STUDIES ON THE RAT OVARIAN AUGMENTATION METHOD FOR FOLLICLE STIMULATING HORMONE

By

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ABSTRACT

The rat ovarian augmentation method for the quantitative determination of follicle stimulating hormone (FSH) has been systematically studied. Assays with different strains and in rats of different ages have been performed, as well as assays with hypophysectomized rats. The effects of variations of the injection schedule and in the amounts of HCG used for augmentation have been examined. Accordingly some modifications of the original method of Steelman & Pohley (1953) have been made. The best results were obtained with 26-28 days old rats of the SS strain (breed of Statens Seruminstitut, originating from the Wistar strain), weighing 45-55 g, and injected twice daily sc for 3 days, with autopsy on the 4th day. The most suitable dose of HCG for augmentation was 20 IU as the total dose per rat. A 3 + 3 design with 3 rats per dose gave a satisfactory precision, the index of precision being between 0.10 and 0.15. The sensitivity was about 2 IU of the 2. IRP-HMG.

The course of the curves for human and ovine FSH are found to be identical.

The reproducibility of the method during 1 year has been studied and is satisfactory.

Since the follicle stimulating hormone (FSH) was discovered many attempts have been made to quantify this hormone.

Evans et al. (1939) used a histological assay based on the production of normal follicles in hypophysectomized female rats, Greep et al. (1942) used
the increase in testicular weight in hypophysectomized male rats and Fevold et al. (1940) used the increase in ovarian weight in immature, intact female rats. Other investigators used the increase in uterine weight in intact, immature mice or rats. When it became known that human chorionic gonadotrophin (HCG) augments the effect of FSH on the ovaries, Simpson et al. (1951), in hypophysectomized HCG-treated female rats showed that a dose-response curve could be obtained, and Steelman & Pohley (1953) found that immature intact HCG-treated rats could be used instead of hypophysectomized rats and that their ovaries were more sensitive to FSH. Later Brown (1955), using the same principle, employed mice instead of rats and got a more sensitive assay. However, the method seems to be highly dependent on the strain of mice used.

Igarashi & McCann (1964) published a quantal assay for FSH using the principle that small amounts of HCG sensitize the mouse uterus to FSH. The method was sensitive but highly unspecific (Uberoi & Meyer 1967). When some two years ago we needed a bioassay to measure the excretion of FSH in urine from human subjects, preliminary experiments indicated that the Steelman-Pohley method was the most promising for the animals we had at our disposal.

In work on bioassays it is well known that even small alterations in the practical performance of the assay can give remarkable improvements. We therefore systematically studied the method, and the purpose of this paper is to present the results obtained. The specificity of the method will be discussed in a following paper (Christiansen 1972).

MATERIALS AND METHODS

Animals

Infantile female rats of the Holtzman strain and of the SS strain (a breed of Statens Serum Institut, originating from the Wistar strain); intact, as well as hypophysectomized rats were used. Altogether 760 rats were used for the assays.

Preparations

A) Human chorionic gonadotrophin. – A commercial preparation, Physex®, containing 1500 IU per ampoule, prepared by LEO Pharmaceutical Products, Copenhagen.

B) Preparations containing follicle stimulating hormone. – 1) Ovine: NIH-FSH-S-1, NIH-FSH-S-2 and NIH-FSH-S-3, kindly supplied by the National Institutes of Health, Bethesda, USA. 2) Human: a) The second international reference preparation for human menopausal gonadotrophin (2. IRP-HMG) established 1964, containing 40 IU FSH and 40 IU luteinizing hormone (LH) per ampoule. b) HMG LEO 63051 and 64021, two commercial preparations made by LEO Pharmaceutical Products, Copenhagen.

The method used was the rat ovarian augmentation test (Steelman & Pohley 1953). In all assays the test material, together with the HCG was dissolved in borate buffer (pH = 9.2) and injected subcutaneously.

