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GEL CHROMATOGRAPHIC SEPARATION OF HUMAN C-PEPTIDE AND PROINSULIN

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SUMMARY

The immunoreactivity of circulating C-peptide is separated into two main peaks on a Bio-Gel column; the faster peak should not be proinsulin but an associated C-peptide without a covalent bond. Proinsulin is in fact eluted in the fraction prior to the faster eluting peak of C-peptide immunoreactivity with 1 *M* acetic acid as the eluting buffer. Therefore the use of gel chromatography to study C-peptide and proinsulin needs to be carefully re-evaluated, although the method has been established as one of the standard methods.

INTRODUCTION

It is now well established that proinsulin is synthesized as a precursor molecule and is converted within the pancreatic beta cell to insulin with release of the connecting peptide and several basic amino acids [1]. C-peptide is released from beta cells in equimolar amounts with insulin [2] and can be detected in the peripheral circulation by radioimmunoassay [3]. The gel chromatographic separation of circulating C-peptide in the peripheral circulation was performed by Block et al. [4] using 3 *M* acetic acid as eluent after acid—alcohol extraction

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[5, 6]. C-peptide immunoreactivity (CPR) in human plasma separates into two peaks on a Bio-Gel column; the faster eluting peak elutes in the region corresponding to proinsulin, and the other peak elutes in the region corresponding to insulin [3, 4]. The first peak should contain in part the immunoreactivity of proinsulin, but the proportion of proinsulin and associated C-peptide in this peak has not been entirely elucidated. The present study was undertaken to determine the nature of circulating CPR after acid—alcohol extraction in man, using the Bio-Gel column by which two analogues of insulin were separated [7, 8].

EXPERIMENTAL

Materials

Reagents. Bio-Gel P-30 (100—200 mesh) was obtained from Bio-Rad, Richmond, CA., U.S.A. $Na^{125}I$ (carrier free) was obtained from The Radiochemical Centre, Amersham, Great Britain. Anti-pig insulin guinea-pig serum M 8309 and porcine monocomponent insulin were generous gifts from Dr. J. Lindholm (Novo Research Institute). Single component insulin was obtained from Drs. R.E. Chance and M.A. Root (Lilly Research Laboratories). The C-peptide assay kit was obtained from Daiichi Radioisotope Laboratories, Tokyo, Japan. All other chemicals were reagent grade.

Subjects. Approximately 1 g of insulinoma tissue was obtained from a patient (aged 53 years, female, fasting blood sugar level = 20—70 mg/dl) as a surgical specimen. Four normal subjects (aged 20—23 years, fasting blood sugar level = 84—95 mg/dl) and four diabetic subjects on diet therapy (aged 58—71 years, fasting blood sugar level = 108—149 mg/dl) were studied. There was no significant difference in obesity between normal and diabetic subjects.

Methods

Tests. All normal and diabetic subjects were given oral glucose tolerance tests (O-GTT). The serum samples taken at 60 min after the O-GTT were frozen at −20°C until used.

Extraction and gel chromatography. A piece of insulinoma tissue was immediately placed in Krebs—Henseleit bicarbonate buffer solution equilibrated with O_2—CO_2 (95 : 5). Within 20 minutes after removal from the patient, the tissue was homogenized in a siliconized glass tube with acid—ethanol (350 ml of 99.5% ethanol, 7 ml of conc. HCl, 153 ml of distilled water) using a Polytron PT 10-35 (Kinematica, Steinhofhalde, Switzerland) for 1 min at setting 7. Subsequent extraction and gel filtration of the samples were the same as the previously reported methods [7].

The extracted sample was dissolved in 3 ml of 1 *M* acetic acid, and centrifuged at 600 *g* for 5 min. The clear supernatant was applied to a Bio-Gel P-30 (100—200 mesh) column (90 × 1.6 cm) equilibrated with 1 *M* acetic acid and eluted with the same elution buffer at 4°C with a flow-rate of 0.14 ml/min. The column was calibrated with porcine [^{125}I]insulin, porcine [^{125}I]proinsulin and synthetic [^{125}I]Arg-Arg-human C-peptide-Lys-Arg. The fraction size was 4.05 ml. Porcine [^{125}I]proinsulin elutes at a V_e/V_0 of 2.4, porcine [^{125}I]insulin at 4.7 and synthetic [^{125}I]Arg-Arg-human C-peptide-Lys-Arg at 4.8.

