Micro Assays for Glucose and Insulin

C. WEINKOVE, E. A. WEINKOVE, B. L. PIMSTONE

SUMMARY

Micro assay techniques for the estimation of blood glucose and plasma insulin in rats and humans are fully described. Only 50 μL of rat blood is needed (5 μL of whole blood for the glucose assay and 10 - 15 μL of plasma for the insulin assay which can be done in duplicate or triplicate). This method has allowed the measurement of glucose and insulin in small animals without the need to sacrifice them. Both assays are sensitive and reproducible. The sensitivity of the rat insulin assay (0,5 ng/ml) was adequate for the measurement of rat plasma insulin, and the interassay coefficient of variation of 10,5% compared favourably with 8,6% found in the conventional assay.

In order to increase the sensitivity of the assay for human plasma insulin, the sample volume is increased to 10 μL and more dilute first antibody is used. Values of plasma insulin from 0,15 to 2 ng/ml (4 - 50 μU/ml) can be determined using heparinised capillary blood from a heel stab, and thus avoiding repeated venepunctures in infants and neonates.

S. Afr. Med. J., **48**, 365 (1974).

During a study of glucose tolerance and insulin release in protein-depleted rats, it became apparent that conventional methods of glucose and insulin assay could not be used since the animals on the low protein diet weighed only 25 - 30 g at the time of investigation. The usual method of estimating blood glucose on the auto-analyser requires a minimum of 0,1 ml of whole blood, and at least 0,2 ml of plasma is required for the plasma immunoreactive insulin determination by means of the conventional techniques.[1] It would thus have been impossible to perform a complete glucose tolerance test with measurement of both insulin and glucose with these older methods without sacrificing animals at varying time intervals. We therefore modified micro assays[2,3] for these determinations as recently reported in an abstract.[4] The use of the micro assay for human plasma insulin determination required further modifications which are fully described in this article.

Groote Schuur Hospital and Department of Medicine, University of Cape Town
C. WEINKOVE, B.SC., M.B. CH.B., F.C.P. (S.A.)
E. A. WEINKOVE, B.SC. HONS
B. L. PIMSTONE, M.D., M.R.C.P.

MATERIALS AND METHODS

Materials

D (+) glucose (M & B laboratory chemicals); 3,3-dimethoxybenzidine dihydrochloride (Eastman Kodak Co.); horseradish peroxidase type VI (approximately 260 purpurogallin units per mg of protein (Sigma Chemical Co.); glucose oxidase, type II, activity 17 300 units per gram (Sigma Chemical Co.); crystalline bovine albumin (BDH biochemicals); guinea pig anti-insulin serum (K5182) and rabbit anti-guinea pig precipitating serum (K7117) (Wellcome Reagents Ltd); twice-recrystallised rat insulin (R170), monocomponent human insulin (12569MC, 24,5 IU/mg) and porcine (S8563) (by courtesy of Dr Lise Heding, Novo Research Laboratories); polyethylene micro test tubes (Beckman Instrument Co.); Beckman Spinco model 152 microfuge; Beckman Spinco micropipettes: 5 μL, 10 μL, 50 μL, 80 μL.

Handling of Rat Blood Samples

As shown in Fig. 1, 50 μL of blood from the tail vein of a rat collected in a heparinised capillary tube is immediately transferred to a micro test tube. Five microlitres of the whole blood is removed with a micropipette and transferred to a clean micro test tube to which is added 80 μL of 2% perchloric acid. After being shaken on a vortex mixer and centrifuged for 90 seconds on a microfuge, 50 μL of the protein-free supernatant is removed and placed into a clean, small test tube for subsequent glucose estimation.

The remainder of the original samples of heparinised whole blood is spun for 90 seconds, and 5 μL aliquots of plasma are transferred to clean micro test tubes and kept at -20°C.

