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Insulin degradation *in vivo*: a high-performance liquid chromatographic analysis

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ABSTRACT

The metabolism of insulin *in vivo* was investigated using an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method. After intravenous injection of A14-[^{125}I]insulin into normals, eight labelled insulin derivatives were found in plasma (peaks 1–8) Two of them (peaks 1 and 7) showed an elution pattern identical with those of reference [^{125}I]monoiodotyrosine and intact A14-[^{125}I]insulin, respectively Of the other six peaks, five (2–6) eluted before and one (peak 8) after insulin. This pattern was highly reproducible in terms of capacity factors and peak heights Radioactivity separated by RP-HPLC was further characterized for its trichloroacetic acid precipitability and immunoprecipitability. Fractions corresponding to peaks 4–6 and 8, which showed an immunoprecipitability higher than 50%, were pooled in order to obtain sufficient radioactivity and were found to be insulin separated by Sephadex G-50 chromatography, containing in its structure, after sulphitolysis, intact A-chain and to be partially rebindable to monocyte insulin receptors These data demonstrate that in blood, products of insulin metabolism circulate which retain a part of the immunological and biological properties of the hormone These products are clearly separated from one another and from intact insulin by RP-HPLC, suggesting that the appropriate use of this technique may allow a further and more accurate qualitative and quantitative characterization of *in vivo* insulin metabolism in physiological and pathological conditions

INTRODUCTION

The *in vivo* metabolism of insulin has been extensively studied in laboratory animals and humans using a variety of different techniques [1]. Experimental data from these studies were used for quantifying the production, distribution or disposal of insulin at the whole organism level. The presence in plasma was observed of degradation products of insulin that were of sufficient molecular size to retain trichloroacetic acid (TCA) precipitability and of smaller, non TCA-precipitated, fragments [2]. More recently, using high-performance liquid chromatography (HPLC) with a reversed-phase (RP) column for the extraction of insulin from

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plasma samples, and comparing the data obtained with those obtained by gel permeation, it has been further suggested that partially metabolized products of insulin exist in the systemic circulation [3].

Nevertheless, *in vivo* insulin degradation remains an incompletely understood process. In fact, no previous study has characterized the sequential steps of hormone breakdown and the exact identity of products derived from insulin metabolism. Recently, the use of HPLC in the detection of degraded insulin has been reported in fibroblasts [4], in human circulating monocytes [5] and in their subcellar fractions [6]. This procedure permits an accurate stepwise evaluation of the intracellular processing of the hormone and a rapid characterization of its degradation products by separating insulin derivatives from one another and from intact insulin. HPLC also allows the recovery of products, so that they may be identified and studied further. Accordingly, the aim of this study was to separate and characterize compounds produced by *in vivo* A14-[^{125}I]insulin metabolism using an RP-HPLC procedure and to study them with respect to their immunochemical and biological properties.

EXPERIMENTAL

Experimental protocol

Three normal subjects were studied after giving informed consent. Thyroid uptake had been blocked by oral administration of saturated potassium iodide. After they had been fasting for 12–13 h, A14-[^{125}I]insulin (100–200 μCi) was injected as a single bolus and blood samples were collected at 3, 7, 12, 15, 20, 30, 40 min and then every 40 min until 2 h. Blood was drawn into tubes containing EDTA, plasma was promptly separated by centrifugation and stored at −20°C until assayed.

Preparation of pure A14-[^{125}I]insulin

Highly purified porcine insulin (Novo Industry, Bagsvaerd, Denmark) was iodinated by the lactoperoxidase method, as described previously [7], and A14-[^{125}I]insulin was subsequently separated from the iodination mixture using RP-HPLC [7]. The specific activity of the tracer was 360 μCi/μg.

A14-[^{125}I]insulin isomer was used in this work because it retains full immunological reactivity [8], biological activity [8,9] and binding to membrane receptors [7,10]. Moreover, it has been widely used to study *in vivo* and *in vitro* insulin metabolism [11].

Extraction of the radioactivity from plasma

A preliminary to all subsequent procedures was the extraction of the radioactivity from the samples using a Sep-Pak C_{18} cartridge (Millipore, Bedford, MA, U.S.A.). The cartridge was activated by elution in sequence with 5 ml of methanol and 10 ml of water and the residual water was purged by passing 20–30

ml of nitrogen. After loading the sample, the Sep-Pak cartridge was washed with 10 ml of water thus washing away unbound components. Elution of bound peptides from the cartridge was then performed with 5 ml of acetonitrile–0.01 *M* phosphate buffer (pH 3 0) (80:20, v/v). The effluent was collected in 1-ml fractions by applying vacuum to the column.

