THE DEMONSTRATION OF PROLONGED ACTION OF LONG-ACTING INSULIN PREPARATIONS IN THE GUINEA-PIG

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SINCE Reiner, Searle and Lang[1] first prepared globin insulin and described its prolonged action in rabbits, the preparation has been used with success by diabetics throughout the world in the control of hyperglycæmia. When the Provisional British Standard for Globin Zinc Insulin was examined for prolonged action in rabbits against the International Standard for Soluble Insulin, Emmens *et al.*[2] concluded that "no routine method of estimating the delayed action of globin zinc insulin can be recommended since a detailed investigation revealed that the rate of recovery of rabbit blood sugar following the decrease induced by globin zinc insulin is biometrically indistinguishable from that induced by soluble insulin unless an impracticably large number of animals is used in a cross-over test".

The United States Pharmacopeia XIV, however, outlines a test for the demonstration of prolonged action of globin zinc insulin in rabbits using as a standard a preparation of U.S.P. Zinc-Insulin Crystals Reference Standard combined extemporaneously with the U.S.P. Globin Reference Standard prior to the test. Dr. Ferry[3] of Burroughs Wellcome and Co. (U.S.A.) Inc., has provided data which show the prolonged action of globin zinc insulin when compared with soluble insulin in rabbits.

The success of American workers in demonstrating a prolonged action with globin zinc insulin in rabbits may in part be due to the type of rabbit used or, in part, to the diet which is fed to the animals.

The details and data which follow describe the use of the guinea-pig for the demonstration of prolonged action of long-lasting insulin preparations.

METHOD

All guinea-pigs used were albinos, either home-bred or obtained from dealers. They were separated as regards sex and given an *ad lib.* diet of greens, diet 18[4] and water. 16 hours prior to the commencement of the test the animals were taken into the laboratory and food and water removed from their cages. For any one test the animals were either all males or all females. On the day of the test the guinea-pigs were weighed to the nearest g. and placed in individual cages. The weight range of the guinea-pigs has been from 200 to 500 g. though for any one test the heaviest has not exceeded the lightest by more than 160 g. Immediately before the injection of the insulin preparation a little over 0·1 ml. of blood was removed from the heart using a 23 S.W.G. $\times \frac{5}{8}$ needle fitted to a 1 ml. tuberculin syringe. The blood sample was straightway fed into an 0·1 ml. graduated micropipette, and the accurately measured 0·1 ml. of blood passed into the deproteinising suspension of Somogyi[5]. Individual reducing sugar con-

centrations were determined by the method of Hagedorn and Jensen[6]. After the collection of the initial blood sample the undiluted insulin preparation was injected subcutaneously above the pelvic girdle using a micrometer syringe. The absolute sensitivity of guinea-pigs is less than that of rabbits and the doses used have ranged from 1·5 to 3·0 I.U./kg. depending upon environmental temperature and weight of animals, though kept constant for any one experiment. Following each injection the time was noted, and at hourly intervals thereafter 0·1 ml. samples of blood were collected in a similar manner.

Since we wished the blood sugar concentrations of guinea-pigs receiving soluble insulin to return at the 6th hour to 90 per cent., or greater, of the initial value it was necessary to exercise each guinea-pig 15 minutes prior to the collection of the blood sample by holding the hind limbs off the bench and allowing the animal to exercise itself with its fore limbs for 30 seconds. This exercising was carried out at each hour whether a blood sample was collected or not. Guinea-pigs ear-marked at the end of the test could be used for a reverse test after a rest period of one week.

The data which follow are derived from tests carried out from January to June, 1953 in a laboratory where the environmental temperature was from 50° to 78° F., though on any one day it never ranged more than 10° F.

Results

The results are presented as mean blood sugar values (as a percentage of the initial) at each hour for which determinations were made.

All data were submitted to variance analyses, and the significance of differences between mean blood sugar values for prolonged action and soluble insulins at each hour was determined by means of Student's t-test[7].

