

A Simple Method for Determining Plasma Insulin in the Presence of Endogenous Insulin Antibodies

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Summary. A simple method for the estimation of plasma insulin in the presence of insulin antibodies is described using dilution and dextran-charcoal separation. In 43 non-insulin resistant diabetics the estimated non-extracted (N.E.) insulin was between 69 and 2444 $\mu\text{U}/\text{ml}$. N.E. insulin appears to be constantly related to values

obtained by acid-alcohol extraction of plasma containing insulin-antibodies, representing about 60% of total insulin.

Key words: Plasma insulin, insulin antibodies, non-extracted insulin, free insulin.

The determination of plasma insulin by radioimmunoassay (I.R.I.) in the plasma of diabetics treated with insulin has necessitated the separation of the endogenous insulin-antibody complex. Heding [1, 2] used acidification, followed by alcohol precipitation and Pearson and Martin [6] gel-filtration, after acidification, to do this. This report describes a method of measuring plasma insulin in the presence of endogenous insulin antibodies by the simple technique of dilution, followed by separation of free and protein-bound insulin with dextran-charcoal. The amount of insulin estimated in plasma by this method represents approximately 60% of total insulin determined by acid-alcohol precipitation. This has been termed non-extracted (N.E.) insulin.

Methods

Plasma collected into lithium-heparin tubes was obtained from diabetic patients, treated with insulin, 12–14 or 24–28 h after the last injection of a short-acting or medium-acting insulin. As the method described has not been quantitated by specific separation of insulin antibodies, the non-specific term “insulin binding”, implying the binding of insulin to plasma proteins is used throughout.

To determine insulin binding in individual plasmas 0.7 ml of ^{125}I -insulin (0.2–0.4 ng), 0.1 ml plasma and 0.1 ml of buffer were incubated for 48 h at 4°C. 2 ml of dextran-coated charcoal was added and after centrifugation the radio-activity was counted in the charcoal precipitate, free and bound counts were calculated and insulin binding expressed as; Percent Binding = bound counts/total counts \times 100. Plasma from normal people and from diabetics who had not received insulin have always had a binding of less than 5% by this procedure in our hands. Plasma insulin was measured by radioimmunoassay using dextran-coated charcoal [3, 5], porcine insulin iodinated with I125 by Chloramine-T,

a pork-insulin standard (Lilly Lot PJ5589-25 I μ /mg) and, a total incubation volume of 1 ml, containing 0.1 ml of plasma. The intra-assay error of the method had a coefficient of 5% and a between assay error coefficient of variation 7.8%, when the final concentration of insulin assayed was between 3 and 40 $\mu\text{U}/\text{ml}$.

Plasma insulin was measured in non-extracted plasma obtained from diabetic patients receiving insulin by diluting each plasma in buffer to three dilutions, either 1/5, 1/10, 1/20, or 1/40. Plasma concentration was equalized by adding an appropriate volume of pooled plasma to the incubation mixture immediately prior to the addition of dextran-coated charcoal. Both undiluted and diluted samples were then assayed for insulin. The bound counts in the blank samples of plasma at each dilution, determined in duplicate, were subtracted from the normal assay samples to which insulin antibody had been added so that the amount of ^{125}I insulin bound to antibody could be determined. In plasma with low binding insulin could be determined with the undiluted sample, but, in most, the estimated level was above the limits of the assay range. By dilution not only was the percentage binding reduced, but the non-extracted insulin was able to be assayed within the standard curve.

Total and “free” insulin were extracted from binding plasmas by the method of Heding (1972). In our hands the performance of this method was similar to that described. Recovery of insulin added to normal plasma was 69% to 82% (mean 75%) and from binding plasma obtained from diabetics on insulin 76% to 109% (mean 95%). Assays of serial dilutions of extracts were linear and binding was always less than 4%.

Results

The assay of insulin in binding plasmas at different dilutions was linear in all cases (Fig. 1). The dilution curve could thus be extrapolated to determine plasma

insulin in the undiluted sample. To three control plasma samples containing insulin but no insulin antibodies and three binding plasmas containing antibodies, standard pork insulin was added to give final added concentrations of 100 $\mu\text{U/ml}$, 500 $\mu\text{U/ml}$ and 1000 $\mu\text{U/ml}$. Samples were then incubated in the normal way and assayed for insulin at appropriate dilution. The re-

covery (79–127%) was similar for both control and binding plasmas (Table 1). The sensitivity of the method determined by the addition of porcine insulin was about 10%, or 25 $\mu\text{U/ml}$ in a sample of binding plasma containing 250 $\mu\text{U/ml}$ N.E. insulin.