Assay for immunoreactive insulin and C-peptide immunoreactivity. Assay for immunoreactive insulin (IRI) and CPR was performed according to previously reported methods [10, 11].

Dilution tests for peaks I and II of CPR. Dilution tests for pooled peak I and peak II of CPR from normal subjects were performed using the C-peptide assay system.

Gel chromatography of CPR peak I obtained from normal subjects after treatment with 7 M urea. The CPR peak I fractions were pooled and lyophilized. The lyophilized sample was dissolved with 1 *M* acetic acid and urea was added to the solution to make a 7 *M* urea solution (total volume = 3 ml). The solution was applied to the same column as mentioned above with the same elution buffer. Each fraction was assayed with the same assay system used for CPR.

Dilution tests for proinsulin. The immunoreactivity that eluted in fraction 24 and which possessed both CPR and IRI activity was tested using the dilution technique with the same assay systems for CPR and IRI.

RESULTS AND DISCUSSION

The two derivatives of insulin were separated on the Bio-Gel column as described in earlier reports [7, 8]. Using the same column with the same elution buffer, the extracted CPR from human serum was also separated into two peaks as shown in Fig. 1. The immunoreactivities of peak I ($V_e/V_0 = 3.0$) and that of peak II ($V_e/V_0 = 4.2$) were studied by the dilution method using the C-peptide assay system. It was demonstrated that both peaks had the same immunoreactivity as that of synthesized human C-peptide as shown in Fig. 2. Peak I of CPR ($V_e/V_0 = 3.0$) was pooled and, after treatment with 7 *M* urea was gel chromatographed using the same column. Most of peak I of CPR (V_e/V_0 = 3.0) was transferred to the position of peak II of CPR (V_e/V_0 = 4.2 and 4.4) as shown in Fig. 3.

The values of peak I ($V_e/V_0 = 3.0$) and peak II ($V_e/V_0 = 4.2$) of CPR in the human peripheral circulation were studied by the same system (Table I). In normal subjects the total CPR level was 4.2 ± 1.5 ng/ml (mean ± S.D.). This

TABLE I

LEVELS OF PEAK I AND PEAK II OF CPR AT 60 min AFTER O-GTT

50-g oral glucose tolerance tests (O-GTT) were given to all normal and diabetic subjects. The serum samples taken at 60 min after O-GTT were frozen at −20° C until used. Extraction and gel filtration of the sera were the same as the reported methods [7]. All values were corrected with the reported recovery rate of extraction of C-peptide (0.607) [12] and the calculated recovery rate of gel filtration of labeled C-peptide (0.717). The results are expressed as mean ± S.D. All *P* values were obtained by a paired Student's *t*-test.

Case	Maximum BS* level (mg/dl) after 50-g O-GTT	Peaks of C-peptide (ng/ml)	
		Peak I	Peak II
Normals (n=4)	131.8 ± 24.6	2.10 ± 0.54	0.33 ± 0.21
	$P < 0.05$	n.s.**	n.s.
Diabetics (n=4)	251.8 ± 47.4	3.21 ± 1.02	0.63 ± 0.39

*BS = blood glucose.
**n.s. = not significant.