The soluble insulin used in all the experiments which follow was the British Insulin Manufacturers' Sub-standard of Crystalline Insulin having a potency of 23·35 I.U./mg.

Typical Analysis

The Provisional British Standard for Globin Zinc Insulin was tested against soluble insulin in a 1-day experiment. The animals were virgin albino females. Table I shows the mean blood sugar values for the globin and soluble insulins, and the significance of the differences.

Table II gives the analysis of variance of the data.

TABLE I

Blood sugars as a percentage of initial values

Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
British Globin Standard ..	79·1	60·2	56·0	60·1	59·5	60·2	8
Soluble insulin	72·1	59·0	62·6	66·0	81·1	96·3	8
P difference	*—	—	—	—	<0·1	<0·01	

Weight range: 230 to 292 g. Dose: 2·5 I.U./kg.

* In this table, and all subsequent tables — indicates that the P difference is >0·2.

Differences between insulins must be tested against the mean square for "between animals within treatments", in this instance 473·20. This value is therefore used as the estimate of the error variance for testing significance of differences between means. Since each mean in Table I is based upon 8 observations the variance of any mean is $\frac{473{\cdot}20}{8} = 59{\cdot}15$. As all means in the table have the same variance, the variance of the difference between any two means is twice the variance of a single mean, i.e., $59{\cdot}15 \times 2 = 118{\cdot}30$. The standard error of the difference is therefore $\sqrt{118{\cdot}30} = 10{\cdot}88$, which is used to calculate "t", for the significance of the differences *P* given in Table I. It will be noted that in order to perform the "t" test it is only necessary to make part of the detailed analysis of variance. Provided the mean squares for between treatments and between animals within treatments are available, the remainder of the analysis is unnecessary for routine purposes.

TABLE II

ANALYSIS OF VARIANCE

Source of variance	Degrees of freedom	Sums of squares	Mean square	P
Between insulins	1	2565·77	2565·77	<0·05 >0·01
Between hours	5	5415·54	1083·11	
Hours × insulins	5	5028·23	1005·65	
Between animals within treatment	14	6624·82	473·20	
Within animals	70	4845·21	69·22	

COMPARISON OF THE "CROSS-OVER" TEST WITH ONE-DAY TESTS

A "cross-over" test on a routine production batch of globin zinc insulin (G.Z.I.) was carried out in male albino guinea-pigs. Table III gives the mean percentage blood sugar values, averaged over the two days, and the significance of differences as before.

TABLE III

BLOOD SUGARS AS A PERCENTAGE OF INITIAL VALUES

Hours after injection	1	2	3	4	5	6	Number of animal observations/ preparation
G.Z.I.	70·1	47·7	49·9	56·7	61·8	68·5	16
Soluble insulin	68·8	55·6	71·6	92·2	97·7	101·5	16
P difference	—	—	<0·01	<0·001	<0·001	<0·001	

Weight range: 256 to 351 g. Dose: 2·0 I.U./kg.

The "cross-over" test therefore demonstrates satisfactorily the prolonged action of globin zinc insulin in guinea-pigs. Since it is normally necessary to wait one week before performing the second half of a "cross-over" test (as with rabbits) several one-day runs were carried out to find out whether they were satisfactory for routine testing. Tables IV (*a*), (*b*), and (*c*) show the results of 3 such trials with 3 different routine production batches of globin zinc insulin.

The 1-day test is therefore quite reliable for the demonstration of prolonged activity.

TABLE IV

BLOOD SUGARS AS A PERCENTAGE OF INITIAL VALUES

(a) Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
G.Z.I.—A	77·0	43·0	43·4	54·6	71·0	78·0	7
Soluble insulin ..	80·0	57·7	69·7	103·9	107·1	108·2	7
P difference	—	—	<0·01	<0·001	<0·001	<0·01	

Weight range: 303 to 363 g. Dose: 2·0 I.U./kg.

