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A14-[^{125}I]MONOIODOINSULIN PURIFIED BY DIFFERENT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURES AND BY POLYACRYLAMIDE GEL ELECTROPHORESIS: PREPARATION, IMMUNOCHEMICAL PROPERTIES AND RECEPTOR BINDING AFFINITY

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) allows the rapid separation of A14-[^{125}I]monoiodoinsulin directly from the iodination mixtures. It remains to be clarified, however, whether the RP-HPLC chromatographic conditions affect the properties of the purified tracer. In this study we prepared A14-[^{125}I]insulin purified by

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polyacrylamide gel electrophoresis (PAGE) and by three different RP-HPLC mobile phases containing, respectively, ammonium acetate (A), sodium perchlorate (B) and trifluoroacetic acid (C). The binding characteristics of all these tracers were examined using an insulin antiserum and insulin cell receptors. The specific radioactivity corresponded to the theoretical maximum for the RP-HPLC-purified tracers and was significantly lower for the PAGE-purified tracers. Significant differences were found in the binding of different tracers to the insulin antiserum: maximum binding ranged from 94 to 99% and was significantly lower for tracers purified by RP-HPLC eluents B and C; antiserum dilution giving 50% tracer binding was lower for tracers purified by RP-HPLC eluent B. The four insulin derivatives showed no difference in non-specific precipitation and in the affinity constant values calculated from the Scatchard analysis. No significant difference was found in the binding of the four insulin derivatives to the human-cultured IM-9 lymphocytes and to the human circulating monocytes. In conclusion, the present work demonstrates that the immunological properties of the A14-[^{125}I]monoiodoinsulin purified by RP-HPLC may be partially affected by the composition of the mobile phase. In order to obtain a fully potent A14-[^{125}I]insulin derivative and to have the possibility of comparing data from different laboratories, the chromatographic conditions must be taken into account.

INTRODUCTION

Preparations of ^{125}I-labelled insulin have found widespread use for the examination of the biological properties of insulin. However, since insulin molecules contain four tyrosine residues (A14, A19, B16 and B26), the iodination of the hormone yields a heterogeneous mixture, consisting of cold insulin and various mono- or disubstituted insulin derivatives. To date, it has been sufficiently established that diiodoinsulins have a somewhat reduced biological activity with respect to the unlabelled hormone [1, 2]. In addition, the four monoiodoinsulin isomers do not exhibit the same binding properties [3, 4].

The A14-[^{125}I]monoiodoinsulin purified by polyacrylamide gel electrophoresis (PAGE) retains full biological activity and represents, therefore, a true advance in biological research as compared with previous labelled insulin derivatives [5].

Lately, high-performance liquid chromatography (HPLC) has been introduced as a single-step procedure to obtain A14-[^{125}I]insulin directly from the iodination mixture [4, 6, 7]; such a tracer has been widely employed in insulin receptor binding studies [3, 4, 8, 9]. However, it remains to be made clear if changes of the HPLC chromatographic conditions (i.e. column matrix, mobile phase, etc.) affect the binding characteristics of A14-[^{125}I]insulin to insulin antisera and cellular receptors [10].

In this study, we describe the immunochemical properties and the binding characteristics of different A14-[^{125}I]insulin derivatives separated by PAGE and HPLC and tested with an insulin antiserum and by binding to human cells. Moreover, the effects of three different mobile phases on such properties are reported.

EXPERIMENTAL

Iodination

Monocomponent crystalline porcine insulin was kindly provided by Novo Industry (Copenhagen, Denmark) and iodination was performed with ^{125}I

(97% isotopic purity, active concentration > 350 mCi/ml) using lactoperoxidase and hydrogen peroxide as oxidizing agents. The final molar ratio of insulin:iodine:hydrogen peroxide was 1:0.013:0.013. The labelling procedure has been reported in detail elsewhere [11].

The distribution of iodine among the various tyrosines of [^{125}I]insulin, labelled according to the described procedure, is as follows: 89% in the tyrosines of the A chain and 6% in the tyrosines of the B chain; the diiodotyrosine content was 5% [11].

Purification of the iodination mixture

Reversed-phase HPLC (RP-HPLC). The isocratic HPLC system and the μBondapak C_{18} (10 μm) reversed-phase liquid chromatography columns (300 × 3.9 mm I.D.) were obtained from Waters Assoc. (Milford, MA, U.S.A.).

