RESEARCH PAPERS

A NEW METHOD OF ASSAY OF HUMAN CHORIONIC GONADOTROPHIN UTILISING MALE TOAD, *BUFO MELANOSTICTUS* SCHNEID

By J. K. Mohanty and P. R. Pabrai

From the Department of Pharmacology, S.C.B. Medical College, Cuttack

Received July 15, 1950

The various methods of assay of human chorionic gonadotrophin may, broadly speaking, be divided into 2 groups—first, based upon primary changes produced by the hormone in the gonads and, second, on secondary changes caused by it in the accessory reproductive organs of test animals. Assays depending on changes in the vaginal epithilium, increase in weight of uterus or of male organs, etc., belong to the latter group and have been found to be totally unreliable and inaccurate. But the single exception of this group of tests is the increase in weight of the prostates of immature hypophysectomised rats (Greep *et al.*[1]), which is unique in that it is affected neither by the follicle-stimulating hormone nor by œstrogens. It is quite an accurate method, although cumbrous, time-taking, and needs a high degree of dexterity in its performance.

Tests based on observations of ovarian hyperæmia, increase in ovarian weight, ovulation in rabbits, formation of corpora lutea, etc., come under the first category. Some of these give a graded, others an " all-or-none," response. Increase in weight of the ovaries of immature female rats is the method of assay recommended in the B.P. Although some of these, including the official method, are fairly accurate in practice, especially when the assay is done in comparison with a reference standard, there are cogent reasons for questioning its inherent accuracy and precision, as for instance, the increase in weight of ovaries of immature rats; the response measured in the B.P. method is most probably a synergistic effect. In rats the effect of this hormone is like that of the luteinising hormone of the anterior pituitary; but the action of the luteinising hormone on ovarian weight is dependent on the presence of follicles in the ovary or of intact functioning hypophysis. So the increase in weight of ovaries, as determined by the official method, might be due to the combined action of the hormone injected and the hormone elaborated by the partially active pituitaries of immature rats. In other words, the reaction is not specific and as such *per se* its precision is limited. Moreover, the method is costly and time-consuming.

The authors, while carrying on work on the sperm test of pregnancy[2], were struck with the high degree of specificity, simplicity and rapidity of reaction of male toads to injections of human pregnancy

urine containing chorionic gonadotrophin. No other hormone except, perhaps, the luteinising hormone of the anterior pituitary shares this reaction, and this anterior pituitary hormone is seldom present in any quantity in the urine to give rise to fallacy and inaccuracy. The reaction is so quick that the intact and functioning hypophysis of the test animal is unlikely to influence it. As such the question of utilising this biological property of the hormone in its assay cropped up. To establish its suitability it was thought worth while to carry on experiments to find out whether the dose-response relationship, particularly in the middle dose ranges, is a linear one when converted into logarithms and probits and to work out the exact conditions and the design of assay, if the regression line is satisfactory. In this paper the results of such an investigation are presented.

METHOD

The standard preparation of human chorionic gonadotrophin was used. Healthy male toads (*Bufo melanostictus*), weighing between 40 to 60 g., caught on the previous evening were utilised. Pilot experiments showed that the maximum dose giving no response and the minimum dose producing 100 per cent. response were 15 and 70 I.U. respectively. Then 7 intermediate dosages, namely, 30, 35, 40, 45, 50, 55 and 60 I.U. were selected for final experiments. In any one day for each dose 12

