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Short Communications

The use of microtiter plates for the simple and sensitive determination of insulin by an ELISA method

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Summary. A method of insulin determination using a commercially available ELISA kit was modified for use in microtiter plates. The adapted assay, based on the binding of porcine anti-guinea pig insulin antibodies to microtiter plates and insulin-peroxidase conjugate as displacer, is sensitive between 0.5 and 30 ng/ml. Since it uses only 10–40 µl of sample material it enables the determination of 5–100 pg of insulin. The rapid (5–6 h), automatable, reproducible and reliable assay makes it possible to determine many samples in a short time.
Key words. ELISA; insulin determination; peroxidase; microtiter plates.

A number of methods are currently available for the determination of immunoreactive insulin (IRI) in biological samples. The most widely used methods are radioimmunoassays, utilizing ^{125}I-insulin as tracer and a variety of methods for the separation of bound from free insulin[1–3].

A new method, the Enzymun-test® insulin[4], was recently made commercially available by Boehringer-Mannheim. This method relies on the competition between insulin-peroxidase conjugate and insulin in binding to insulin-antibodies coated onto plastic tubes, and a colorimetric determination of the bound peroxidase conjugate. We have adapted and modified this assay for the use in microtiter plates. By using microplate technology and this modified ELISA system hundreds of samples could be cost-effectively determined in a very short time. This modification may enable clinical and research laboratories to determine human or rat insulin easily by utilizing readily available, automatable systems while avoiding the problematic use of isotopes.

Materials and methods. Preparation of plates. For the routine determination of insulin, round-bottom microwell plates (Microwell module U-16 Nunc, immuno quality 80 PCS, elevated absorption capacity) were used. Anti-porcine insulin guinea pig serum (Novo Biolabs M8309) was diluted with 45 ml distilled water to give a final dilution of 1:9000. 100 µl of the antibody solution was pipetted (using a 12 channel pipette) into the wells. The plates were evaporated to dryness at 28 °C for 24–30 h in a Dynatech microtiter incubator (under circulating air). Once dryed, the plates were sealed and stored at 0–4 °C until used.

Assay. 10–40 µl samples were deposited in the wells and 200 µl of phosphate buffer 40 mM pH 6.8 including 0.25% BSA (RIA grade, Sigma) were added. Plates were covered and samples were incubated 2 h at 37 °C in a Dynatech microtiter incubator. Following the incubation, the samples were withdrawn and the plates washed once with 250 µl cold (0–4 °C) tap water. 100 µl of insulin-peroxidase conjugate solution (Bovine insulin-peroxidase, Sigma, 250 U/mg protein at final concentration of 5 mU/ml in phosphate buffer 40 mM pH 6.8, BSA 0.25%), which was prepared at least 1 h before the experiment and stored in dark at 25 °C, was added and incubated for 2 h in the dark at 25 °C.

Following the incubation, the samples were withdrawn, washed once with 250 µl of cold tap water and dried.

100 µl of chromogene (ABTS®, 9.1 mM, Boehringer-Mannheim) were added and incubated at room temperature (20–25 °C) for 1 or 2 h. The absorbance at 405 nm was read using the MR700 microplate reader (Dynatech) using 100 µl chromogene as blank. For the determination of rat or human insulin levels, rat insulin (Novo Biolabs) or human insulin (Boehringer-Mannheim) prepared in phosphate buffer 40 mM pH 6.8, BSA 0.25%, were used as the respective standards. Standard curves were run in parallel to experimental points at all times.

Radioimmunoassays (RIA) of human and rat insulin were conducted using the NOVO insulin determination kit (Novo Biolabs, Denmark) according to the manufacturer's instructions.

Results and discussion. The Enzymun-test® insulin (Boehringer-Mannheim)[4] was modified and simplified so that smaller quantities of plasma could be rapidly analyzed using microtiter plate technology.