In 43 samples obtained from diabetics on insulin there was a significant correlation between percentage

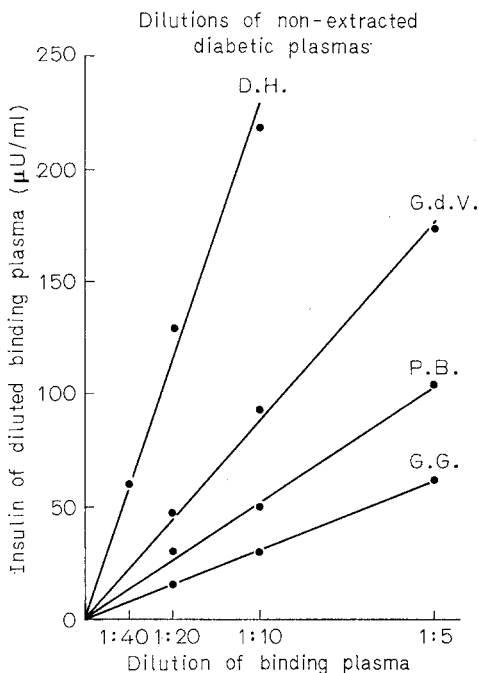


Fig. 1. The assay of N.E. insulin at different plasma dilutions

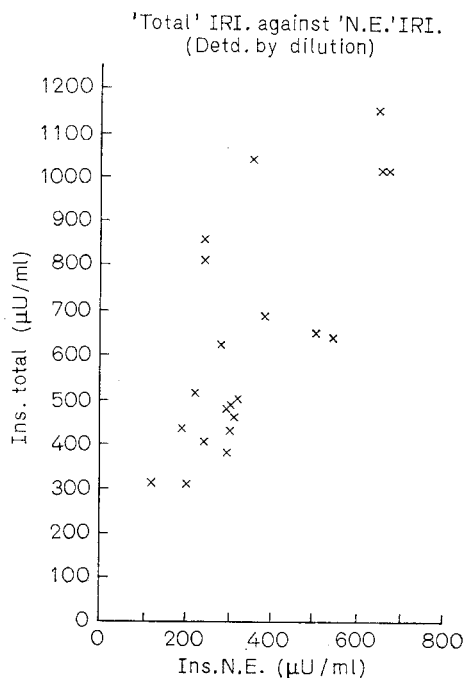


Fig. 3. The relation between total I.R.I. (Heding) and N.E. insulin in 23 diabetics receiving insulin ($r = 0.92$; $p < 0.001$)

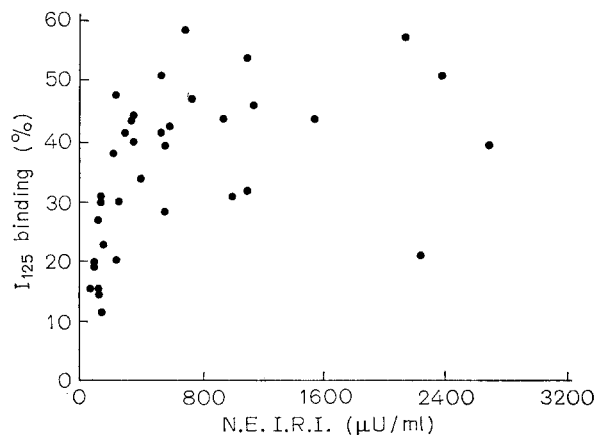


Fig. 2. The relation between N.E. insulin and % binding of ^{125}I insulin ($r = 0.738$; $p < 0.001$)