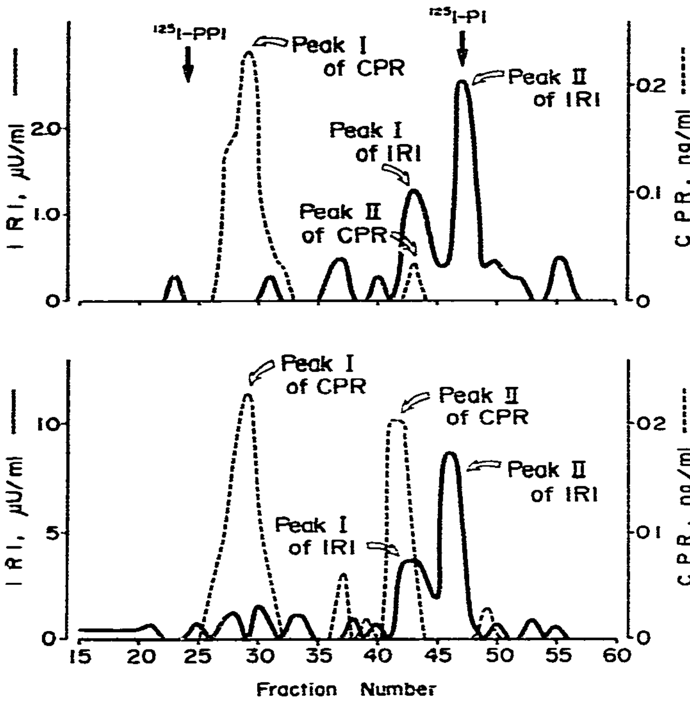

Fig. 1. Elution profiles of extracted human serum C-peptide (CPR) and insulin (IRI) on the Bio-Gel column (4.05 ml fraction size) obtained from a normal young adult and an adult onset diabetic patient on diet therapy. Upper panel: a normal adult (N.M., 23 years old, male). Lower panel: an adult onset diabetic patient (M.W., 68 years old, female). Detection level for CPR is 0.02 ng/ml and that for IRI is 0.2 μU/ml. PPI = porcine proinsulin, PI = porcine insulin.

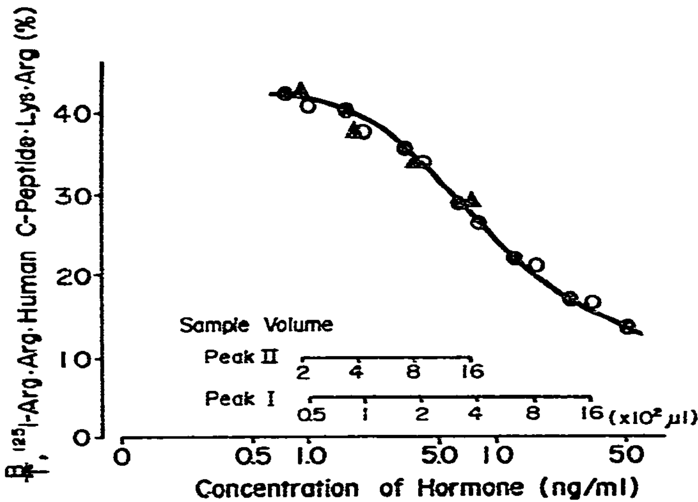

Fig. 2. Comparison of the immunoreactivities of peak I, peak II obtained from normal subjects, and synthesized human C-peptide in the CPR assay. Closed circle (•) represents synthesized human C-peptide. Open circle (○) represents peak I of CPR, and closed triangle (▲) represents peak II of CPR. All values are the mean from paired determinations. B/T = percent bound.

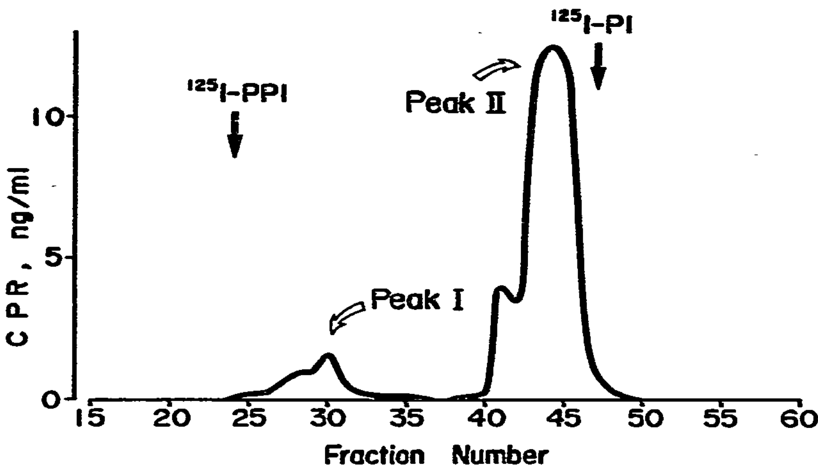

Fig. 3. Elution profile of pooled peak I of CPR obtained from normal subjects, after treatment with 7 *M* urea on the Bio-Gel column (4.05 ml fraction size). Detection level for CPR is 0.05 ng/ml. PPI = porcine proinsulin, PI = porcine insulin.