A 5-μl volume of the iodination mixture was injected into the RP-HPLC system and eluted at room temperature at a flow-rate of 1 ml/min; UV detection at 214 nm was carried out using a 441 ion UV absorbance detector (Waters Assoc.) and the following eluents: (A) 0.01 *M* sodium phosphate buffer–2-propanol–acetonitrile (68:10:22), pH 3, containing 0.15 *M* ammonium acetate; (B) 0.01 *M* sodium phosphate buffer–acetonitrile–isopropanol (60:13:17), pH 2.2, containing 0.1 *M* sodium perchlorate; (C) acetonitrile–0.1% trifluoroacetic acid (34:66).

The 1-ml samples were collected in an LKB Ultrorac 7000 fraction collector (Bromma, Sweden) and aliquots were counted in a Packard Auto Gamma 500 C counting system (Warrenville, IL, U.S.A.). Acetonitrile and 2-propanol were successively evaporated. Authentic monoiodinated A14-insulin (Novo Industry) was used as a reference tracer.

Polyacrylamide gel electrophoresis. All chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Electrophoresis of the iodination mixture was performed according to the method described by Linde et al. [5].

After freezing, gels were cut in a gel slicer into 1.5-mm slices, and radioactivity (ca. 90%) was recovered by elution from each gel slice overnight at 5°C in 0.5 ml of 0.1 *M* ammonium bicarbonate (pH 8) containing 0.5% bovine serum albumin (BSA).

Determination of specific radioactivity

The concentration of immunoreactive insulin in A14-[^{125}I]insulin preparations was calculated according to the method described by Hunter [12]. The specific radioactivity was expressed as μCi ^{125}I per μg equivalents of immunoreactive insulin (μCi/μg).

Antibody dilution curves

A 100-μl volume of a solution containing A14-[^{125}I]insulin (27 pmol/l in 0.04 *M* phosphate buffer, pH 7.4, 0.5% BSA) was incubated in triplicate for 23 h with 100 μl of porcine insulin guinea pig antiserum at serial dilutions (from 1:1250, corresponding to an excess of binding capacity, to $1:1280 \cdot 10^3$) and 200 μl of 0.04 *M* phosphate buffer (pH 7.4)–0.5% BSA. A 1-ml quantity of 2.1% (w/v) dextran-coated charcoal in 0.04 *M* phosphate buffer (pH 7.4) was then added (10 min, 4°C) and, after centrifugation (2500 *g* for 10 min),

the A14-[^{125}I]insulin count-rate of 0.7 ml of the supernatant was measured in triplicate together with that of a sample containing total radioactivity in the same volume and medium. Non-specific precipitation of the tracer by dextran-coated charcoal was calculated after incubation of 100 μl of A14-[^{125}I]insulin with 300 μl of phosphate buffer for 23 h.

Standard curves

Standard solutions of unlabelled porcine insulin were prepared by dissolving crystalline monocomponent porcine insulin to achieve a final concentration of 1288 pmol/l, as confirmed by measuring the optical density of the solution at 277 nm by a Zeiss PM Q II spectrophotometer (Oberkochen, F.R.G.). Serial dilutions (10—1288 pmol/l) were used for the inhibition curves. Standard solutions (100 μl) were incubated in triplicate with 100 μl of A14-[^{125}I]insulin (0.0027 pmol), 100 μl of antiserum and 100 μl of phosphate buffer for 23 h. Bound/free tracer was separated by dextran-coated charcoal and counted as described above. A Scatchard analysis of the curves was then determined.

Cells used in the binding studies

IM-9 Human lymphocytes. These lymphocytes were a gift from Dr. I.D. Goldfine (Cell Biology Laboratory, Mount Zion Hospital, San Francisco, CA, U.S.A.) and were grown in suspension as previously described [13]. This cell line is known to have high-affinity insulin receptors and has been widely employed for insulin binding studies [14—16].

Human monocytes. Blood samples (150 ml) were collected in an acid citrate—dextrose solution from eight healthy volunteers (six males and two females, aged 25—42 years, with no sign of metabolic or endocrine disorders) after an overnight fast. Mononuclear leukocytes were then prepared by centrifugation of a Ficoll-Hypaque gradient, according to the method of Boyum [17]. The monocyte percentage in the mononuclear cell preparation was evaluated by the latex bead phagocytosis method [18].