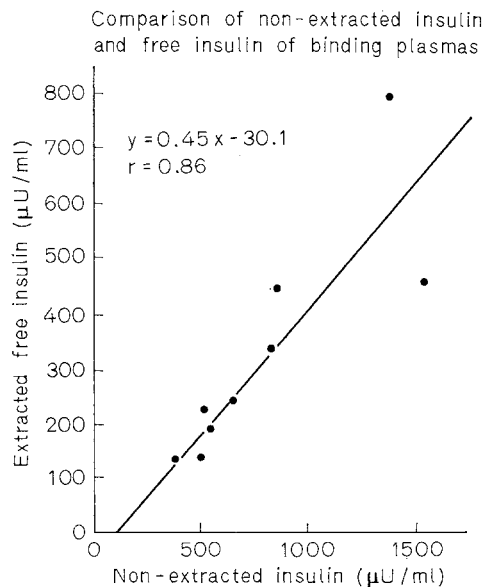


Fig. 4. The relation between “free” I.R.I. (Heding) and N.E. insulin in eight diabetic subjects receiving insulin

binding and N.E. insulin (Fig. 2), but above a binding of 50% no further increase in binding was found, despite increasing insulin levels. Plasma insulin measured as N.E. insulin in the 43 diabetics studied was between 69 and 2440 $\mu\text{U/ml}$.

In 23 binding plasmas extracted by acid alcohol total insulin was found to be closely related to N.E. insulin ($r=0.92$ — Fig. 3) with a ratio of 1.75:1 (N.E. I.R.I. = 57% total insulin). In 8 samples in which "free" insulin was also estimated by simple alcohol precipitation N.E. insulin was greater than the "free" fraction, but constantly related, so that "free" I.R.I. represented about 40% of N.E. insulin (Fig. 4). Con-

firmation that the bound/free ratio of insulin remains relatively constant in non-resistant, insulin-dependent diabetics was obtained by directly comparing "free" and total I.R.I. from extractions of the same sample. There was a significant correlation ($n=8$; $r=0.89$; $p<0.01$) between them, "free" representing approximately 25% of the total I.R.I. This relation was illustrated by the study of a young diabetic woman who injected herself with approximately 3000 units of crystalline insulin zinc suspension in a suicidal attempt. N.E. insulin total and "free" insulin and % binding determined 2 to 7 days after this are shown in Fig. 5. At the time of initial presentation, 48 h after the

Table 1. Recovery of insulin added to non-extracted binding plasma and control plasma

	Insulin added $\mu\mu$	Experiment 1.		Experiment 2.		Experiment 3.	
		Insulin obtained $\mu\mu/\text{ml}$	Recovery %	Insulin obtained $\mu\mu/\text{ml}$	Recovery %	Insulin obtained $\mu\mu/\text{ml}$	Recovery %
	0	383		216		263	
Insulin							
Binding	100	513	106	320	99	359	99
Plasma	500	858	97	885	124	703	92
	1000	1376	99	1260	104	998	79
	0	57		54		70	
Control							
Plasma	100	199	127	171	111	160	94
	500	545	98	465	84	620	109
	1000	1105	105	973	92	1095	102

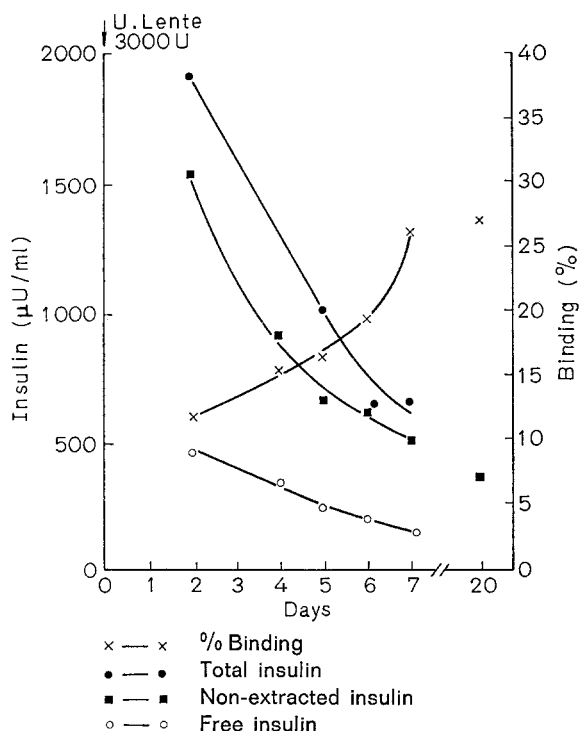


Fig. 5. Changes in N.E. insulin ■—■; total I.R.I. ●—●; "free" I.R.I. o—o; and % binding ^{125}I x—x; following the injection of 3000 units Ultra-Lente insulin in a diabetic female

episode, she had severe but easily treatable hypoglycaemia, which persisted until 96 h; no further insulin was required until 10 days after the episode. The parallel changes in N.E. insulin, total and "free" insulin are all apparent, with a reciprocal increase in % binding to decreased plasma insulin.