Between the injections the solutions were stored at +4°C.

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26-28 days old female rats of the SS strain. Mean values and total doses per rat are referred to. 5 rats per dose, 10 rats as controls. Augmented with a total of 20 IU HCG sc per rat, 5 injections sc during 3 days, the volume per injection being 0.5 ml. Autopsy on the 4th day. The ordinate indicates the weight of both ovaries. The vertical bars indicate the standard deviations. The mean values in the different curves have been displaced a little in order to show the standard deviations separately.

The rats were killed with ether, the ovaries immediately removed and dissected free of surrounding fat tissue and weighed on a torsion balance to the nearest 0.5 mg. Statistical calculations were done according to McArthur et al. (1966).

**RESULTS**

Fig. 1 shows the assays with two different strains of rats, the Holtzman and the SS strains. The regression lines are almost identical. The standard deviation (the vertical bars in Fig. 1) is remarkably smaller in the assay with the SS strain than in the assay with the Holtzman rats. At a total dose of 400 µg NIH-FSH-S-1, ovarian weights 3.6 times above the controls are obtained with the SS rats and 3.3 times above the controls with the Holtzman rats. The weight of both ovaries is referred to, since preliminary studies have shown that there is no difference between the response of the right and left ovary to FSH.

In the following assays only SS rats have been used.

With regard to the variations in the age of the rats used, the possibilities are limited. Rats less than 3 weeks old cannot be used as they are still suck-
Fig. 2.

\[ X \quad X \] 20-22 days old female rats of the SS strain. \[ \bullet \quad \bullet \] 26-28 days old female rats of the SS strain. Mean values and total dose per rat is referred to. 5 rats per dose. Augmented with 20 IU HCG sc total per rat. 6 injections sc during 3 days, the volume per injection being 0.5 ml. Autopsy on the 4th day. The vertical bars indicate the standard deviations.

Fig. 3.

26-28 days old female rats of the SS strain. \[ \bullet \quad \bullet \] augmented with 20 IU HCG total per rat. \[ X \quad X \] augmented with a total of 40 IU HCG per rat, and \[ \triangle \quad \triangle \] augmented with a total of 80 IU HCG per rat. 10 rats per dose. See also legend to Fig. 2.

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The test material used is HMG LEO 64021. 5 rats per dose. See also legend to Fig. 3.

ling, while rats more than 4 weeks old cannot be used because they might have already reached the age of puberty. Fig. 2 shows 2 dose-response curves, the one with rats of 20–22 days, weighing 35–45 g, and the other with rats of 26–28 days, weighing 45–55 g. The conditions are otherwise identical. The assay using the 26–28 days rats is certainly the better one, as the standard deviation is smaller and the slope steeper. Using 400 µg NIH-FSH-S-2 as the total dose an ovarian response of 3.4 times the controls is obtained with the 26–28 days rats and of 2.7 times with the 20–22 days rats. The latter seem to have reached the maximal ovarian response under these conditions with the 400 µg dose.

In the following assays rats aged 26–28 days are used.

Fig. 3 shows assays with 26–28 days old female rats of the SS strain. The FSH preparation is NIH-FSH-S-3. The 3 dose-response curves represent assays with 20 IU, 40 IU and 80 IU HCG, respectively as the total dose used for augmentation. The standard deviation is lowest and the slope steepest in the assay with 20 IU HCG. Using 400 µg NIH-FSH-S-3 an ovarian stimulation of 3.1, 2.7 and 2.4 times the controls is obtained with 20, 40 and 80 IU HCG, respectively. In a similar assay (Fig. 4) but using a commercial HMG preparation it is seen that 20 IU HCG is sufficient to bring about augmentation, has the lowest standard deviation, and produces the steepest slope. With a total dose of 0.1 ampoule of HMG LEO 64021, an ovarian response of 3.3, 2.5 and 2.2 times the controls is obtained with 20, 40 and 80 IU HCG, respectively. Consequently a dose of 20 IU HCG was chosen for the further studies.
26–28 days old female rats of the SS strain. Variations in the number of sc injections per day. ▲—▲ 1 daily injection for 3 days. ⋄—⋄ 2 daily injections for 3 days. ×—× 3 daily injections for 3 days. Autopsy on the 4th day. Mean values and total dose per rat are referred to. Augmented with 20 IU HCG total per rat. 5 rats per dos. In the last 2 cases the volume per injection is 0.5 ml, in the first one the volume is 1.0 ml. The vertical bars indicate the standard deviations.