Insulin receptor binding studies

For binding studies, IM-9 lymphocytes were collected by centrifugation and resuspended at a concentration of 1.5—2.0 · 10^6 cells per ml in fresh medium with 10% foetal calf serum and 20 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7.8. The specific insulin binding was then measured in triplicate samples incubated with the four A14-[^{125}I]-insulin preparations (33 pmol/l) and native insulin at increasing concentrations (0– 3.3 μmol/l), as previously described [19].

Human mononuclear cells, at a concentration of 40 · 10^6 cells per ml, were incubated under similar conditions to the IM-9 lymphocytes. Specific binding was calculated as previously described [20] and normalized to 10^7 monocytes per ml.

RESULTS

Values of specific radioactivity ranged from 240 to 280 μCi/μg (67– 78% of the theoretical maximum) for the isomer purified by PAGE and from 340 to

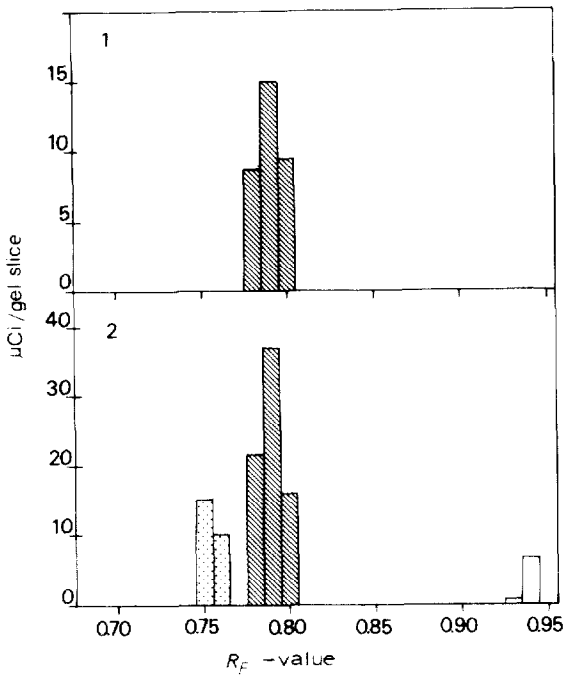

Fig. 1. Distribution of radioactivity in polyacrylamide gels for pure A14-[^{125}I]insulin (panel 1) and for the iodination mixture (panel 2). Gel slices corresponding to R_F 0.75, 0.79 and 0.94 contain A19-[^{125}I]monoiodoinsulins (plus B16 and B26), A14-[^{125}I]monoiodoinsulins and ^{125}I, respectively. The radioactivity recovered from the gel accounts for ca. 90% of the applied radioactivity. A14-[^{125}I]monoiodoinsulin represents ca. 70% of the total recovered activity from the iodination mixture.

360 μCi/μg (94–100% of the theoretical maximum) for A14-[^{125}I]insulin separated by RP-HPLC.

Pure A14-[^{125}I]monoiodoinsulin showed a single radioactive band (R_F value of 0.79, Fig. 1, panel 1) on PAGE, while the iodination mixture showed three bands of activity with R_F values of 0.75, 0.79 and 0.94, corresponding to A19-monoiodoinsulins (plus B16 and B26), A14-monoiodoinsulins and ^{125}I (Fig. 1, panel 2), respectively.

When pure standard A14-[^{125}I]monoiodoinsulin and the A14-[^{125}I]monoiodoinsulin purified by PAGE were chromatographed by RP-HPLC, they both showed the same retention time when using eluent A (26 min), B (19 min) and C (12 min) (Fig. 2, panels 1–3). RP-HPLC analysis of the iodination mixture exhibited a main peak of radioactivity (ca. 70% of the total injected radioactivity) in the fraction eluting with a retention time similar to that of A14-[^{125}I]insulin standard. This behaviour is examplified in Fig. 2, panel 4, for eluent A. When these fractions were analysed by PAGE, and A14-[^{125}I]insulin content equal to or above 95% was found. The other two peaks of radioactivity observed on RP-HPLC purification of the iodination mixture corresponded to A19-monoiodoinsulin and ^{125}I, as confirmed by the rechromatography by PAGE.