Glucose Assay

For each assay fresh standard solutions containing 0, 50, 100, 200 and 400 mg/100 ml glucose are made up. These standard solutions as well as the internal standard are treated in the following manner: 5 μL of solution + 80 μL of 2% perchloric acid are mixed, spun and 50 μL of the supernatant transferred to clean test tubes. The glucose reagent is made up as described by Lavine *et al.*[2] One millilitre of the freshly prepared glucose reagent is then added to the test tubes containing the supernatants, mixed and placed in a water bath at 30°C for 30 minutes. Absorbance is read in a spectrophotometer at 436 nm against a blank containing glucose reagent alone and plotted against glucose concentration of the standards.

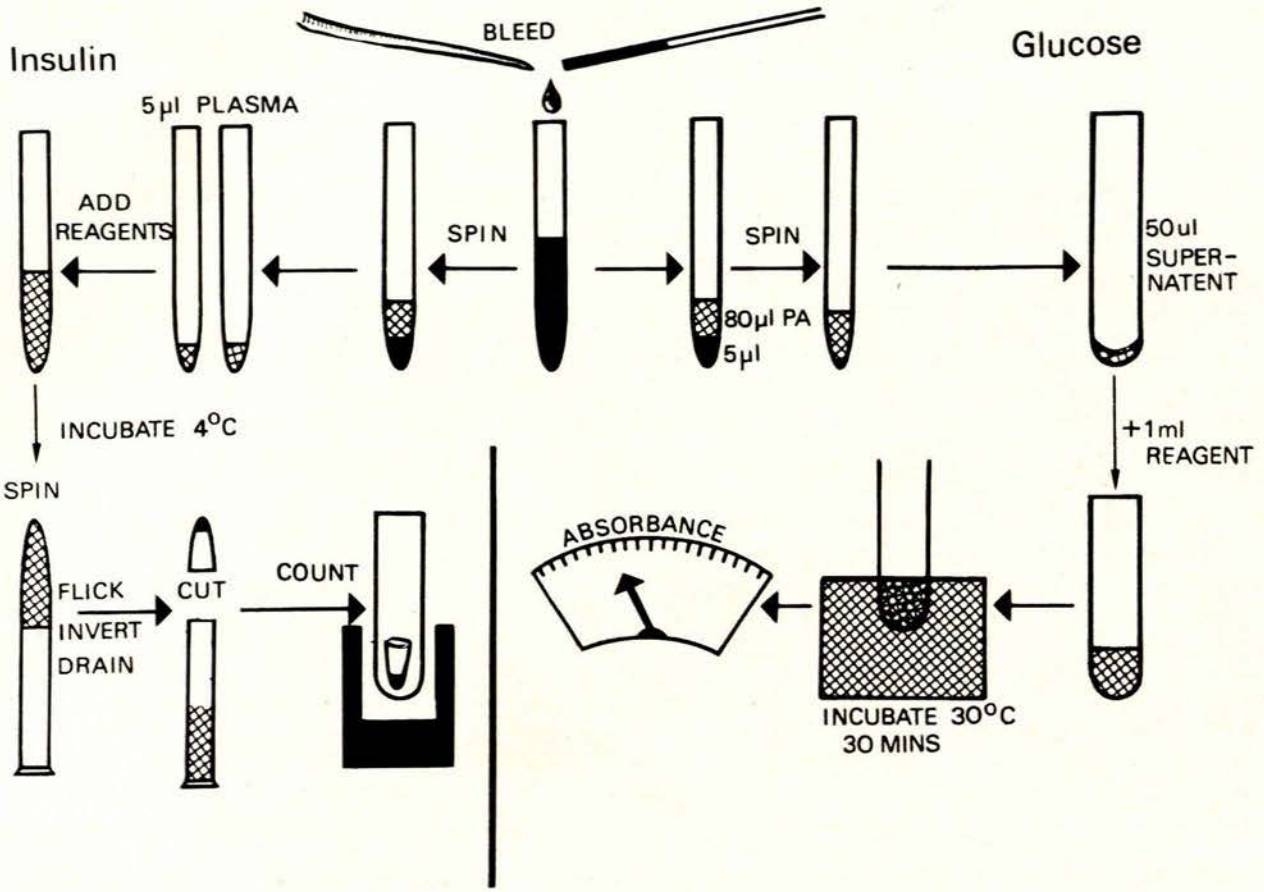

Fig. 1. Schematic outline of the methods used to determine blood glucose and plasma insulin on a 50 µL sample of whole rat blood. PA = perchloric acid.