High-performance liquid chromatography

HPLC-grade chemicals were obtained from Merck (Darmstadt, F.R.G.). Deionized, distilled water, purified with a Milli-Q system (Millipore), was used for reagent preparation. Before use all eluents were degassed under vacuum.

The isocratic HPLC system was from Waters Assoc. (Milford, MA, U.S.A.) and consisted of a Model 510 pump, a Model U6K injector with a 2-ml loop and a Model 441 UV detector. The RP-HPLC analysis was performed using a Waters Assoc. μBondapak C_{18} (average particle size 10 μm) column (300 mm × 3.9 mm I D) and as mobile phase 0.01 *M* sodium phosphate buffer–isopropanol–acetonitrile (67.11 22, v/v), containing 0.15 *M* ammonium acetate (11.56 g/l of eluent) and adjusted to pH 3.0 with hydrochloric acid.

Samples were eluted at 1.0 ml/min and 1.0-ml fractions were collected with an LKB (Bromma, Sweden) Model 7000 Ultrapak collector. Radioactivity was counted with an Auto Gamma 500 C counting system (Packard, Downers Grove, IL, U S A.).

Characterization of radiolabelled products from the RP-HPLC elution

Fractions from the RP-HPLC elution with the same capacity factors were pooled, desalted and lyophilized. Aliquots of lyophilized material were resuspended in 0.04 *M* phosphate buffer with 0 5% bovine serum albumin (BSA) (pH 7.4) and tested for their TCA precipitability, immunoprecipitability and Sephadex G-50 gel permeation. TCA precipitability of the samples was checked using 0.5% (w/v) BSA and 10% (w/v) TCA (final concentrations). Immunoprecipitable radioactivity was measured as previously described [12] using a modification of the double-antibody immunoprecipitation technique of Hales and Randle [13]: guinea pig anti-insulin antibodies in excess (dilution 1·500) were added to samples and the antigen–antibody complexes were precipitated with rabbit anti-guinea pig γ-globulins.

Sephadex G-50 Fine (Pharmacia, Uppsala, Sweden) gel permeation was performed using a 60 cm × 0.9 cm I.D. column which was eluted with 1 *M* acetic acid containing 0.1% BSA at a constant flow-rate [14]. In order to evaluate the state of insulin A-chain, further analysis of degradation products was effected with oxidative sulphitolysis and rechromatography. Lyophilized material was dissolved in 0.5 ml of a solution containing 0.03 *M* sodium tetrathionate, 0.075 *M* sodium sulphate, 8 *M* urea and 2 mg of native insulin. The conversion was obtained by incubation of the samples at 5°C for 6 h [15] Standard A14-^{125}I-labelled A-chain was prepared as reported previously [15] The resulting mixtures were characterized by RP-HPLC [15].

To study the residual binding ability of degradation products to insulin receptors, mononuclear leukocytes were prepared as described previously [5]. Cells ($33 \cdot 10^6$) were resuspended in binding buffer (50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–120m*M* NaCl–1.2 m*M* $MgSO_4$–2.5 m*M* KCl–15 m*M* $NaC_2H_3O_2$–10 m*M* glucose–1 m*M* EDTA–1% BSA, pH 7.8) and then incubated with radiobelled products from RP-HPLC elution. After 120 min at 15°C, cells were centrifuged in a Beckman (Fullerton, CA, U S.A.) microfuge (2 min at 8000 *g*), the supernatants were discarded and the pellets were counted to assess cell associated radioactivity [16]. Non-specific binding was determined in the presence of 2 μM unlabelled insulin and was subtracted from the total tracer binding. The incubation of cells with fractions from RP-HPLC elution caused no increase in the non-specific binding Results were expressed as a percentage of the binding to mononuclear cells of intact A14-[^{125}I]insulin treated identically.

