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An Ultra-Micro Immunoassay for Insulin (38123)

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In our laboratories, samples containing 1.0 micro unit (μU) insulin could be assayed by the standard double antibody (1) or by the insulin immunoassay using cellulose powder (2). An assay was needed to determine samples containing less than 1.0 μU of insulin, from small spontaneously diabetic Chinese hamsters (3, 4). Previous work by Clark (5) suggested that a micro assay could be developed to meet our requirements. This paper details an ultra-micro procedure capable of measuring as little as 0.02 μU of insulin. Recovery studies on the two procedures compare favorably.

Materials and Methods. This section describes preparation of reagents and procedures for both micro and macro assays. All reagents and standards were diluted with a 1% solution of bovine serum albumin[1] (1% BSA).

The 1% BSA for the micro procedure was prepared by adding 10 g bovine serum albumin and .01 moles of sodium borate to a liter of distilled water, pH adjusted to 7.0 with 1 *N* HCl. Preparation of 1% BSA for the macro procedure was described by Morgan and Lazarow (1). All reagents and standards were stored at −20°.

Insulin standards. Standards for both procedures were prepared from glucagon free insulin, lot PJ4609 (Lilly[2]) and made up in the appropriate 1% BSA. Since each procedure required different buffers, 2 standard solutions of insulin (512 μU/ml) were prepared in the appropriate buffers. These working solutions were further diluted with the appropriate 1% BSA to give 2 sets of standards containing 256, 128, 64, 32, 16, 8, 4, 2 and 1 μU/ml.

Normal guinea pig serum (NGPS). Serum was obtained from normal healthy guinea pig blood.

Anti insulin serum guinea pig (AISGP). Serum was obtained by immunizing guinea pigs with regular insulin (Iletin) as described by Morgan and Lazarow (1). A 1:100,000 dilution of AISGP was used for the macro procedure, prepared by adding 1.0 ml of NGPS and 0.01 ml of AISGP to a liter of the appropriate 1% BSA. A 1:128,000 dilution of AISGP without NGPS was used for the micro procedure.

^{125}I insulin. ^{125}I labeled insulin specific activity 50 mC/mg or greater was purchased from Abbott Laboratories. Two solutions were prepared containing 10 μU/ml each, one for each procedure.

Anti guinea pig serum rabbit (AGPSR). AGPSR was purchased from Antibodies Incorporated.[3] To insure maximum precipitation, highest possible titer was secured. AGPSR for the micro procedure was used undiluted, and 1:3 dilution was used for the macro procedure.

Collection of plasma for the micro method. Approximately 0.1 ml of blood was taken from the orbital sinus of Chinese hamsters (6). It was collected via a capillary tube,[4] in small disposable test tubes containing 5 lambda of heparin solution.[5] The test tubes were kept in an ice bath prior to and after collection of

[1] Nutritional Biochemicals, Cleveland, Ohio. Bovine serum albumin, Fraction V.

[2] Eli Lilly and Company, Indianapolis, Indiana. Glucagon free insulin.

[3] Antibodies Incorporated, Route 1, Box 1482, Davis, California 95616. Anti-Guinea Pig Gamma Globulin Rabbit 6.96 mg/ml.

[4] Scientific Products, Evanston, Illinois. 8 × 32 mm heparinized tubes.

[5] The Upjohn Company, Kalamazoo, Michigan. 1000 USP units of heparin.

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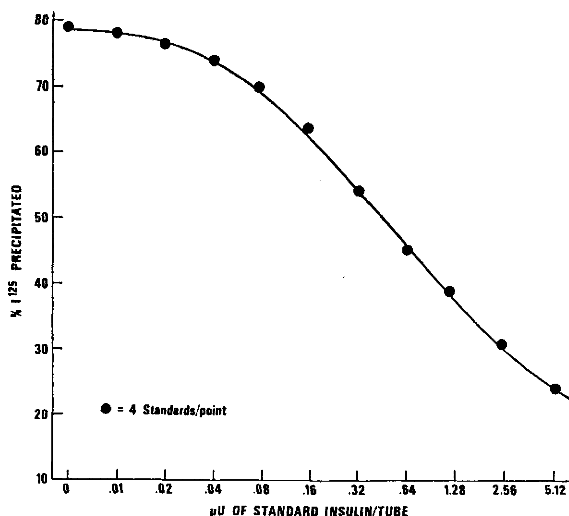

FIG. 1. A Typical Standard Curve for the Micro Immunoassay.

blood; plasma was assayed immediately or in some cases stored at −20° for assay at a later date.