Insulin Immunoassay Micro Method

The technique is based on the double antibody technique of Morgan and Lazarow.[1] The method used for the estimation of plasma insulin in the rat is described and minor modifications for the determination of human plasma insulin will be described later. Standard solutions of rat insulin are made up to concentrations of 0, 0,625, 1,25, 2,5, 5, 10, 20 and 40 ng/ml. Since, as can be seen from Table I, only 5 µL of plasma and standard solution are used, the volumes of the other reagents are reduced. All dilutions are made in 0,05M veronal buffer pH 8,6, containing 0,25% bovine albumin. In the case of second antibody and carrier plasma, the same buffer is used containing 0,01M EDTA. The reagents are added in the concentration and sequence shown in Table I.

On day 7, the micro test tubes with their caps securely closed are spun for 5 minutes, inverted and 'flicked' as one would flick down a thermometer, and allowed to drain for 1 hour at 4°C. The tapered ends containing the precipitate are cut off above the fluid level with a garden pruner, dropped into test tubes and counted in an automatic gamma scintillation spectrometer. The amount of bound labelled hormone, corrected for non-specific binding is expressed as a percentage of the total labelled hormone added. A typical standard curve is shown in Fig. 2.

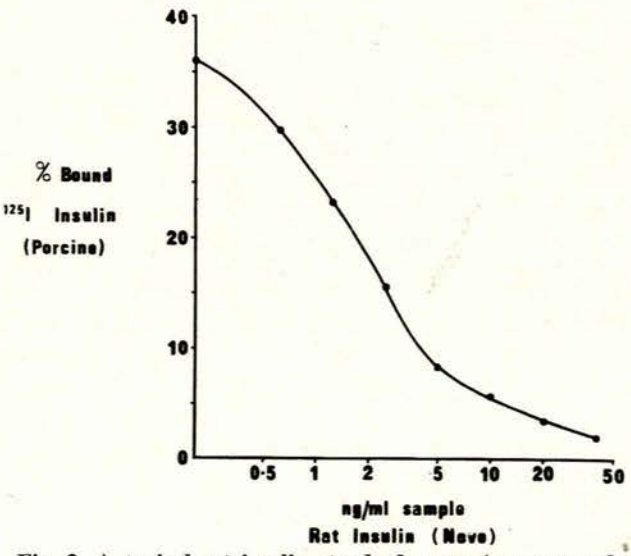

Fig. 2. A typical rat insulin standard curve (see text and Table I for details).

TABLE I. SCHEMATIC OUTLINE SHOWING THE SEQUENCE AND FINAL CONCENTRATION OF REAGENTS USED IN THE MICRO ASSAYS, AS COMPARED WITH THE CONVENTIONAL ASSAY FOR PLASMA INSULIN (ALL INCUBATIONS AT 4°C)

		Micro assay					
		Rat		Human		Conventional assay	
Day added	Reagent added	Vol. (μL)	Final conc.*	Vol. (μL)	Final conc.*	Vol. (μL)	Final conc.*
1	Standard or sample	5	1/21	10	1/11	50	1/35
	Anti-insulin serum	50	1/315 000	50	1/440 000	100	1/420 000
	Buffer	—	—	—	—	450	
2	Insulin ^{125}I†	50	190 pg/ml	50	180 pg/ml	100	30 pg/ml
5	Carrier plasma (guinea pig plasma)	50	1/4 100	50	1/4 200	100	1/9 000
	Rabbit anti-guinea pig plasma	50	1/33	50	1/34	100	1/72

* Final concentrations for reagents added on days 1 and 2 represent dilutions before addition of day 5 reagents.
† Approximately 20 pg of porcine insulin labelled with ^{125}I according to the method of Hunter et al.[5] to a specific activity of 250 - 300 μC/μg.

