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Haemolysis affects insulin but not C-peptide immunoassay

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Summary. Venous blood was taken at the end of a glucose infusion test in 19 individuals and divided into four aliquots, 3 of which were variably haemolysed by repeated passage through a 23-gauge needle to simulate traumatic venepuncture. Plasma insulin (measured by both a charcoal separation and a double-antibody method), C-peptide, and haemoglobin were measured in each aliquot, and haemolysis was also assessed visibly. A significant loss of immuno-assayable plasma insulin was found in samples with only a trace of visible haemolysis, with up to 90% lost in severely haemolysed samples. Plasma C-peptide was unaffected by haemolysis. This represents an additional advantage for the use of plasma C-peptide in assessing insulin secretion.

Key words: Insulin, C-peptide, radio-immunoassay, haemolysis.

The products of haemolysis are known to interfere with immunoassays of insulin which use charcoal separation [1]. It has been suggested that a sample must be visibly pink before assay results are seriously affected [1]. There have been no reports of the effect of haemolysis on immunoassayable C-peptide. The increasing use of insulin and C-peptide assays in research and clinical practice makes it important to know the extent to which haemolysis affects these measurements and could produce seriously misleading results.

Subjects and methods

Blood samples were obtained from 19 relatives of Type 2 (non-insulin-dependent) diabetic patients who were volunteers in a study of familial aspects of Type 2 diabetes. Subjects attended from home and were fasting. An 18-gauge cannula (British Viggo, Swindon, UK) was inserted into an antecubital vein and was used for the infusion of 278 mmol/l glucose at the rate of 5 mg glucose · kg ideal body weight · min^{-1} for 1 h. A second 18-gauge cannula was inserted into a distal arm vein and was used for sampling at 5-min intervals. The hand was warmed with an electric heating pad to 'arterialise' the sampled blood. After 60 min of the glucose infusion, 20 ml of blood was carefully drawn from the free-flowing distal cannula. Four 5-ml aliquots were immediately put into lithium heparin tubes on ice. One sample was left undisturbed and the other three were immediately and variably haemolysed by passage through a 23-gauge needle 2, 4 or 6 times respectively. The samples were then centrifuged at 4 °C at 2500 rpm for 15 min and plasma separated immediately and stored at −20 °C. After traumatisation all samples were handled in an identical fashion. All hormone measurements were performed in the same assay.

Assays

Insulin was measured by two separate assays which differ in their method of separating bound from free insulin. The charcoal separation assay [1] was performed using Novo Human MC insulin (Novo Research Laboratories, Bagsvaerd, Denmark) as standard and Wellcome RD10 (Wellcome Research Laboratories, Beckenham, Kent, UK) guinea pig anti-porcine insulin antiserum. Between-assay coefficient of variation (CV) was 12.9% at 8 mU/l ($n=89$) and 9.9% at 28 mU/l ($n=16$). The double-antibody [2] radioimmunoassay was performed using Novo Human MC insulin as standard, Wellcome RD10 antiserum and Wellcome Sac-Cel donkey anti-guinea pig, solid-phase antiserum. Between-assay CV was 18.2% at 2 mU/l, 5.9% at 39 mU/l and 12.1% at 80 mU/l (all $n=20$). The CV for duplicates of the insulin assays in the study were 12.4% for charcoal separation and 7.6% for double-antibody ($n=76$). C-peptide was assayed by a charcoal separation assay using Novo Human C-peptide synthetic standard and antiserum M1230, and ^{125}I labelled tyr-C-peptide [3]. Between-assay CV was 11.5% at 0.24 nmol/l and 9.1% at 0.8 nmol C-peptide/l ($n=30$). The CV for the duplicates of the C-peptide assays in the study was 5.3% ($n=76$). Hormone assays were performed on plasma rather than serum and fibrin in the thawed samples was removed by shaking of the plasma followed by centrifugation.

Plasma haemoglobin (Hb) was assayed using the Crosby and Furth [4] modification of the method of Bing and Baker [5]. The degree of visible haemolysis was assessed by an experienced observer (MAB) and graded as follows: Grade 0, no visible haemolysis; Grade 1, minimal haemolysis; Grade 2, moderate haemolysis; Grade 3, severe haemolysis.