Collection of plasma for the macro method. Blood was collected from numerous Chinese hamsters, the plasma pooled and assayed immediately or stored at −20°.

Ultra-micro procedure. Standards, unknown plasma samples, and blanks were run in quadruplicate. Reactions were carried out in 0.5 ml centrifuge tubes.

First reaction. Solutions were added in the following order: (1) 0.01 ml insulin standards, or unknown plasma samples, or 1% BSA for blanks. (2) 0.02 ml of a 1:128,000 dilution of AISGP. (3) 0.02 ml of a solution of ^{125}I labeled insulin containing 10 μU/ml. Contents of tubes were mixed on a Vortex mixer. Tubes were covered with saran wrap and incubated at 2° for 36 hours.

Second reaction. Solutions were added to the tubes containing the products of first reaction in the following order: (1) 0.02 ml of a 1:100 dilution of NGPS. (2) 0.02 ml of undiluted AGPSR. Contents of tubes were mixed on a Vortex mixer, covered with saran wrap and stored at 2° for 4 hr. After the second incubation, tubes were centrifuged for 3 min at 2000 rpm and the supernatant carefully transferred by means of dropping pipettes into disposible test tubes suitable for gamma counting.

Washing the precipitate. 0.3 ml of 1% BSA were added to the 0.5 ml centrifuge tube containing precipitate. Tubes were mixed on a Vortex mixer and centrifuged for 3 min at 2000 rpm. Supernatant was carefully removed by dropping pipette and discarded. 0.5 ml centrifuge tubes containing precipitate were placed in test tubes suitable for gamma counting. Supernatant and washed precipitate were counted for 10 min each in the Packard Auto Gamma Spectrometer.

Modification of Morgan and Lazarow's double antibody procedure (1). Insulin standards, unknown plasma samples and blanks were run

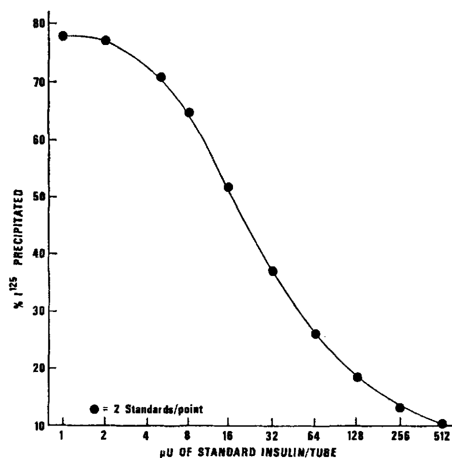

FIG. 2. A Typical Standard Curve for the Modified Immunoassay for Insulin.

in duplicate. The reaction was carried out in 13 × 100 mm disposable culture tubes.

First reaction. Solutions were added as follows: (1) 1.0 ml of insulin standards, or 1% BSA for blanks, or 0.5 ml of unknown plasma samples plus 0.5 ml of 1% BSA. (2) 0.5 ml of a 1:100,000 dilution of AISGP. (3) 0.5 ml of a solution of ^{125}I labeled insulin containing 10 μU/ml. Contents were mixed on a Vortex mixer covered and allowed to react for 24 hr at 2°.

Second reaction. 0.3 ml of a 1:3 dilution of AGPSR were added to each tube. The tubes were mixed on a Vortex mixer, covered and stored for 18 hr at 2°. Upon completion of the 18 hr reaction, the tubes were centrifuged at 2000 rpm for 3 min and the supernatant carefully decanted into disposable tubes suitable for gamma counting. Supernatant and precipitate were counted in the Packard Gamma Spectrometer for 1 min.

