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ABSTRACT

A simplified method for measuring free, total and C-peptide in insulin dependent diabetics with circulating insulin antibodies was studied The free insulin and C-peptide were extracted from the plasma with 25 per cent PEG solution (polyethylene glycol, Carbowax 6000) Total insulin was extracted from the plasma with 25 per cent PEG solution after dissociation of the antibody-antigen complex with dilute HCl Aliquots of the extracts were used in the insulin and C-peptide radioimmunoassay system The sample from normal controls and diabetics on chlorpropamide (CP) the insulin and C-peptide were determined without prior extraction

(*) Paper presented at XI Congreso de la Asociacion Latinoamericana de Sociedad de Biologia y Medicina Nuclear - ALASBIMN, held in Santiago-Chile, Oct 08 - 11, 1989

DETERMINAÇÃO RADIOIMUNOLÓGICA DE INSULINA LIVRE, TOTAL E C-PEPTÍDEO EM DIABÉTICOS TRATADOS COM INSULINA*

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RESUMO

Descrevemos um método simples para a medida de insulina livre, total e C-peptídeo em diabéticos insulino-dependentes com anticorpos antiinsulina circulantes. Insulina livre e C-peptídeo são extraídos do plasma com uma solução de PEG 25% (polietileno glicol, Carbowax 6000). Insulina total é extraída do plasma com solução de PEG 25% após dissociação do complexo antígeno-anticorpo com HCl diluído. Alíquotas dos extratos são usadas nos sistemas de radioimunoensaio para insulina e C-peptídeo. A concentração em amostras de plasma de indivíduos normais e diabéticos tratados com clorpropamida (CP), insulina e C-peptídeo foram determinados sem extração prévia.

(*) Trabalho apresentado no "XI Congreso de la Asociación Latinoamericana de Sociedades de Biología y Medicina Nuclear-ALASBIMN", Santiago-Chile, de 08 a 11 de outubro de 1989.

INTRODUCTION

The demonstration of an impaired insulin secretion in Diabetes (Yalow and Berson, 1960[a]) has focused considerable attention on the regulatory mechanisms in the function of the beta cell Since these authors (1960[b]) introduced the radioimmunologic method for the determination of plasma insulin from normal and diabetics subjects, this assay has been widely used, either in its original form or in form modified Nevertheless plasma insulin from diabetic patients treated with commercial insulin preparations for periods longer than few weeks has circulating insulin antibodies (Berson and Yalow, 1962) which interfere with the insulin radioimmunoassay The antibodies must be removed from the diabetic plasma before immunoreactive determination can be performed In this situation, besides since C-peptide is secreted from pancreatic beta cells in equimolar concentrations with insulin (Horwitz et al, 1975) C-peptide levels can be used as a guide to endogeneous insulin production

Widespread availability of the human C-peptide immunoassay has been hindered by several factors First, because of the cross-reactivity between human C-peptide and all the other C-peptides studied (Rubenstein et al, 1970), human C-peptide must be used for standard, radioactive label and as antigen for the production of antibodies Synthetic C-peptide was used as the standard because no natural peptide was available (Yanahara et al, 1970) Natural human C-peptide is limited in its availability because extensive post mortem degradation takes place in the pancreas (Natain et al, 1975) Second, human C-peptide has a relatively low molecular weight (3021 daltons) and thus tends to be a poor antigen When coupled to large proteins, immunization with C-peptide does not consistently result in a high titer antiserum rabbits or guinea-pigs Probably a lack of rigid secondary or tertiary structure (Frank and Veros, 1968, Markussen, 1971) renders this peptide poorly immunogenic Third, in order to iodinate the C-peptide to high specific activity a tyrosyl residue must to be added because there is no tyrosine or histidine residue in any of the known natural peptides (Melani et al, 1970) As C-peptide has no biological activity, the radioimmunoassay is the only analytical technique available for its determination Despite of the difficulties, a number of investigators have succeded in developing assays suitable for measuring the concentration of human C-peptide serum (Melani et al, 1970, Block et al, 1972, Faber et al, 1976, Heding, 1975) However, there are many problems with these methods Heding (1975) has drawn attention to additional potential sources of error in the determination of serum C-peptide She has shown that there is considerable variation in the displacement of the tracer from different C-peptide antisera by

sera from long-term insulin treated juvenile diabetics As certain antisera gave zero values with these serum samples, it seems probable that some antisera to human C-peptide are capable of reacting to varying degrees with substances in serum that are different from C-peptide and proinsulin (Heding,1975) Similar results were obtained by Faber et al, 1976 Further, Heding (1975) and Kuzuya et al (1977) have obtained evidence that immunoreactive serum C-peptide may exist in more than one form Thus, since the immunoreactivity of this peptide differed markedly when assayed with various antisera, this could explain partially at least the wide range in serum C-peptide concentrations reported by different investigators