Statistical analysis

The non-traumatised sample was taken as the baseline sample. The plasma insulin and C-peptide in the traumatised samples are expressed as percentages of the values in the baseline sample. Plasma

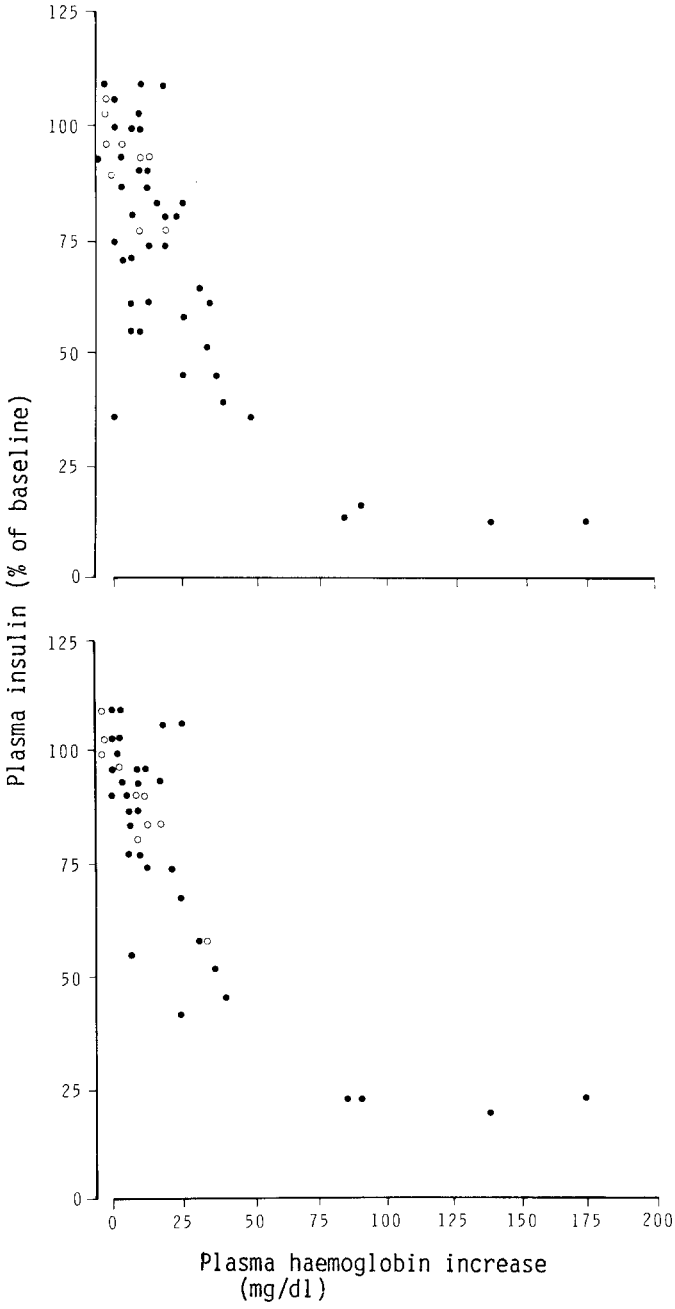

Fig. 1. Relationship between the incremental plasma haemoglobin from the baseline non-traumatised sample and the plasma immunoassay result expressed as a percentage of the baseline sample. O signifies more than one result. Top: Charcoal separation assay. Bottom: Double-antibody assay

haemoglobin is expressed as an increment from the baseline value. The coefficient of variation of an assay was calculated from the assay precision determined from the duplicate values in the assay using the formula:

$$\text{Coefficient of variation} = \frac{\sqrt{\frac{\Sigma\,(\text{difference}^2)}{2n}}}{\text{Mean}}$$

The effect of different grades of visible haemolysis on the insulin assay results was assessed by Student's t-test.

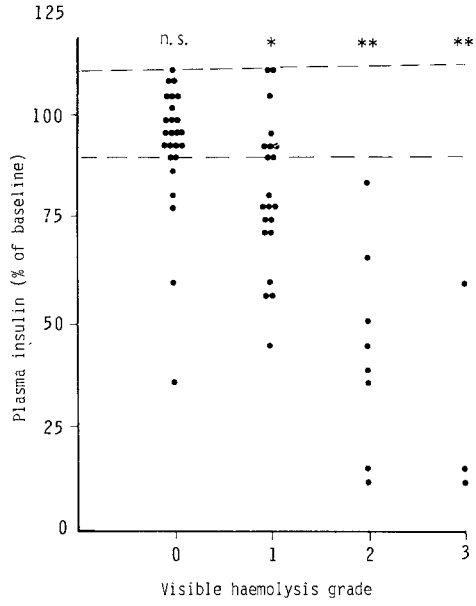

Fig. 2. Relationship between the degree of visible haemolysis and the immunoassayable insulin in the sample in the charcoal phase separation assay. The dotted lines indicate the within-assay coefficient of variation 12.4%. Grade 0: no visible haemolysis; grade 1: minimal; grade 2: moderate; grade 3: severe. $*p < 0.01$; $**\ p < 0.001$