(b) Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
G.Z.I.—B	70·6	63·6	49·2	55·3	55·7	72·3	8
Soluble insulin ..	66·8	64·7	63·9	86·1	94·2	112·7	8
P difference	—	—	—	<0·01	<0·001	<0·001	

Weight range: 232 to 301 g. Dose: 2·5 I.U./kg.

(c) Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
G.Z.I.—C	69·6	46·1	52·3	43·5	52·4	55·8	7
Soluble insulin ..	59·0	44·5	53·4	80·5	93·1	100·0	7
P difference	—	—	—	<0·01	<0·01	<0·01	

Weight range: 223 to 303 g. Dose: 2·0 I.U./kg.

THE PROLONGED ACTION OF PROTAMINE ZINC INSULIN

Table V shows the prolonged action in guinea-pigs of a commercial sample of protamine zinc insulin.

TABLE V

BLOOD SUGARS AS A PERCENTAGE OF INITIAL VALUES

Protamine Zinc Insulin. (P.Z.I.)

Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
P.Z.I.	114·9	92·5	71·0	59·7	56·2	62·3	5
Soluble insulin ..	82·5	63·2	66·8	79·4	97·6	108·8	5
P difference	<0·05	<0·1	—	—	<0·02	<0·01	

Weight range: 258 to 366 g. Dose: 2·5 I.U./kg.

DISCUSSION

The above results clearly indicate that the guinea-pig is a satisfactory test animal for the demonstration of the prolonged action of long-acting insulin preparations, whereas in the rabbit[2], the prolonged action of globin zinc insulin cannot be demonstrated. This difference can be either one of species or of sensitivity to soluble insulin. The latter reason is more probably correct for Ferry[3] believes that the retardation effect is less evident in rabbits when a small dose is given than when a large dose is given. Since the guinea-pig is less sensitive than the rabbit, weight for weight, in its response to soluble insulin a much larger dose of the long-acting insulin has to be given to produce a response, thus increasing the

amount given of the agent producing the prolonged action. The ratio of this agent to the dose of insulin is, however, always the same whether a large or small dose is given. Experiments with globin zinc insulin have been conducted recently in our laboratory on fed rabbits where it has been necessary to increase the dose of insulin given to produce a response. A significant prolonged action has been demonstrated. It thus seems probable that a minimum amount of the prolonging agent, not directly dependent on the amount of insulin injected, has to be present in the tissue fluid at the site of injection, or in the serum, to produce a satisfactory prolonged action. Further experiments are in progress to clarify this problem.

It would be valuable to conduct parallel trials in diabetics and the guinea-pig and rabbit with all the prolonged-acting insulins to ascertain the most satisfactory animal test for the control of insulin preparations used clinically.

SUMMARY

1. A method is described using small groups of guinea-pigs for the demonstration of the prolonged action of globin insulin and protamine zinc insulin.

2. A 1-day test gives results which compare favourably with those of a 2-day "cross-over" test.

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DISCUSSION

The paper was presented by MR. G. A. STEWART.

DR. F. HARTLEY (London) said that hitherto workers in this country had failed to demonstrate prolongation of action in rabbits, and, as the authors pointed out, that differed from American experience. Even clinically, experience in this country by no means confirmed the extent of the prolongation which seemed to have been found in humans in the United States. The author had only recorded one set of results with protamine zinc insulin in guinea-pigs and the impression might be given that globin zinc insulin gave a similar prolongation to that given by prototamine zinc insulin. That was contrary to clinical experience, and amplification of Table 5 by the inclusion of results beyond the 6-hour

period might well have brought out the more prolonged action of protamine zinc insulin. He would like to have seen tests carried out in guinea-pigs with soluble insulin. Was it certain that a delayed action would not have been demonstrated in some of the animals?

MR. G. A. STEWART, in reply, said that soluble insulins had been tried in guinea-pigs. They had not demonstrated any delay action, nor did the curves differ. The test described was only the demonstration of the prolonged action of globin zinc solution and was not a quantitative assay. He agreed that if the tests reported in Table V were continued for a longer period protamine zinc insulin would show a greater prolongation of the effect than globin zinc insulin.