RESULTS

Preliminary studies performed to characterize the elution pattern from the Sep-Pak C_{18} cartridge using the products derived from the cellular metabolism of insulin [5] have shown that final degradation products of insulin (^{125}I or [^{125}I]monoiodotyrosine) were unretained in the cartridge, whereas intermediate degradation derivatives and intact insulin were retained. The recovery of the radioactivity from the Sep-Pak C_{18} column was always 98% for each product tested. Plasma radioactivity obtained after A14-[^{125}I]insulin injection was also separated by the Sep-Pak C_{18} column into two aliquots: that of low polarity was retained in the column and that of high polarity eluted freely from the column. To characterize the latter material further by RP-HPLC, samples were deproteinized with 25% poly(ethylene glycol) (PEG). All radioactivity remained in the supernatant.

The RP-HPLC elution profile of unretained radioactivity showed the presence of a single peak (Fig. 1, shaded peak 1) with a capacity factor of 0.5, superimposable on that of ^{125}I and [^{125}I]monoiodotyrosine used as standards, clearly indicating that this peak was composed of final degradation products. The RP-HPLC characterization of radioactivity retained in the Sep-Pak C_{18} cartridge showed at all times the presence of seven peaks (Fig 1, peaks 2–8) The samples corresponding to peak 7 coeluted with intact insulin used as a standard. Of the other six peaks, five (peaks 2–6) were less hydrophobic than intact insulin and one (peak 8) was more hydrophobic. The product pattern shown in Fig. 1 was highly reproducible with only minor quantitative differences among the various patients studied. On increasing the length of time from A14-[^{125}I]insulin injection, a rapid decrease of peak 7 (insulin peak) was observed (Fig. 1) This loss of label from the A14-[^{125}I]insulin peak was quantitatively accounted for by the accumulation of labelled material in the other peaks in RP-HPLC. In particular, the iodide peak (peak 1) increased rapidly and accounted for the majority of plasma insulin degradation products at all times studied (Table I).

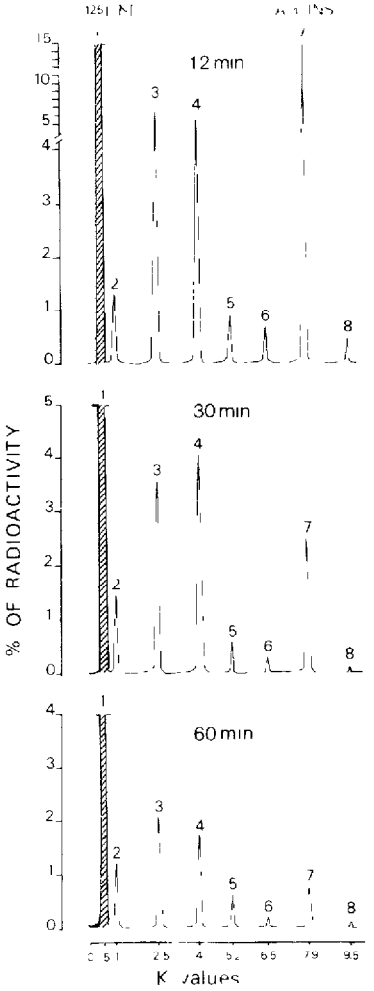

Fig 1 RP-HPLC elution patterns of plasma radioactivity at different times after A14-[^{125}I]insulin injection Peak 1 (shaded) was obtained by RP-HPLC of the radioactivity unretained in the Sep-Pak C_{18} cartridge Peaks 2–8 represent the RP-HPLC elution profile of the radioactive material retained in the Sep-Pak C_{18} cartridge and eluted as described under Experimental Peak 1 coeluted with standard [^{125}I]monoiodotyrosine (^{125}I-M), and peak 7 with standard A14-[^{125}I]insulin (A14-INS)

In order to assess the performance of the RP-HPLC procedure, samples obtained at 12, 30 and 60 min from one patient were examined in triplicate on three successive days. The relative standard deviations (R.S D s) of the capacity factor for A14-[^{125}I]insulin peak (peak 7) were less than 3% both within and between assays. The within-assay R.S.D.s. of the insulin peak heights ranged from 4 1 to 5.2% for high (12-min sample) and low (60-min sample) concentrations, respectively. The between-assay R.S.D. was 6.3% The within- and between-assay