TABLE I

SHOWING DATA OF EXPERIMENTS ON HUMAN CHORIONIC GONADOTROPHIN ON MALE TOADS

Experiment No.	No. of toads giving positive results on different dosages (12 toads for each dose in each experiment)							Remarks
	30 I.U.	35 I.U.	40 I.U.	45 I.U.	50 I.U.	55 I.U.	60 I.U.	
I	1	1	3	5	6	10	12	Slope (b) = 10·365 ±(S.E.) 1·987
II	0	1	3	6	8	10	12	$\dot{p}$ = 0·0965
III	0	3	2	4	6	8	10	Regression equation : y = 10·365 x—12·244
IV	0	1	3	5	9	10	11	M.E.D. = 46·083 I.U. ±(S.E.) 1·0006
V	0	2	4	6	8	8	11	χ^2 = 2·712(P = 0·84)
VI	1	1	3	4	7	10	10	
VII	1	1	4	7	11	10	10	
VIII	0	0	2	6	7	11	12	
IX	0	1	3	6	8	9	8	
X	1	1	3	6	8	8	11	
Total	4	12	30	55	78	94	107	
No. of positives per cent.	3·33	10·00	25·00	45·83	65·00	78·33	89·17	

animals were used, and the effects of all the 7 dosages were studied simultaneously. The experiment was repeated on 10 different but not on consecutive days. In all 120 toads were used for each dosage. The

allotment of animals to different dosages was made at random. Lest the toads in a day's catch should not be homogeneous, they were first sorted out into 6 groups of 14 members each. Then 2 members from each group were allotted to the individual doses by a process of strict randomisation. Each animal was given a number, written with enamel paint on a small tin plate and tied to one of its hind limbs. Preliminary experiments showed that the accidental death of any toad was unlikely, which expectation prove true in subsequent work, so no additional animal was taken in any group in anticipation of replacement of any member dying in course of the experiment. The toads used for each dosage, after their weights having been recorded, were placed in lots of 3 under a bell jar, which was kept a little tilted to allow entry of air. All the 4 jars containing the test animals of 1 dose group were kept close together, each jar being duly marked with the respective group number.

The preparation of gonadotrophic hormone was dissolved in 0·6 per cent. sodium chloride solution giving a concentration of 5 I.U./ml. The required quantity of the solution was injected into the dorsal lymph sac of the toads from a 50-ml. burette graduated to 0·1 ml., and fitted with a hypodermic needle by means of a thin rubber tube. The solution flowed freely under gravitation and the flow was regulated by the stopcock of the burette. A small hæmostat was applied on the skin at the point of puncture for a minute or so to prevent leakage of fluid when the animal was replaced in its jar. The order of administration of the dosages, the quantity of the solution injected and other relevant data were systematically recorded simultaneously. The dose was proportional to the body weight and was given in volumes of 120, 140, 160, 180, 200, 220 and 240 ml./kg., of toad for each dosage respectively (*vide supra*).

Microscopic examination of cloacal samplings was made half an hour after injection and every ½ hour thereafter till the samples showed sperms. A sample showing no sperms within a period of 4 hours was considered to be negative. The order of examination of cloacal samplings was the same as the order of administration of the hormone. The experiments were carried out in 1950 from February to April, but the pilot experiments were performed in the latter part of 1949. The data of the experiments are presented in Table I.

Statistical Calculation

The method of statistical calculation was that described by Emmens[3] for probit assays. The dosages in I.U. were converted into common logarithms and the percentage of animals showing positive response into empirical probits. When the empirical probits were plotted graphically against log-dosages, a straight line, showing the regression of response on dose, was obtained. From the empirical regression line, expected probits were read back, and from the latter corrected probits were calculated to work out the final regression line (Figure 1).

Mathematically, the equation for the regression line, the slope (b) and its standard error, the median effective dose (M.E.D.) and its standard error, etc., were calculated. The χ^2 test showed a fair degree of homogeneity of assay animals. The relevant statistics of assay and parameters of the population of the test animals are also presented in Table I.