One patient with severe insulin resistance requiring 1500 units of insulin a day was studied. N.E. insulin was 99000 $\mu\text{U/ml}$, total I.R.I. 200000 $\mu\text{U/ml}$ and "free" I.R.I. 13000 $\mu\text{U/ml}$.

Discussion

The estimation of plasma N.E. insulin in insulin-binding plasma obtained from diabetic subjects receiving insulin by simple dilution and dextran-charcoal separation gives consistent results. The validity and reproducibility of the method were verified by dilution and recovery studies. Whether more prolonged incubation would lead to increased dissociation of the endogenous insulin-antibody complex is uncertain, but comparison of 5 day versus 2 day incubates on two occasions did not reveal any evidence that this occurred.

The range of fasting plasma insulin measured as N.E. insulin by immunoassay in 45 non-insulin resistant subjects whose insulin dose was between 24 and 96 units a day was 69 to 2440 $\mu\text{U/ml}$. Com-

parative values for total insulin in similar patients by other methods are 6—4347 $\mu\text{U/ml}$ alcohol precipitation [2] and 700—6000 $\mu\text{U/ml}$ by gel-filtration [6]. There was a close relation between N.E. insulin and both the total and "free" insulin determined by Heding's alcohol, acid-alcohol precipitation method. N.E. insulin represented 57% of total insulin and "free" insulin 40% of N.E. insulin. The implied constant relation between free and total insulin was confirmed directly in a small number of plasma samples and in one patient where serial estimates of N.E., total and free I.R.I. were made after a suicide attempt. These results suggest that in non-insulin resistant diabetics antibodies produced by exogenous insulin have relatively constant dissociation between free and bound insulin. This is in agreement with the lack of any relation between acute insulin sensitivity and antibody titres and the commonly observed constancy of insulin dose in stable diabetic subjects, which appears unrelated to antibody titre [2, 4, 6]. Extrapolation of these "in vitro" results to clinical situations must be balanced by the possibility of marked changes in the kinetics of the insulin-antibody complexes. However, it is of interest that in the patient with severe insulin resistance "free" insulin represented only 7% of total I.R.I.

Previous reports of the measurement of insulin by direct immunoassay in the presence of insulin antibodies have used double-antibody precipitation [4] and chromato-electrophoresis [7]. With the former method an increased recovery of added insulin was observed at high levels, but with the present method recovery was linear up to 1000 μU . insulin per ml. Whether this is due to the absorption of endogenous human insulin antibodies by charcoal and their failure to precipitate with the "second" anti-globulin antibody is uncertain. As observed by Moxness *et al.* [4] there was a close relation between the percentage binding to dextran-charcoal and N.E. I.R.I. up to estimated I.R.I. values of 750 $\mu\text{U/ml}$. Above 50% binding the estimated binding did not increase, despite greatly increased I.R.I., which in one case of insulin resistance was 99000 $\mu\text{U/ml}$. Further work is required to establish the relation between I.R.I. and insulin antibody titre determined specifically by immunoelectrophoresis, as separation by dextran-charcoal does not differentiate between specific antibody binding and other plasma protein binding of insulin. The serial studies on the patient who injected herself with a huge

amount of insulin in a suicidal attempt showed that there was a reciprocal relation between percentage binding and N.E. I.R.I. over a ten day period of observation. Her recovery after the injection of approximately 3000 units of insulin zinc-suspension crystalline was no doubt due to the buffering effect of the insulin antibodies as well as to the delayed absorption of the insulin.

We conclude that the estimation of N.E. insulin by the dilution technique using dextran-charcoal separation provides a useful clinical estimate of plasma insulin in the presence of insulin antibodies. The method is simple and uses only 0.2 ml of plasma for duplicate determinations of both N.E. insulin and percentage binding of added I^{125} insulin to plasma protein.

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