Modification for the Micro Assay of Human Plasma Insulin

The micro assay described above was not sensitive enough for the detection of basal insulin values in humans. To increase sensitivity the following two modifications were made (Table I): (*i*) unknown and standard samples were added in a volume of 10 μL, other volumes being kept constant; (*ii*) the first antibody was made more dilute to increase the sensitivity. A typical standard curve is shown in Fig. 3.

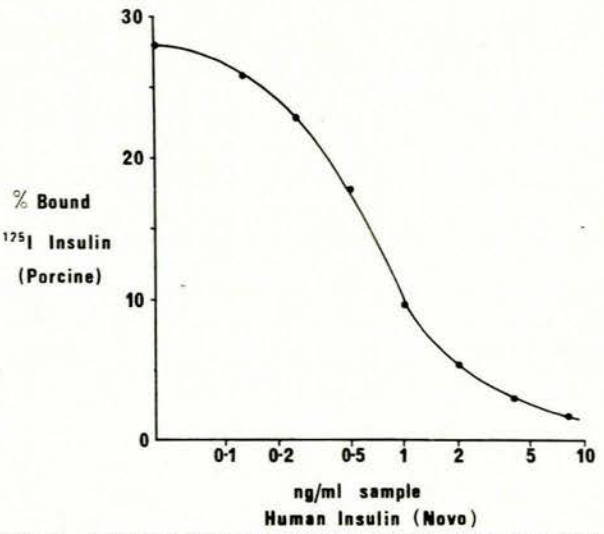

Fig. 3. A typical human insulin standard curve (see text and Table I for details).

RESULTS

Glucose Assay

Absorbance was found to be linear up to 400 mg/100 ml. Our internal standard over a 3-month period showed an interassay coefficient of variation of 4,6% (N = 12).

Insulin Micro-Immunoassay

As can be seen from Fig. 2 our insulin assay could detect a plasma insulin level of 0,5 ng/ml in the rat. The curve shown covers the physiological range of rat plasma insulin, which very rarely drops below 0,5 ng/ml or rises above 10 ng/ml. Our internal standard over a 3-month period showed an interassay coefficient of variation of 10,5% (N = 12).

In the case of human plasma insulin samples, using a 10-μL sample volume with a more dilute first antibody, we were able to obtain sensitivity of 0,15 ng/ml (4 μU/ml) and could measure plasma insulins up to 2,0 ng/ml (50 μU/ml), as shown in Fig. 3. Our internal standard showed an interassay coefficient of variation of 5,2% (N = 5).

Plasma insulin was estimated for the same samples in the micro and conventional assays. There is a good correlation between the results for both the human and the rat insulin assays (Fig. 4).

DISCUSSION

A method has been described whereby small volumes of blood may be used for the measurement of glucose and insulin. In fact, only 50 μL of whole blood is necessary for both assays in rats. The glucose assay utilising 5 μL of whole blood gives consistent results, with an interassay coefficient of variation of 4,6%. The insulin assay done on 5 μL of plasma showed an interassay coefficient of variation of 10,5%, which compares favourably with 8,6% found in our conventional assay. The sensitivity of the micro assay for insulin is 0,5 ng/ml,