Immunochemical properties

When the four A14-[^{125}I]monoiodoinsulins purified from the iodination

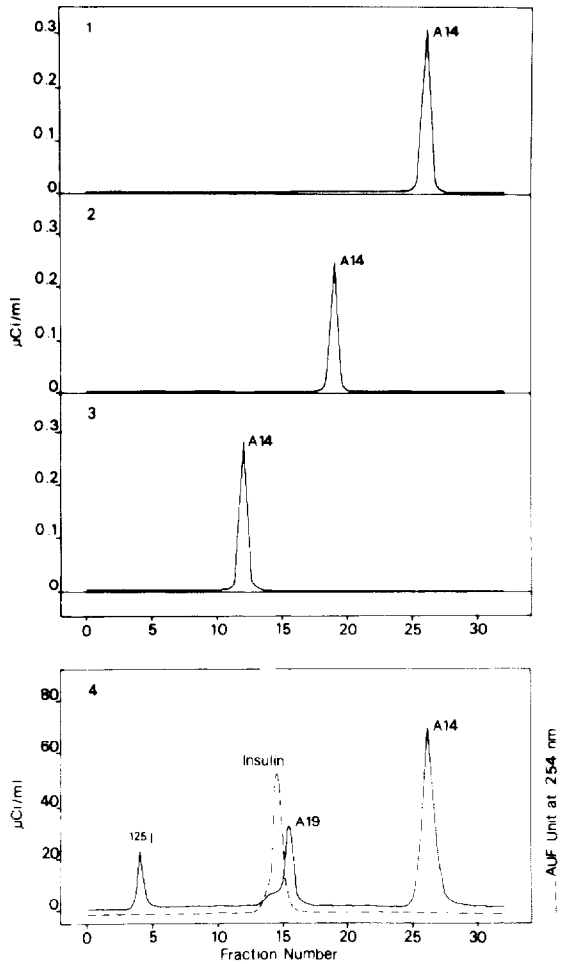

Fig. 2. RP-HPLC analysis of pure A14-[^{125}I]monoiodoinsulin and of the iodination mixture. Panels 1—3: elution of simultaneously injected pure A14-[^{125}I]monoiodoinsulin and A14-[^{125}I]monoiodoinsulin purified by PAGE using eluent A (panel 1), eluent B (panel 2) and eluent C (panel 3). The two tracers coeluted, whatever the eluent used. Panel 4: elution of the iodination mixture using eluent A. About 98% of the radioactivity was recovered. The fraction corresponding to A14-[^{125}I]monoiodoinsulin accounted for 70%. Unreacted insulin (dotted line) partially coeluted with A19-[^{125}I]monoiodoinsulins. B-chain-bound radioactivity was 5% and eluted between A14- and A19-[^{125}I]monoiodoinsulins (not shown in the figure).

mixture by PAGE and RP-HPLC eluents A, B and C were assayed for their immunochemical and binding properties, the highest maximum binding to our insulin antiserum was obtained for A14-[^{125}I]insulin purified by RP-HPLC eluent A (Table I). The values observed using the tracers purified by RP-HPLC eluents B and C were significantly lower (Table I). The antiserum dilution giving 50% binding of the tracer was significantly lower for the isomer separated using eluent B, with respect to the tracer purified by PAGE and RP-HPLC eluent A (Table I). Non-specific precipitation was not statistically different for the four tracers.

TABLE I

BINDING AFFINITY OF THE FOUR DIFFERENTLY SEPARATED A14-[^{125}I]MONOIODOINSULINS TO A GUINEA PIG ANTI-INSULIN SERUM

Values represent mean ± S.D. of four replicates.

Tracer purification	Maximum binding (%)	50% Binding (antiserum dilution × 10^3)	Non-specific precipitation (%)
PAGE	96 ± 2	1:580 ± 80**	2.1 ± 0.4
HPLC eluent A	99 ± 1*	1:590 ± 99**	1.7 ± 0.7
HPLC eluent B	95 ± 4	1:455 ± 20	1.7 ± 0.6
HPLC eluent C	94 ± 2	1:495 ± 60	2.3 ± 0.8

*HPLC eluent A versus HPLC eluents B and C: $P < 0.05$.
**PAGE and HPLC eluent A versus HPLC eluent B: $P < 0.05$.