After several methods of binding different insulin antibodies to activated or non-activated microtiter plates had been tried, the present method was adopted, as it was found to be the most reproducible and reliable. Using the NOVO porcine anti-guinea pig insulin antibodies and high absorption microtiter plates, maximal binding to the plates was achieved at antibody dilutions of 1/6000–1/8000, and no detectable binding at 1/32000 dilution (data not shown). Using 1/8000–1/9000 dilution of this antibody, very high (0.8–1.2 OD) extinction values were obtained at 405 nm within 2 h of incubation with chromogene (in the absence of displacer insulin). The displacement with rat insulin resulted in typical insulin standard curves (fig. 1). Although reliable insulin values could be determined up to 30 ng/ml, a better resolution, using linear plotting, is obtained between 0.5 and 10 ng/ml of rat insulin, after 1 or 2 h of incubation with chromogene (fig. 1, inset). This range of determination, which resulted in curves of corr. coeff. $\geqq 0.95$ (fig. 1, inset), enabled rapid calculation of the experimental points, without the necessity of a computer interface to the spectrophotometer.

From the comparison between the present method and the commercially available RIA kit (fig. 2), it can be seen that qualitatively similar curves were obtained for human (fig. 2A) or rat (fig. 2B) insulin with both methods. Furthermore, good correlation lines are obtained when the samples are compared in both assay methods, resulting in essentially identical results ($y = 1.003\,x - 0.08$, $r = 0.995$, $p < 0.01$, $n = 18$ (fig. 3).

Serial dilutions of rat or human plasma samples from 1:1 to 1:10 in phosphate buffer 40 mM pH 6.8, BSA 0.25% resulted in linear curves with a maximum coefficient of variation of 15%. The sensitivity of the assay defined as the concentration of insulin reducing 5% of the initial OD was 0.5 ng/ml, thus indicating the possible measurement of as little as 5 pg of insulin.

When the expected values for added insulin were compared to the ones measured, a good recovery (85–135%) was obtained (date not shown). Statistical analysis of the quality control experiments (table) revealed acceptable precision, with a coefficient of variation usually between 10 and 15%. As a routine assay, we currently use 20 µl of rat, mouse or primate plasma, 2 h incubation at 37 °C and color yield at room temperature for 2 h. Qualitatively similar results could be obtained by reducing the sample to 5 µl or increasing to 40 µl. In addition, similar results were obtained by incubating the samples with the plates for 18 h at 0–4 °C and then processing as described. As shown in the figure 1 inset, reliable coloration already developed after 1 h of incubation at