TABLE I

RELATIVE AMOUNTS OF RADIOACTIVITY IN THE PEAKS 1–8 SEPARATED BY RP-HPLC

Results of a representative study are reported

Time (min)	Relative radioactivity								Retention time (cpm/ml)
	1	2	3	4	5	6	7	8	
3	14 00	0 16	0 26	0 30	0 11	0 31	84 70	0.16	18532
7	28 22	0 50	2.31	1 82	0 21	0 72	65.50	0 72	6903
12	67 46	1 38	7.42	5 97	0 90	0 76	15 55	0 56	8510
15	75 01	1 31	7 00	5 55	0.72	0 83	9 07	0 51	9401
20	82.08	1 29	5 83	4 32	0 82	0 55	4 69	0 42	10155
30	87 04	1 43	3 66	4 11	0 80	0 32	2 49	0 15	9733
40	89 40	1 27	3 55	2 71	0 91	0 25	1 77	0 14	8996
80	95 08	1 01	1 50	1 42	0 26	0 15	0 46	0 12	7637
120	97 99	0 51	0 63	0 41	0 11	0 09	0 20	0 06	6946

R.S D s of the capacity factors and peak heights for the other products of degradation were very close to those of intact insulin.

All peaks from the RP-HPLC elution were tested for their TCA and immunoprecipitability (Table II) Peaks 1–3 had TCA and immunoprecipitability low-

TABLE II

TCA PRECIPITABILITY (TCA) AND IMMUNOPRECIPITABILITY (IMP) OF PRODUCTS FOUND IN PLASMA AFTER A14-[^{125}]INSULIN INJECTION AND OF STANDARD [^{125}I]MONOIODOTYROSINE (^{125}I-M) AND A14-[^{125}]INSULIN (A14-INS)

The values are means ± S D of three patient studies

Peak	Precipitability (%)	
	TCA	IMP
1	0	0
2	10 ± 2 1	0
3	30 ± 4 2	10 ± 5 0
4	87 ± 5 4	50 ± 6 2
5	90 ± 4 0	65 ± 4 1
6	90 ± 5 1	75 ± 4 3
7	97 ± 4 3	90 ± 3 0
8	93 ± 6 0	90 ± 3 2
^{125}I-M	0	0
A14-INS	98 ± 4 0	92 ± 2 3

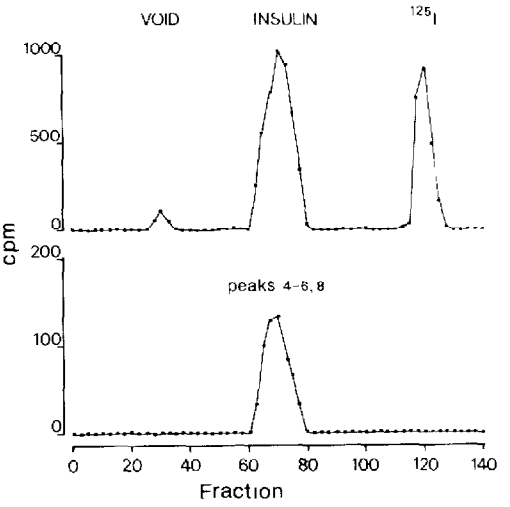

Fig 2 Sephadex G-50 chromatography of standard A14-[^{125}I]insulin (top) and of material from peaks 4–6 and 8 (bottom)

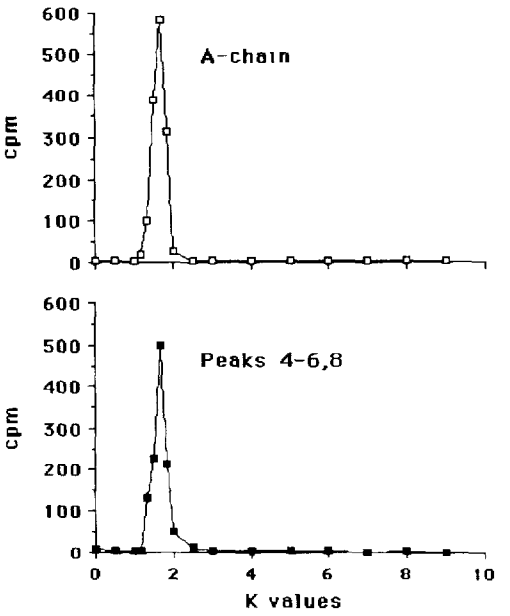

Fig 3 RP-HPLC elution profiles of standard A14-^{125}I-labelled A-chain and of labelled sulphitolysed material from peaks 4–6 and 8

TABLE III

RECEPTOR BINDING (%) OF A14-[^{125}I]INSULIN (A14-I) AND OF POOLED RADIOACTIVITY FROM PEAKS 4–6 AND 8

Mononuclear cells from each patient were incubated with A14-[^{125}I]insulin and with pooled radioactivity from peaks 4–6 and 8