Since the development of a reproducible radioimmunoassay for human C-peptide, this technique has provide a simple method for evaluation of the beta cell function in insulin-requiring diabetics despite the presence of circulating insulin antibodies (Block et al, 1972, Block et al, 1973) However, although insulin-antibodies do not interfere with the human C-peptide assay, they do bind endogeneous proinsulin which has an insulin moiety and greatly retard its clearance from the circulation Because most C-peptide react to a greater or lesser extend with human proinsulin, this peptide may become a major determinant of serum total C-peptide immunoreactivity in many insulin-requiring diabetic patients (Horwitz et al, 1976) In this regard Kuzuya et al (1977) have evaluated the use of polyethylene glycol for precipiting insulin antibodies from sera followed by measurement of C-peptide in the supernatant This simple procedure can also be used for the determination of free insulin levels and has considerable potential for the evaluation of beta cell function in insulin requiring diabetic patients In contrast to insulin very little C-peptide is metabolized by the liver (katz and Rubenstein, 1973) Thus, the simultaneous measurement of insulin and C-peptide concentrations in peripheral blood enables "in vivo" estimation of both hepatic insulin extraction and insulin secretion by the pancreas

The purpose of our study was to evaluated the feasibility of the polyethylene glycol precipitation method (Nakagawa et al, 1973; Kuzuya et al, 1977) to remove insulin-antibodies from the insulin-treated diabetics plasma in the radioimmunoassay system for the measurement of free, total and C-peptide levels

MATERIAL AND METHODS

Precipitation of insulin-antibodies in plasma, with polyethylene glycol (PEG)

- <u>Extraction of free insulin and C-peptide</u> Free insulin and C-peptide were routinely extracted from plasma with PEG as follows

Pipette 300 µl of plasma into a test tube Pipette 300 µl of cold 25 per cent PEG solution (Carbowax 6000) Mix immediately (Vortex type for 1 min) Centrifuge (2 500 to 3 000 rpm for 30 min) in a refrigerated centrifuge model PR-2 (International Equipment Co) Use 100 µl of the upper phase, which contains the free insulin and/or C-peptide, directly in the respective radioimmunoassay

- Extraction of total insulin Total insulin was extracted from plasma with PEG as follows Pipette 300 µl of plasma into a test tube Pipette 60 µl of 1 ON of HCl with a precision pipette and mix Allow the mixture to stand at room temperature for 1 h Pipette 60 µl of 1 0 N NaOH with a precision pipette into the plasma-acid mixture and mix Pipette 420 µl of cold 25 per cent PEG solution into the test tube mix for 1 min Centrifuge (2 500 to 3 000 rpm for 30 min) The supernatant fluid should be clear If turbidity is noticed, slight adjustment of pH to 8 0 ± 0 3 is necessary Use 100 µl of the upper phase, which contains the total insulin, in the radioimmunoassay

RADIOIMMUNOASSAY

A modification of the polyethylene glycol method of Desbuquois and Aurbach (1971) was used for the insulin radioimmunoassay The ethanol method of Heding (1975) was used for the C-peptide radioimmunoassay

- Insulin radioimmunoassay The radioimmunoassay procedure was as follows First reaction (overnight, 4°C) to duplicates of 100 µl of standard solutions (containing from 3 to 200 µU of insulin per ml) or samples was added 100µl of antiinsulin guin-pig serum diluted 1 30 000 It was obtained in our laboratory from pork insulin Second reaction (overnight, 4°C) 100 µl of ^{125}I-insulin (50pg) were added It was performed in our laboratory by iodinating pork insulin with ^{125}I according to the general method of Hunter and Greenwood (1962) (chloramine T) with modifications recommended by Freychet et al (1971) After another incubation period at 4°C, 300 µl of cold 25 per cent PEG solution was added in order to separate the free and antibody-bound insulin After centrifugation at 4°C the precipitates were counted

- C-peptide radioimmunoassay (reagents donated by Dr Lise Heading from Novo Lab , Denmark) First reaction (overnight, 4°C) to duplicates of 100 µl of standard solutions (containing from to 0 05 to 1 0 pmole of human synthetic C-peptide per ml or samples was added 100 µl of antisera to human C-peptide M-1230 The antisera M1230 was obtained from a guinea-pig repeatedly immunized with a preparation of synthetic human C-peptide coupled to albumin (Faber at al, 1976)

Second reaction (overnight, 4°C) 100 µl of ^{125}I-Tyrosylated C-peptide (0 03 pmol) was added This tracer was radioiodinated according to the chloramine T method of Hunter and Greenwood (1962) modified by Naithani et al (1975) After another incubation period at 4°C, 1,6 ml of ethanol was added in order to separate the free and the antibody-bound C-peptide After centrifugation at 4°C the precipitates were counted