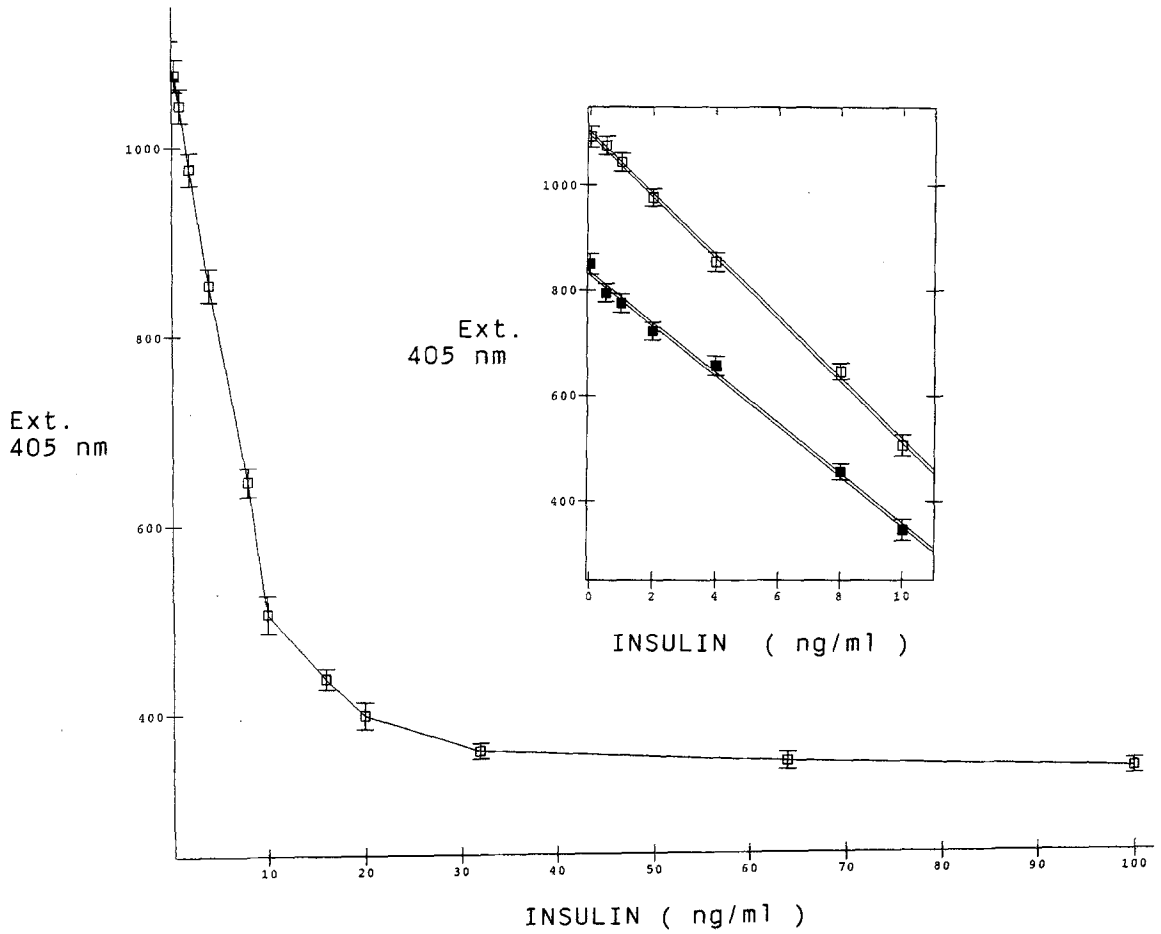

Figure 1. Rat insulin standard curves using microtiter plates. Determinations were performed using 20 µl of rat insulin standard (NOVO) as described at 1 h (■) or 2 h (□) of color development at 20 °C. *Inset:* linear fitting of 0–10 ng/ml of rat insulin. Yielding at 1 h a linear curve ($y = -48\,x + 830$, $r = 0.97$) and at 2 h a linear curve ($y = -58\,x + 1100$, $r = 0.99$).

Variation between and within assays

Expected (ng/ml)	Between assays (n = 12) Detected (ng/ml) Mean	Range	SD	C.V. (%)	Within assay (n = 8) Detected (ng/ml) Mean	Range	SD	C.V. (%)
20	19.92	15.5 -24.9	2.18	10.9	20.29	16.8 -22.9	2.0	9.9
16	16.35	12.5 -20.6	2.5	15.4	16.26	14.0 -17.6	1.23	7.6
10	10.16	8.61-12.7	1.42	14.0	9.73	8.3 -11.4	0.9	9.3
8	8.82	7.3 -10.7	0.9	10.5	8.06	7.7 - 9.8	0.8	10.0
4	4.33	3.4 - 5.7	0.6	14.5	4.01	3.2 - 5.1	0.55	13.8
2	1.65	1.25- 2.3	0.3	18.7	1.98	1.7 - 2.5	0.25	12.9
1	1.06	0.78- 1.2	0.12	12.0	1.12	0.85- 1.3	0.15	14.0

