding tracers prepared by low-pressure methods. Addition of serum albumin to the column eluate immediately after the HPLC separation was examined. The tracers were stored frozen

until the measurement of binding affinity (Table VI, C), which showed that the binding activity was retained compared to the reference tracers. The added albumin probably binds to Si-C_{18} derivatives in the eluate (tracers stored frozen without albumin showed decreased binding affinity). The use of isolation procedures under conditions with minimum hydrophobic interaction between the polypeptide and the Si-C_{18} derivatives, such as gel chromatography in ethanol-containing buffer or removal of the organic modifier by extraction with cyclohexane, resulted in retained binding affinity as shown in Table VI, A and B.

The relative biological activity of isolated milligram amounts of monoiodoinsulin with low specific activity (Table VII) showed consistency with the binding affinities of the corresponding mono[^{125}I]iodoinsulin tracers (Table VI, values in parentheses).

The absolute biological activity of insulin, A14 and A19 monoiodoinsulin (Table VIII) was in agreement with our previously determined activities for the corresponding isomers prepared by low-pressure methods[3]. The observation that A14 monoiodoinsulin and insulin have the same biological activity in isolated adipocytes and A19 monoiodoinsulin has about half this activity is widely accepted[1–3,6–13,23], although Jørgensen *et al.*[4] found evidence for about 20% higher potency of A14 monoiodoinsulin relative to insulin. The values for the four isomers agreed well with the relative biological activities and the binding affinities of corresponding monoiodoinsulin tracers prepared by disc electrophoresis/ion-exchange chromatography (shown in parentheses in Table VI). There was a minor discrepancy in the biological activity of B26 monoiodoinsulin determined relatively and absolutely, but the conclusion that the monoiodoinsulins purified by RP-HPLC retain full biological activity is still valid.

The increasing number of reports describing the reduction of the biological activity of peptides and proteins (often enzymes) after HPLC purification demonstrates the need to consider the potential risks. The reasons for the reduced biological activity are frequently the irreversible denaturating effect of the organic modifiers used[20,21] or a direct instability in acidic and/or organic solvents[23,32,33]. In some cases the biological activity can be restored[34]. Böhlen *et al.*[22] reported that the loss of biological activity in the isolation of bovine pituitary fibroblast growth factor can be prevented by using highly polar organic solvents such as ethylene glycol or glycerol. Sharifi *et al.*[19] found that a residue from the acetonitrile used gave 20% inhibition of 3T3 cell protein synthesis, whereas residues from isopropanol and ethanol did not inhibit the synthesis. Vacuum concentration of *n*-propanol-containing fractions from an RP-HPLC fractionation of human fibroblast interferon (Hu IFN) leads to 99% loss of IFN activity[20]. The activity was retained when the HPLC fractions were stored at −20°C or when the fractions were first extracted with cyclohexane to remove *n*-propanol and then vacuum concentrated, in accordance with the present results.

Until now it had not been demonstrated that column bleeding can be responsible for the decrease in biological activity. The effect on the biological activity of other proteins is often ascribed to the denaturation effect of the organic modifiers in the eluent, but since it is known that the insulin molecule is quite stable during, *e.g.*, acid ethanol extraction from the pancreatic glands, this is unlikely for insulin and monoiodoinsulin.

The detection of silicon and C_{18} molecules in the RP-HPLC column eluate in various amounts depending on the column support and buffer system explains the fluctuating reduction of binding affinity and biological activity of insulin and monoiodoinsulins.

Using isolation methods with minimum hydrophobic interaction between insulin and silica-C_{18} degradation products, the biological properties were retained. We cannot exclude that other types of RP-HPLC column available now or in the future will have the same tendency for bleeding, but it should be emphasized that the bioactivity of any compound purified by HPLC should always be compared to that of the same compound purified to a similar degree using more "physiological" methods.

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