level is compatible with the previously reported result [4]. The value of CPR peak I was 2.1 ± 0.54 ng/ml (mean ± S.D.), and that of peak II was 0.33 ± 0.21 ng/ml (mean ± S.D.) at 60 min after a 50-g O-GTT. In diabetics, the total CPR value was 5.2 ± 2.4 ng/ml (mean ± S.D.). This level is compatible with the previously reported result [4]. The value of peak I for diabetics was 3.21 ± 1.02 ng/ml (mean ± S.D.), and that of peak II was 0.63 ± 0.39 ng/ml (mean ± S.D.) at 60 min after the 50-g O-GTT. There was no significant difference between the value for normal subjects and that for diabetics, although the absolute value of CPR in diabetics was more than that of normals. In the earlier reports [7, 8]

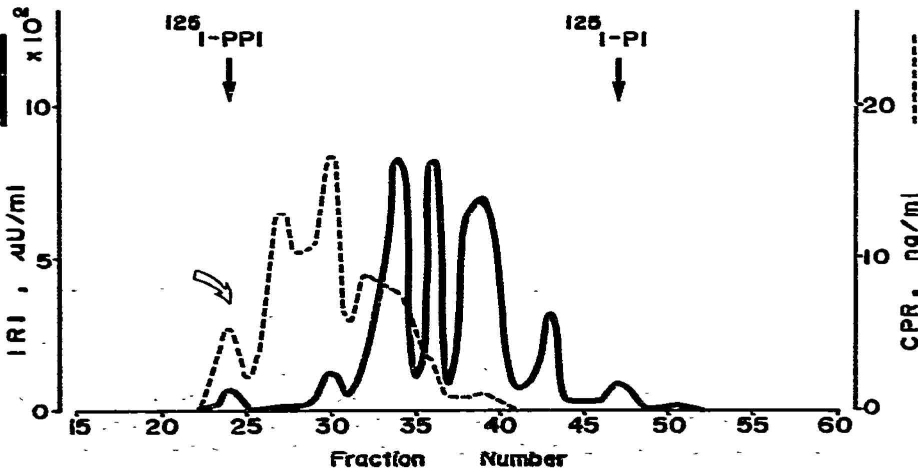

Fig. 4. Elution profile of extracted human C-peptide (CPR) and insulin (IRI) on the Bio-Gel column (4.05 ml fraction size) obtained from human insulinoma tissue (T.O., 53 years old, female). Open arrow shows the peak which has both CPR and IRI. Detection level for CPR is 0.4 ng/ml and that for IRI is 4 μU/ml. PPI = porcine proinsulin, PI = porcine insulin.

there was a significant difference between normals and diabetics in the values for insulin derivatives. Therefore, the involvement of the insulin secretion system in glucose stimulation could precede that of the C-peptide secretion system in the course of this disease.

Gel chromatographic separation of IRI and CPR was performed using the same methods after extraction from the insulinoma tissue (Fig. 4). There were possibly intermediates of insulin molecules, besides peak I (V_e/V_0 = 4.2) and