Recovery studies. Reliability of the micro procedure was checked by recovery studies, and by direct comparison of the 2 assay procedures. Different pools of plasma were used for individual experiments. To 1.0 ml plasma, 0.1 ml of 1% BSA was added. To a second 1.0 ml plasma 125 μU of insulin in 0.1 ml of 1% BSA were added. The 2 aliquots were assayed by the micro procedure. Three more recovery studies were done with the micro and macro procedures. For the first study a pool of Chinese hamster plasma was divided into 2 aliquots. To the first, 0.1 ml of 0.9% saline was added to each ml of plasma. To the second, 125 μU of insulin in 0.1 ml of saline were added to each ml of plasma and assayed. The last 2 studies were done exactly as the first one mentioned except 50 μU of insulin were added to each ml of plasma.

Calculations. Calculations are based on the isotope dilution principle and are the same as for other immunoassay procedures. The percent of radioactivity in the precipitate (% PPT) was calculated by the following formula.

$$\frac{\text{Count/min precipitated} \times 100}{\text{Count/min in supernatant} + \text{count/min precipitated}} = \%\ \text{PPT}$$

Results. Figures 1 and 2 are typical standard curves. Per cent of counts precipitated are shown on the ordinate, and microunits of insulin standard per tube are on the abscissa. Insulin content of the unknown may be read directly from these curves, since the per cent of counts is known. The result is multiplied by the appropriate factor to give micro units of insulin per ml of plasma.

Figure 3 is a composite standard curve for the micro procedure representing 5 micro assays. The standard errors of the means of the individual points are extremely small (ranging from ± 1.1 to ± 2.6).

The results in Table I show that in two

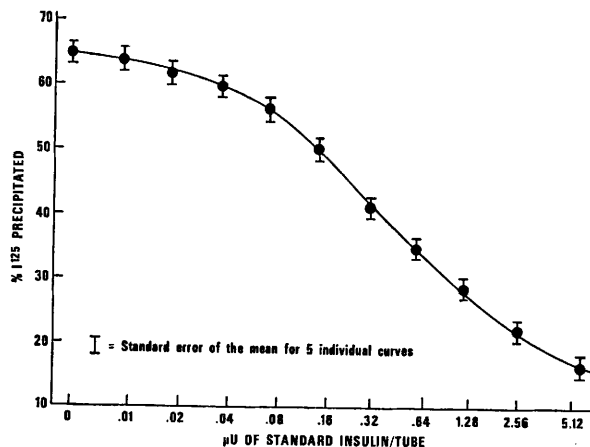

FIG. 3. A Composite Standard Curve for the Ultra-Micro Immunoassay for Insulin.

TABLE I. Recovery of Insulin Added to Chinese Hamster Plasma by the Micro Procedure.

Exp. no.	Plasma + 1% BSA	Plasma + 125 μU Insulin	% of Added insulin recovered
	μU/ml	μU/ml	
1	88 ± 1.9[a] (4)	160 ± 12.0 (4)	75
2	135 ± 1.5 (4)	261 ± 1.5 (4)	82

[a] Standard Error of the Mean. ( ) No. of Samples/Point.

separate studies 75%–82% of the added insulin was detected when the micro procedure was used. Data presented in Table II are obtained from recovery studies using both assays. In all three studies, the insulin detected by the micro procedure was comparable to that detected by the standard macro procedure.

Discussion. In Clark's thesis (5), procedures are described to measure 2 ranges of insulin, from 12.5–200 μU of insulin and from 0.001 to 0.05 μU. Conditions such as concentrations of antibodies, labeled insulin and incubation time were varied to adjust for varying insulin concentration. However, standard curves, and recovery studies were not shown and, therefore, it is difficult to evaluate the sensitivity and reproducibility of his assays. Further, the range from 12.5 to 200 μU was not adequate for our purposes. The second procedure exceeded our requirements and was very time consuming, since it required a 72 hr incubation.