Observation For both radioimmunoassay systems, ^{125}I-insulin, ^{125}I-Tyr-C-peptide, antiinsulin and anti-C peptide sera were diluted in phosphate buffer (0 04M, pH 7 4) containing human albumin (1g/l) All standards and samples were dissolved and diluted in phosphate buffer (0 04M, pH 7 4) containing NaCl (6g/l) human albumin

Calculations The count-rates for each of the standards and unknown samples were expressed as a percentage of the mean count-rates of the "zero" samples The standard curves were prepared by plotting the values obtained for the standards (expressed as percentages) as the logarithm of concentration on linear log-graph paper The concentration was read directly from the curve for each of the plasma samples

If plasma is used in this procedure without pretreatment, the concentration was read from the standard curve with no further calculation Free insulin and C-peptide concentrations were calculated by multiplying the reading from the standard curve by 2 0 The factor 2 0 is used to correct for the dilution of plasma with PEG

The total insulin in the PEG extract was calculated by multiplying the reading from the standard curve by 2 8 which corrects for the dilution of plasma with PEG

The antibody-bound insulin may then be calculated Bound insulin = total insulin - free insulin

THE SAMPLES

They were collected prior (0) and up to 3 hours (30-60-90-120-180 min) after glucose load in 8 non diabetics, 4 maturity onset diabetics on chlorpropamide and 4 maturity onset insulin-dependent diabetics

The samples from non diabetics control and diabetics on chlorpropamide (CP), the insulin and C-peptide were determined without prior extraction

RESULTS

1 <u>Non diabetics</u> - Basal insulin values were between 0 012 to

0 087 pmol/ml The peak insulin levels at the 30 or 60 minutes sample had a mean values of 0 522–0 656 pmol/ml respectively Fasting C-peptide concentration ranged between 0 10 and 0 33 pmol/ml After the administration of glucose, the levels rose 6 to 16 fold, the mean maximum level of 1 768 pmol/ml being observed at the 90 min sample C-peptide levels were significantly greater at all times (Table 1), except for basal values

2 Maturity onset diabetics on chlorpropamide (CP) – The results of the oral glucose tolerance tests are shown in Table 2 The insulin levels were significantly greater at 180 min sample corresponding to a delayed mean peak of the same magnitude as that in the controls The (CP) patients had normal insulin but not C-peptide responsiveness to glucose load

3 Maturity-onset insulin dependent diabetics – The basal free C-peptide levels were at the limit of sensitivity of the assay and no response to either glucose could be detected (Table 3)

TABLE 1

Insulin and C-peptide levels (mean and SD) during GTT in 8 non diabetics

Time	Insulin		C-peptide	
min	pmol/ml	SD	pmol/ml	SD
0	0 034	0 009	0 212	0 023
30	0 522	0 071	1 393	0 106
60	0 656	0 102	1 712	0 065
90	0 490	0 039	1 768	0 064
120	0 409	0 037	1 625	0 093
180	0 268	0 055	1 300	0 162

TABLE 2

Insulin and C-peptide levels (mean and SD) during GTT in 4 maturity onset diabetics on chlorpropamide treatment (CP)

Time	Insulin		C-peptide	
min	pmol/ml	SD	pmol/ml	SD
0	0 073	0 027	0 202	0 069
30	0 170	0 072	0 430	0 121
60	0 373	0 092	0 445	0 089
90	0 596	0 155	0 737	0 335
120	0 548	0 090	0 642	0 196
180	0 619	0 070	0 535	0 145

TABLE 3

Total, free insulin and C-peptide levels mean and SD during GTT in 4 maturity-onset insulin dependent-diabetics

Time	Total insulin		Free insulin		C-peptide (Free)	
min	pmol/ml	SD	pmol/ml	SD	pmol/ml	SD
0	1 622	0 778	0 035	0 014	0 041	0 005
30	1 542	0 704	0 071	0 022	0 115	0 031
60	1 541	0 516	0 098	0 040	0 110	0 025
90	1 454	0 464	0 138	0 073	0 113	0 039
120	1 966	0 706	0 124	0 063	0 156	0 049
180	1 237	0 518	0 131	0 076	0 162	0 057

DISCUSSION

The antisera to human C-peptide M1230 revealed a mean intra-assay coefficient of variance of 3 2 and 9 6 per cent respectively (Faber et al, 1976) It is interesting to note that, with the same antisera, our results intra-assay was in good agreement with those found by these authors, but in the repeated determinations of C-peptide in different assays in the same control plasma the values were systematically lower at each new assay When the storage was avoided a good results were obtained We determined the concentration of C-peptide on the radioimmunoassay system in plasma sample instead serum as indicated in the literature For these studies only a fresh plasma was used

These results indicated that radioimmunoassay system for C-peptide was shown to be quite suitable for the measurement of low and high plasma levels such as those obtained in normal controls, diabetics or insulin dependent diabetics

The C-peptide radioimmunoassay had opened up the possibility of continuously monitoring beta cell function during the evolution of the diabetic syndrome irrespective of the mode of therapy

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