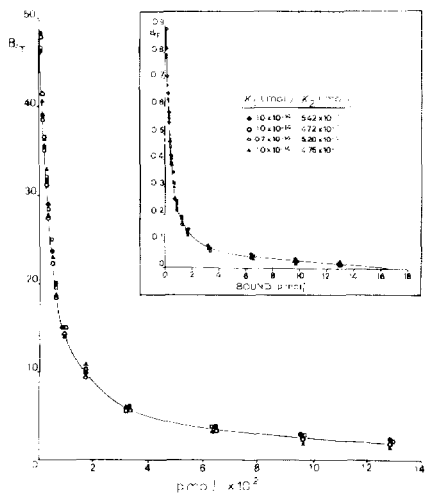

Fig. 3. Standard curves obtained using A14-[^{125}I]monoiodoinsulin purified by PAGE (•), HLPC eluent A (○), eluent B (□) and eluent C (▲) as a function of unlabelled insulin. The points represent means of four replicates. Insert: Scatchard plots used to estimate the average affinity constants of insulin to antibodies using the four preparations. The curves are fitted for univalent insulin reacting with two orders of antibody binding sites at high (K_1) and low (K_2) affinity constants.

Similar values of the bound/total radioactivity were obtained with the four tracers at low and high native insulin concentrations. The apparent affinity constant (K) for the anti-insulin serum was calculated from the dose–inhibition curves (Fig. 3) and the Scatchard analysis (Fig. 3, insert). The Scatchard plot is curvilinear, probably owing to the reaction of univalent insulin with two or more antibody binding sites. Alternatively, since the antiserum used was heterologous and polyclonal, it cannot be excluded that the present antiserum binds to different classes of insulin epitopes. The affinity constants at high-affinity (K_1) and low-affinity (K_2) antibody receptor sites were similar for all tracers (Fig. 3).

Receptor binding properties

The binding of the four insulin tracers to human-cultured IM-9 lymphocytes was not significantly different (Table II). Non-specific insulin binding was 3—5% of the total binding and was similar for all four preparations. Scatchard analysis of the binding data showed a curvilinear shape, compatible with either two different classes or a single receptor class of the negative cooperativity type [21] (Fig. 4). Again, a non-significant difference was found in receptor affinity or in receptor capacity among the four A14-[^{125}I]insulins.

Similar with IM-9 lymphocytes, no significant difference in receptor binding was seen among the four tracers using human circulating monocytes (Table II). Non-specific binding ranged from 8 to 18% of the total binding, and was similar for the four preparations.

TABLE II

BINDING OF THE FOUR DIFFERENTLY PURIFIED A14-[^{125}I]MONOIODOINSULINS TO IM-9 HUMAN LYMPHOCYTES AND HUMAN MONOCYTES

Values represent mean ± S.D. of six separate experiments.

Tracer purificaton	IM-9 Lymphocytes (% bound A14-[^{125}I]insulin per 0.1 mg protein)	Human monocytes (% bound A14-[^{125}I]insulin per 10^7 monocytes)
PAGE	2.86 ± 0.48	3.96 ± 0.47
HPLC eluent A	3.06 ± 0.18	4.55 ± 0.54
HPLC eluent B	3.31 ± 0.25	4.53 ± 0.72
HPLC eluent C	2.97 ± 0.46	3.37 ± 0.13

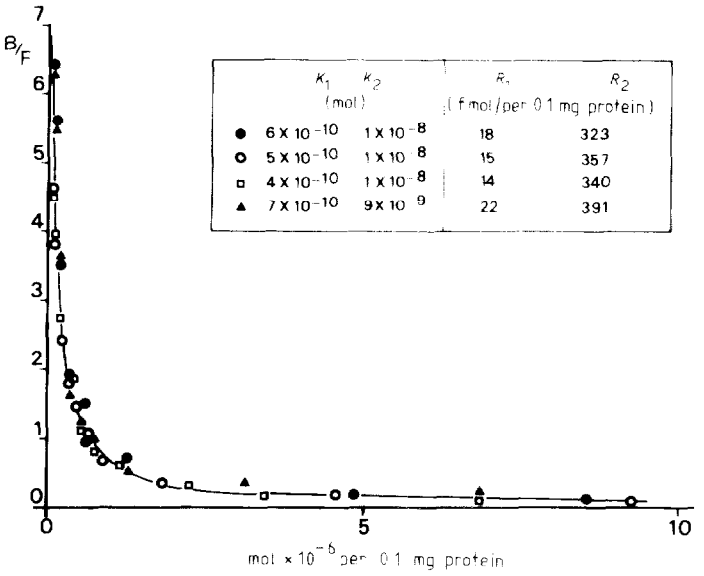

Fig. 4. Scatchard plots for insulin receptors in IM-9 human lymphocytes with A14-[^{125}I]-monoiodoinsulin purified by PAGE (●), HPLC eluent A (○), eluent B (□) and eluent C (▲). Insert: the average affinity constants (K) and binding capacities (R) were estimated assuming that insulin interacts with two classes of receptors: high-affinity (K_1), low-capacity (R_1) and low-affinity (K_2), and high-capacity (R_2) receptors.