The assay reported in this paper covers a range which satisfies our requirements and is intermediate between those ranges reported by Clark (5). Further, it has the advantage of a shorter incubation time and is capable of measuring a broader range of insulin samples. As an example a 250 fold difference in insulin content can be assayed, compared to a 50 fold difference for Clark's (5) most sensitive procedure.

The ultramicro assay described in this communication is capable of measuring insulin in samples containing from 5.12 μU to 0.02 μU. Although 0.02 μU of insulin in a sample may be detected, the assay is most sensitive as judged by the slope of the curve (Fig. 1) for samples containing 0.04–2.56 μU of insulin. The assay appears to be highly reproducible. This is indicated by the composite curve (Fig. 3), since the standard errors of the means of individual points are extremely small in the sensitive part of the curve (approximately 2% of the mean). Further, recovery studies suggest that the assay consistently detects a satisfactory amount of added insulin. In two separate experiments (Table I), 75% and 82% of added insulin was detected. The data in Table II demonstrate that the micro procedure detected as much added insulin as the widely used standard procedure.

When 0.02 ml of the usual dilution of

TABLE II. Comparison of Recovery of Added Insulin, Double Antibody Macro Procedure *vs.* Double Antibody Micro Procedure.

Exp. no.		Macro procedure		Micro procedure	
		Insulin, μU/ml	% Recovered	Insulin, μU/ml	% Recovered
1	Plasma + Saline	95 ± 8.4[a] (3)	—	87 ± 10.9 (4)	—
	Plasma + 125 μU	167 ± 10. (8)	76	174 ± 6.9 (8)	82
2	Plasma + Saline	173 ± 1.6 (6)	—	184 ± 10.8 (4)	—
	Plasma + 50 μU	203 ± 4.3 (4)	91	210 ± 18. (4)	90
3	Plasma + Saline	163 ± 4.8 (6)	—	165 ± 2.5 (6)	—
	Plasma + 50 μU	198 ± 3.3 (6)	93	200 ± 5.8 (4)	93

[a] Standard Error of the Mean. ( ) No. of Samples/Point.

AISGP (1, 2, 5) was used for the micro assay, the curve was very flat, the per cent precipitated ranged from 30 to 58. This was very insensitive and would not allow detection of a difference between 0.04 and 2.56 μU. In order to determine the concentration giving the most sensitivity, the assay was carried out using dilutions ranging from 1:1,000 to 1:400,000. Numerous experiments suggested that 1:128,-000 provided the most sensitive curve.

To insure sufficient count above background, it is essential to use freshly assayed labeled ^{125}I insulin with a specific activity greater than 50 mc/mg. Labeled ^{131}I insulin may be substituted for ^{125}I insulin provided its specific activity is greater than 50 mc/mg.

Several preparations of AGPSR were tried, but in general gave less satisfactory results than the preparation described in the Materials Section. Lower titer AGPSR did not give maximum precipitation, therefore resulting in a flat curve.

The normal plasma insulin value in fasted Chinese hamsters is approximately 50 μU/ml (7). The application of this procedure permits the use of a sample as small as 1.0 lambda. The preliminary results of this procedure demonstrate that the assay is sufficiently sensitive to determine plasma insulin levels in very small preweanling and weanling Chinese hamsters (3, 4), serial bleeding for glucose and insulin tolerance tests, and pancreatic insulin levels in small embryos (5). Ultimately, it may be applicable for the assay of individual beta cells in tissue culture systems.

It must be pointed out that the assay is tedious and time consuming. Extreme care must be taken when pipetting such minute volumes. It is also highly important that all glassware be specially cleaned. Glassware must be hand washed with detergent and thoroughly rinsed. Therefore, the procedure is not recommended for routine use, but only when the sample size or insulin content require extreme sensitivity.

Summary. This paper describes in detail an ultra-micro double antibody assay that is capable of determining insulin in samples containing from .02 to 5.12 microunits (μU) of insulin; however, the assay is most sensitive for samples containing .04–2.56 microunits. This method compares favorably with the widely used double antibody procedure of Morgan and Lazarow (1).

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