Patient	A14-I	Peaks 4–6 and 8	%[a]
1	5.2	1.8	34.6
2	4.7	1.4	29.8
3	6	2.1	35

[a] Receptor binding of peaks 4–6 and 8 has a percentage of A14-[^{125}I]insulin binding.

er than 30 and 10%, respectively. In contrast, peaks 4–6 always had a TCA precipitability higher than 80% and an immunoprecipitability higher than 50% Peak 7 had TCA and immunoprecipitabilities identical with those observed with standard A14-[^{125}I]insulin. Finally, peak 8 showed values very similar to those of peak 7 Taken together, these data further suggest that the material in peak 7 is undegraded A14-[^{125}I]insulin. In contrast, fractions corresponding to peaks 1–3 are composed of final degradation products of insulin and peaks 4–6 and 8 of material which retains, almost in part, immunochemical characteristics of the hormone. To examine further the question of size, composition and biological activity of the immunoprecipitable intermediate products of insulin degradation, lyophilized radioactivity from peaks 4–6 and 8 was pooled. The Sephadex G-50 column elution of this pool showed a single peak in the region of A14-[^{125}I]insulin, indicating that it is composed of products of size the same as or very similar to that of intact insulin (Fig. 2). As we used insulin labelled on A14-tyrosine, we were able to evaluate the state of the A-chain. Thus, we performed the oxidative sulphitolysis of pooled material from peaks 4–6 and 8. The RP-HPLC of the sulphitolysed mixture showed a single peak of radioactivity corresponding to that of standard A14-^{125}I-labelled A-chain (Fig. 3), their capacity factor being 1.7. A14-^{125}I-labelled A-chain was not TCA or immunoprecipitable. Finally, we found that the binding value of immunoprecipitable intermediate products was, on average, 30% of that of intact insulin (Table III).

DISCUSSION

We have developed a procedure for the characterization of plasma radioactivity after bolus injection of A14-[^{125}I]insulin, and we have demonstrated that *in vivo* insulin degradation is a time-dependent process that results in several degradation products rapidly amassing in plasma. They have markedly different polarity compared with intact insulin This may be due to either a major change

in surface charges or a loss of hydrophobic residues. For peak 8, which elutes after insulin, it is possible to hypothesize a conformational change which revealed more of the hydrophobic core of the insulin molecule. This seems unlikely for the other derivatives of insulin metabolism because they were markedly less hydrophobic than intact insulin; it is probable that they have lost some hydrophobic residues

Four intermediates (peaks 4–6 and 8) showed an immunoprecibitability higher than 50% and coeluted with the insulin peak in Sephadex G-50 gel permeation chromatography Moreover, this study supports the notion that the labelled material of peaks 4–6 and 8 still contains intact insulin A-chain in its structure. These observations, in agreement with the results obtained when insulin degradation was studied in cell systems [11], suggest that the degradative pathway produces material which retains much of the antigenic structure of intact insulin and is composed of intact A-chain linked to other portions of the insulin molecule. Moreover, the presence of intact A-chain in its structure strongly suggests that the initial products of *in vivo* insulin metabolism are altered in the B-chain. In addition, this study shows that no significant amount of free A-chain is found in plasma as a product of *in vivo* insulin degradation.

Finally, we have demonstrated that insulin-sized degradation products of insulin possess, on average, a significantly lower binding affinity to monocytes than intact insulin. It is interesting that the loss of binding activity of this material is more severe than that of immunoprecipitability. This result confirms that the insulin–receptor affinity depends on the integrity of a small insulin region containing some sites which are different from the antibody recognition sites [17] and suggests that it is involved in the degradation process faster than the antibody recognition sites.

In conclusion, the developed RP-HPLC method allows a rapid and efficient separation from one another and from intact insulin of derivatives produced by *in vivo* insulin metabolism and permits their recovery so that they may be characterized further. Hence the appropriate use of this procedure should be an interesting aid to a more accurate qualitative and quantitative characterization of *in vivo* insulin metabolism in physiological and pathological conditions. These investigations could be of particular interest as insulin degradation is dependent on the formation of an insulin–receptor complex and the substrate for insulin metabolism is cell-bound insulin [11]. Hence it is clear that the study of *in vivo* insulin degradation may give important insights into the cellular mechanisms of insulin processing at the whole organism level

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