Middle-term stability

The four tracers were retested after 30 and 60 days of storage at −55°C in 0.04 *M* phosphate buffer (pH 7.4) containing 0.5% BSA. The characteristics of their binding to both the insulin antiserum and the cell receptors remained unchanged.

DISCUSSION

The use of HPLC has been introduced recently for the separation of an iodination mixture of insulin [3, 4, 6–9]. This method represents a real advantage for fractionating the heterogeneous labelling mixture, as it quickly provides separation of the unlabelled insulin and of the iodinated peptides with various degrees of substitution and positions of the iodine. HPLC, however, introduces some new chromatographic conditions such as high pressure, exposure to organic solvents and type of column support. The importance of these variables on the characteristics of the tracer is still not clearly understood. Moreover, since A14-[^{125}I]insulins purified under different HPLC chromatographic conditions have been used in biological works, we believe that the results of such studies cannot be compared to one another until it is proved that the separation conditions do not influence the tracer binding properties.

Doubts regarding the biological integrity of A14-[^{125}I]monoiodoinsulin purified by HPLC were recently cast by Welinder et al. [10]. These authors reported a reduced affinity to adipocytes of A14-[^{125}I]insulin purified by HPLC compared with the tracer isolated by gel electrophoresis–ion-exchange chromatography. Moreover, after 4 h of incubation in various HPLC buffers and organic modifiers, the A14-[^{125}I]monoiodoinsulin separated by the conventional method showed a lower binding affinity to adipocytes. The authors concluded that the non-physiological conditions under which HPLC is performed, reduce the binding affinity of the insulin tracer.

In this study, we performed the purification of A14-[^{125}I]insulin by PAGE and by HPLC using three different mobile phases: in eluent A, a non-toxic chemical, ammonium acetate, was added to the phosphate buffer and organic modifier, while perchlorate and trifluoroacetic acid were present in eluents B and C, respectively. In each case, an excellent isolation of the A14-monosubstituted insulin derivative from the iodination mixture was obtained, and HPLC purification of the tracer was simpler and faster than PAGE separation.

The specific activities of the tracers purified by HPLC were higher than that of the tracer prepared by PAGE and reached the theoretical maximum. Since purification was performed from the same iodination mixture, such a result was not the consequence of differences in isotopic purity or the carrier-free properties of [^{125}I]monoiodoinsulin [5]; instead, the result means that the HPLC separation of A14-[^{125}I]monoiodoinsulin provides a tracer essentially free from uniodinated insulin.

As far as the binding affinity to a guinea pig anti-porcine insulin serum is concerned, similar values for the maximum binding and for the antiserum title were observed for A14-[^{125}I]monoiodoinsulin purified by PAGE and HPLC eluent A, while lower values were found for the tracers purified by HPLC eluents B and C. On the contrary, the binding affinities of A14-[^{125}I]mono-

iodoinsulin to human-cultured IM-9 lymphocytes and human circulating monocytes were superimposable for the tracers purified by PAGE and by HPLC, whatever the eluent used. There are other examples showing that a modification of the structure of the insulin molecule affects the antibody response but not the biological action of the hormone [22]. This may be due to the fact that receptor assays and anti-insulin antibody assays explore the integrity of different sites of the insulin chains. In fact, the insulin—receptor affinity depends on the integrity of a small region containing some sites of the A and B chain which are different from the antibody recognition sites [23]. The receptor binding sites under our experimental conditions are not affected by the RP-HPLC procedure. On the other hand, the interaction of some component of the mobile phase to the immunologically determinant sites of the A and B chain may modify the reaction of the insulin molecule with its antibodies. Our results disagree with those obtained in a previous study in which a reduced binding affinity of the HPLC-purified tracer to adipocytes was reported [10]. We used different column matrices, mobile phases and cell receptor models, and this may explain the different results.

In conclusion, the present work demonstrates that A14-[^{125}I]monoiodoinsulin purified by HPLC eluent A is actually an excellent choice for both immunological studies and receptor binding characteristics. Moreover, each tracer obtained under a new HPLC condition should be tested accurately and compared with a reference tracer in order to examine its immunochemical and biological potency. A14-[^{125}I]monoiodoinsulin purified by HPLC eluent A is a good option as a reference tracer.

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