Results

Insulin assays

For all samples in the study, including haemolysed samples, the plasma insulin (geometric mean ± 1 SD) was 9.0 (range 4.7–17.2) mU/l by charcoal immunoassay and 15.1 (range 8.9–25.7) mU/l by double antibody assay. The relationship between the plasma insulin values as measured by the different assays is: double-antibody plasma insulin = 1.06 charcoal-separation plasma insulin + 5.9 mU/l, $r^2 = 0.76$. Both insulin assays showed a marked loss of immunoreactive insulin with sample haemolysis (Fig. 1). There was no correlation between the reduced insulin assay result produced by haemolysis and the initial plasma insulin value. There was significant loss of plasma insulin with Grade 1 (minimal) visible haemolysis ($p < 0.01$) or greater in both insulin assays (Fig. 2). Samples which were traumatised but in which no haemolysis was visible (Grade 0) were not significantly different from baseline, although 2 of the samples gave unusually low results (reduction from 23.9 to 14.6 and 23.9 to 8.8 mU/l respectively).

C-peptide assays

For all samples, the plasma C-peptide (geometric mean ± SD) was 1.03 (range 0.89–1.32) nmol/l. There was no reduction of assayed plasma C-peptide at any level of haemolysis (Fig. 3).

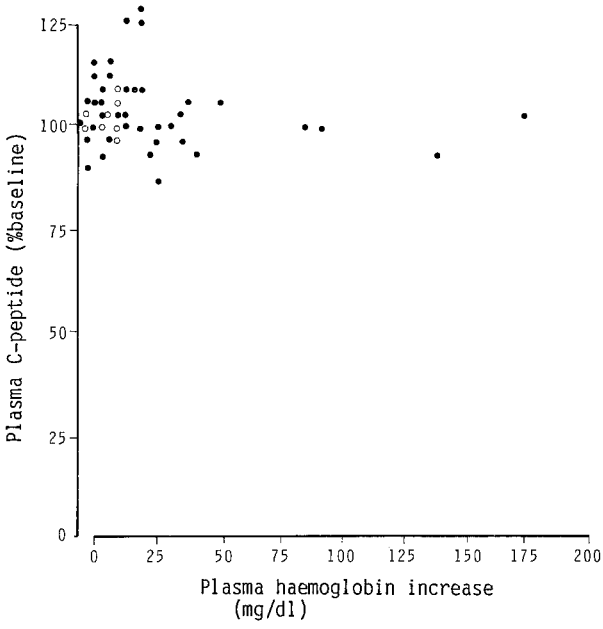

Fig. 3. Relationship between the incremental plasma haemoglobin from the baseline non-traumatised sample and the plasma C-peptide immunoassay result expressed as a percentage of the baseline sample. O signifies more than one result

Discussion

This study shows that even slightly visible haemolysis has a marked effect on reducing immunoreactive plasma insulin, and that in severely haemolysed samples up to 90% of the insulin may be lost. Plasma C-peptide was unaffected by haemolysis. The study was designed to quantify the effect of haemolysis on assays, and does not study the mechanism by which the measured insulin is reduced.

Albano et al. [1] showed that in haemolysed samples the labelled insulin used in the assay tubes was probably denatured as well as the insulin to be measured, but the mechanism was uncertain. The addition of reduced glutathione, which is present in red cells and has the potential for disrupting sulphydryl links, did not appear to be responsible for the insulin loss [1]. The reduction in immunoreactive insulin was found with two different methods of separating bound from free insulin. The two insulin assays gave slightly different quantitative results and this may relate to the use of insulin-free plasma as the diluent for the standard in the charcoal separation assay only. By studying relatives of diabetic patients we have included both normal and genetically affected subjects. A sub-population with an abnormal immuno-reactive insulin is unlikely, as variant insulins are extremely rare [6].

The findings have practical implications. Special care should be taken to avoid traumatising samples intended for insulin assay. If haemolysed samples are all that is available, C-peptide should be used to assess endogenous insulin secretion, although C-peptide is labile if plasma is not stored at −20 °C or if samples are repeatably frozen and thawed. Particular care is needed in the investigation of spontaneous hypoglycaemia, where a spuriously low plasma insulin value may mask the diagnosis of an insulinoma. As an index of B-cell function, C-peptide assay has several advantages over insulin assay; including the absence of hepatic extraction, linear metabolic clearance rate kinetics, low cross reactivity of many antibodies with proinsulin, together with high affinity giving greater precision at fasting concentrations. The lack of susceptibility to products of haemolysis represents an additional practical advantage.

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