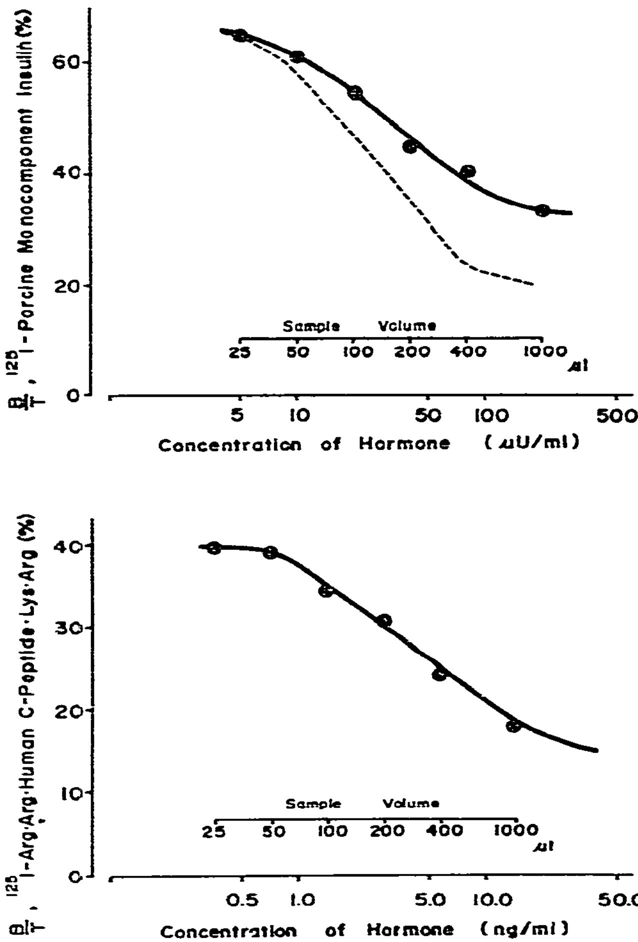

Fig. 5. Comparisons of the immunoreactivities of the peak which has the immunoreactivities of CPR and IRI obtained from human insulinoma tissue, porcine insulin and synthesized human C-peptide in the IRI (upper panel) and CPR (lower panel) assay systems. Upper panel: antiserum is anti-pig insulin guinea-pig serum M 8309. Standard hormone is porcine monocomponent insulin (lot No. 834098). Single component porcine insulin (lot No. 615-1082B-108-I) was used as labeled hormone after iodination with ^{125}I. Closed circles (•) represent the peak substance obtained from human insulinoma, which was eluted at the position of porcine [^{125}I]proinsulin. The dotted line (— — —) represents the immunoreactivity of standard hormone. Lower panel: C-peptide assay kit obtained from Daiichi Radioisotope Laboratories was used. Closed circles (•) represent the same peak substance as mentioned above. The solid line (—) represents the immunoreactivity of standard hormone. All values are the mean from paired determinations. B/T = percent bound.

peak II (V_e/V_0 = 4.8) of IRI [7, 8] and peak I (V_e/V_0 = 3.0) of CPR. Also a peak (V_e/V_0 = 2.4) which had both IRI and CPR activities and whose eluting position (V_e/V_0 = 2.4) corresponded to that of porcine [^{125}I] proinsulin, was detected (Fig. 4, open arrow). The immunoreactivity of this peak was studied by the dilution method using the C-peptide and insulin assay systems (Fig. 5). The immunoreactivity of this peak substance, corresponding to that of porcine [^{125}I] proinsulin, showed almost the same reactivity as that of human proinsulin [9] (Fig. 5, upper panel), and the same peak showed the same immunoreactivity as that of synthesizes human C-peptide [4, 11] (Fig. 5., lower panel). In their reported study [9, 11], natural human proinsulin was obtained from Drs. Steiner and Rubenstein. In the report of Block et al. [4], who carried out their work in the same place as Drs. Steiner and Rubenstein, the immunogenicity of human proinsulin is also almost the same as that of human C-peptide in their assay system. These results are compatible with our results (Fig. 5). Therefore the peak substance should be human proinsulin itself.

From the above-mentioned results, the following conclusions can be made: (1) There are two groups of C-peptide immunoreactivity in human peripheral circulation: one elutes rapidly (V_e/V_0 = 3.0) and is the associated form of C-peptide, the other is a slower peak (V_e/V_0 = 4.2). And the association of C-peptide is not by means of a covalent bond. (2) The involvement of the insulin secretion system could precede that of the C-peptide secretion in the course of diabetes under the load of glucose stimulation. (3) Proinsulin should be eluted at the position (V_e/V_0 = 2.4) which precedes peak I of CPR on the column. Therefore the gel chromatographic study for proinsulin and C-peptide needs to be re-evaluated carefully, although this method has been established as one of the standard methods.

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