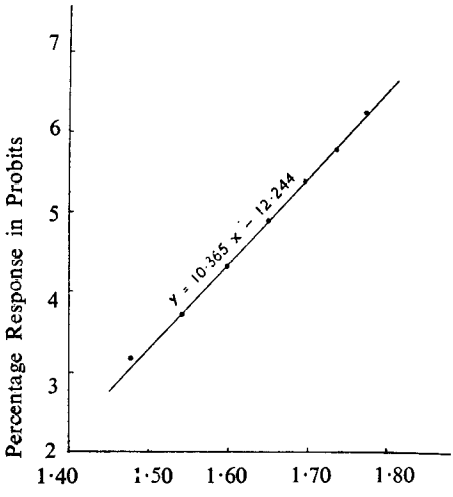

FIG. 1. Regression line relating response of toads to injections of human chorionic gonadotrophin

RESULTS

Figure 1 shows that the regression of response on dose in long-probit scale is a linear function, which fits in very well with the observed values. The value of b., i.e. the slope of the line is 10·365 ± (S.E.) 1·987 and that of σ is 0·0965. This denotes a satisfactory steepness of the regression line, which in turn implies that the animals are fairly uniform in their response. The value of χ^2 with six degrees of freedom is 2·712 (P=0·84). In other words, the χ^2 test reveals that there is no significant departure from homogeneity in the population of the test animals. The M.E.D.=46·083 I.U. ± (S.E.) 1·0006.

DISCUSSION

Although the assay is based on quantal response, the statistics and parameters, as worked out in the present series of experiments, reveal a high degree of inherent accuracy and precision of the method. The reaction is specific for human chorionic gonadotrophin and is not influenced by other hormones, viz., follicle-stimulating hormone, equine gonadotrophin œstrogens, etc., as reported previously by the authors[2]. The luteinising hormone of anterior pituitary is likely to respond positively, but this, for reasons already given, will seldom create fallacy or inaccuracy in routine assays or even in academic investigations. The population of the species of toads as found in nature shows a fair degree of homogeneity.

Whether seasonal variation will affect the results of assay is not yet definitely known, but from their experience with this reaction for over a year the authors are of opinion that it is unlikely that this factor will make any significant difference in the results, particularly when the assay is performed in comparison with a reference standard. However,

further work in this connection, including comparative study with other methods of biological standardisation of this hormone, is in progress.

This method of assay of human chorionic gonadotrophin utilising male toad, *B. melanostictus,* compares very favourably with other methods, indeed it has advantages over them due to its high degree of specificity, simplicity of performance, comparative cheapness, and very satisfactory precision and accuracy. With proper designing and selection of the test animals, the accuracy of the test is likely to be enhanced further. As such it commends itself for adoption in routine work, particularly in countries of South and South-East Asia, where this species of toad is found in abundance. Other species of toads and some species of frogs also give this reaction, but their suitability for use in assay is yet to be studied.

Summary

1. The suitability of assaying human chorionic gonadotrophin, utilising the reaction of sperm ejaculation by the male toad, *B. melanostictus,* is reported.

2. Experiments carried out to establish the dose-response line in the log-probit scale, and to find out the parameters of the population, are described.

3. The results of these experiments show that the regression line is steep, the slope (b) of the line being 10·365±(S.E.) 1·987; M.E.D. = 46·083 I.U.±(S.E.) 1·0006; the χ^2 test showed a fair degree of homogeneity of the population of toads as caught in nature.

4. The test is highly specific, precise, simple and cheap; as such it commends itself for routine use in the assay of human chorionic gonadotrophin.

The authors express their indebtedness to Dr. B. Mukerji, Director, Central Drugs Laboratory, Calcutta, to Dr. S. Swarup, Professor of Statistics, the All-India Institute of Hygiene and Public Health, Calcutta, to Lt.-Col. P. Papatla, Principal, and Dr. S. M. Banerji, Professor of Pharmacology, S.C.B. Medical College, Cuttack.

References

1. Greep, van Dyke and Chow, *Endocrinology,* 1942, **30,** 635.
2. Mohanty and Pabrai, *Ind. med. Gaz.,* 1950, **85,** 43.
3. Emmens, *Principles of Biological Assay,* Chapman and Hall, London, 1948.