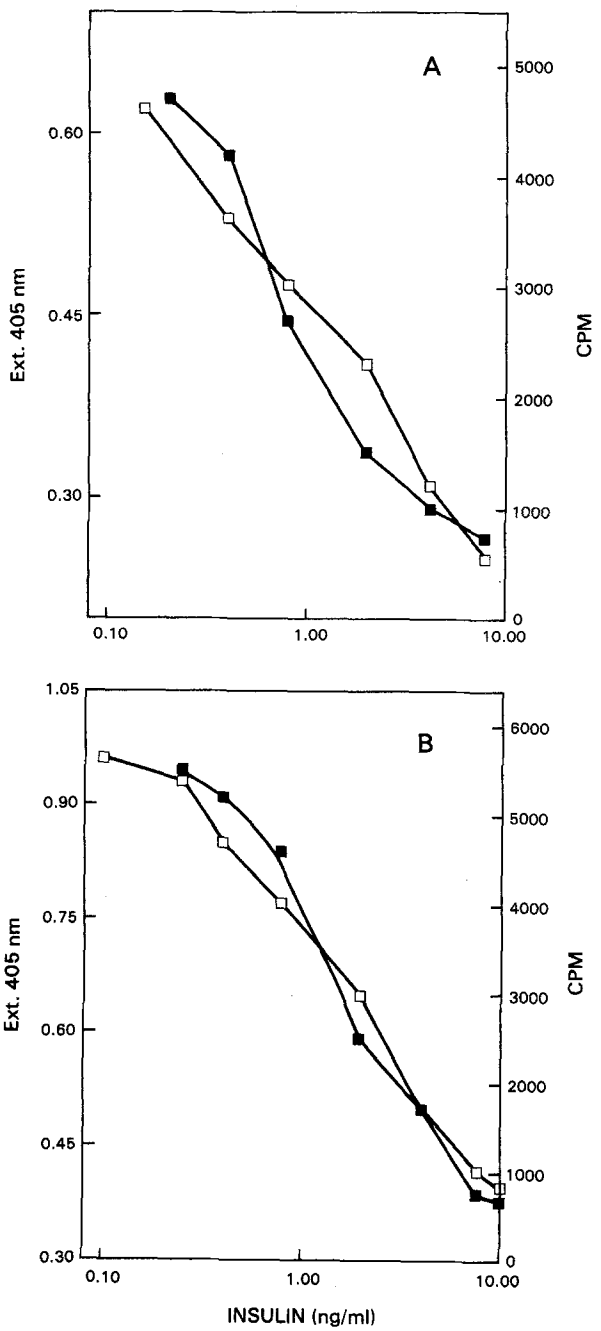

Figure 2. Comparison between ELISA (□) and RIA (■) methods for the determinations of human (A) or rat (B) insulin. Data from representative experiments done in duplicate, after 1 h of color development and RIA as specified by the manufacturer (NOVO Biolabs, Denmark).

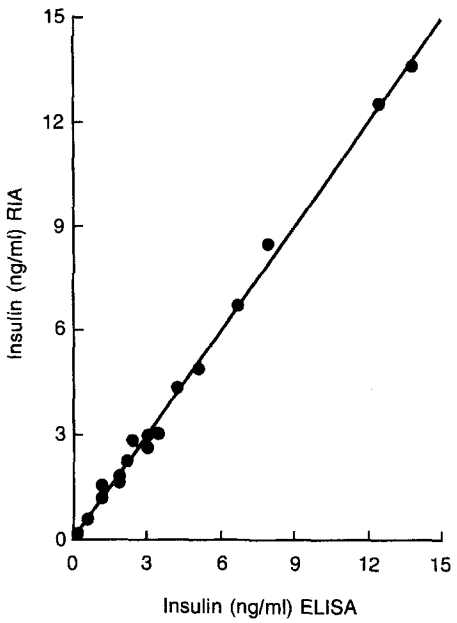

Figure 3. Comparison between ELISA and RIA determination of insulin in rat plasma. Data from a representative experiment done in duplicate after 1 h of color development and RIA as specified by the manufacturer (NOVO Biolabs, Denmark). Serum samples were obtained by decapitation following the treatment with saline or oral glucose load (1.5 g/kg p.o.) (n = 18). Regression analysis results in a linear relationship ($y = 1.003\,x - 0.08$, $r = 0.995$, $p < 0.01$).

room temperature, although steeper curves were obtained at 2 h. After 3 h of color development, the color was stable overnight at 0–4 °C, thus enabling the determination the following day. Preliminary results seem to indicate that this assay is not affected by hemolysis.

Using 96-well microtiter plates, which enable rapid and automatable pipeting, washing and spectrometric determination of relatively small samples, without any extraction or separation techniques, it was possible accurately (standard error of assay $\leq \pm 5\%$) and rapidly (5–6 h) to determine a very large number of samples from human, rat or mouse insulin.

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