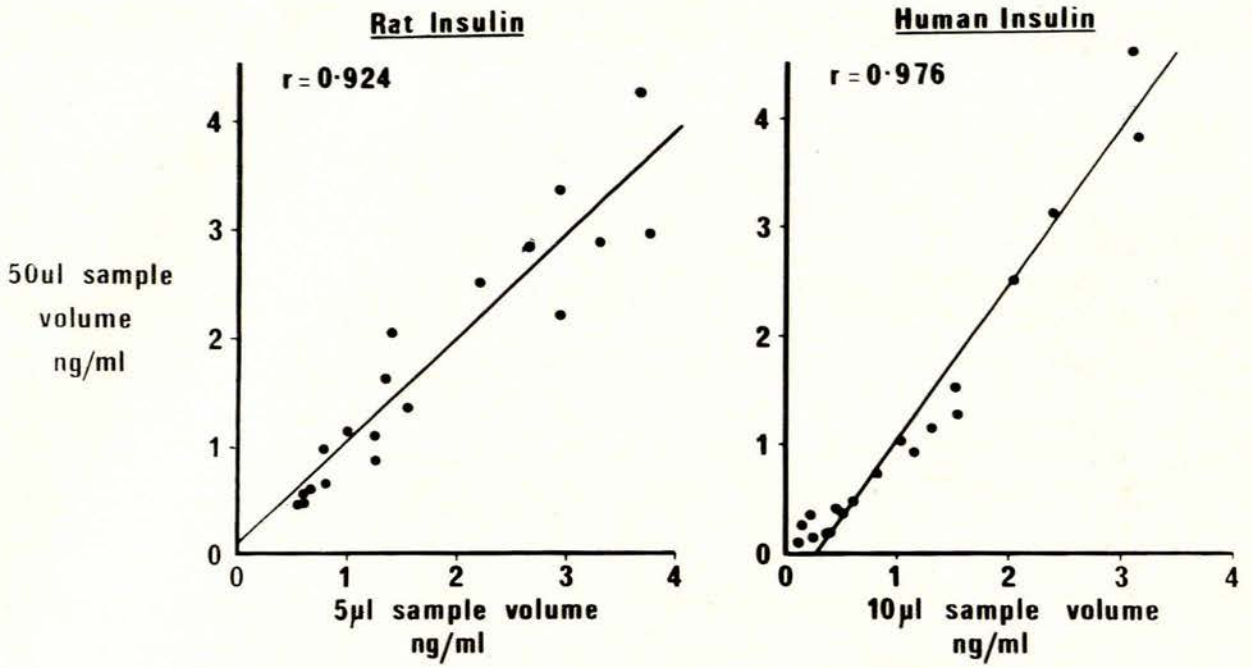

Fig. 4. Comparisons of plasma insulin values as determined by the micro assay and conventional assay.

which is within the normal range for the rat which has fasting levels of insulin much greater than that of humans. After a glucose load the rise in insulin may be up to 10 times that at the fasting level. Therefore, in developing the micro assay we have tried to incorporate this range of expected insulin values into the standard curve. We are now able, by using these assays, to do glucose tolerance tests on malnourished rats without the need to sacrifice them.

In humans where the fasting plasma levels are much lower, we were forced to adjust the insulin micro assay to a different range of values by increasing the volume of sample added and by using more dilute first antibody. Plasma human insulin values, as in the rat insulin micro-assays, showed a good correlation with values obtained using the conventional assay. Although the micro assay is more time-consuming, we feel that in neonates this is justified as repeated venepuncture is avoided. A heparinised capillary tube of blood (100 µL) from a heel stab is quite adequate for both the glucose and plasma insulin estimation.

This work was supported by grants from the Association of Round Tables of Southern Africa through the South African Medical Research Council, the South African Sugar Association and the University of Cape Town Staff Research Fund. We wish to thank Mr A. Isaacs for technical assistance and Dr Lise Heding (Novo Research Laboratory) for donating purified rat and human insulin.

REFERENCES

1. Morgan, C. R. and Lazarow, A. (1963): Diabetes, **12**, 115.
2. Lavine, R. L., Chick, W. L., Like, A. A. and Makdise, T. W. (1971): *Ibid.*, **20**, 134.
3. Tiernan, J. R., Kemball, M. L. and Soeldner, J. S. (1968): J. Lab. Clin. Med., **71**, 345.
4. Weinkove, C., Furnival, E. A. and Pimstone, B. L. (1973): S. Afr. Med. J., **47**, 668.
5. Hunter, W. M. and Greenwood, F. C. (1962): Nature (Lond.), **194**, 495.