The injection schedule is the main factor on which the steepness of the regression line depends. Of course the ideal method would have a continuous supply of the hormone. Fig. 5 shows 3 assays using 1, 2 and 3 injections daily for 3 days with autopsy on the 4th day. The standard deviation in the assay using 6 injections is slightly smaller than in the other assays, the regression line being more linear and steeper. With a total dose of 400 µg NIH-FSH-S-3 an ovarian response of 1.7, 2.9 and 2.5 times the controls is obtained with 3, 6 and 9 injections, respectively. This means that there is no advantage in giving 9 injections instead of 6 injections for 3 days, whereas 6 injections are much better than 3 injections. It was therefore decided to use 6 injections in 3 days for the further investigations.

In Fig. 6 2 assays are shown with hypophysectomized 26–28 days old rats, augmented with 20 and 100 IU HCG, respectively. The sensitivity, standard deviation and steepness of the curves are about the same as in the assay with intact rats.

Using the ventral prostate weight method for LH (Greep et al. 1942), it has been shown that there is a great difference in the course of the curves for human and ovine LH, the human LH producing a steep curve and the ovine
Experiments with 26–28 days old hypophysectomized female rats of the SS strain. Two daily injections sc during 3 days, the volume per injection being 0.5 ml. 24 hours rest from hypophysectomy to the start of injections. Autopsy on the 4th day. ●—● augmented with a total of 20 IU HCG per rat. ×—× augmented with a total of 100 IU HCG per rat. Mean values and total dose per rat are referred to. 9–12 rats per dose. The vertical bars indicate the standard deviations.

Dose-response curves for 3 different FSH-containing preparations, 2 of human and one of ovine origin. See also legend to Fig. 2. ●—● 2. IRP-HMG. ×—× HMG LEO 63051. ▲—▲ NIH-FSH-S-3.
Study on the reproducibility of the Rat Ovarian Augmentation Test during 1 year. The interval between the assays is 2 months. 5 rats per dose. See also legend to Fig. 2.

LH a flat curve (Christiansen 1967). This difference could not be demonstrated in the Steelman-Pohley assay for FSH, as the 3 dose-response curves in Fig. 7 do not differ significantly from parallelism.

The reproducibility of the assay is shown in Fig. 8 and Table 1. The FSH preparation is 2. IRP-HMG. 26–28 days old rats of the SS strain were used. For augmentation 20 IU HCG, given in 2 daily injections for 3 days sc was used. Autopsy was performed on the 4th day. The assays have been performed during 1 year with an interval of 2 months. The slopes have been checked for parallelism and linearity. No significant difference from parallelism and linearity has been found. Fig. 8 shows the dose-response curves graphically and Table 1 gives the data. The relative potency has been calculated in terms of assay number one. The reproducibility is satisfactory in the 6 assays as the relative potency is about 1.0, but in assay number 2, performed in the month of May, the relative potency is 1.47 and the fiducial limits, both upper and lower are higher than 1.0. Table 1 also shows the influence on the precision of the assay by reducing the number of rats per dose. This has been done mathematically from the same 7 assays by using the computer first to remove one rat per dose and then 2 rats per dose by randomization. Three rats per dose instead of 5 only slightly decrease the precision of the assay.
Table 1.

Data for the curves presented in Fig. 8 (see legend to Fig. 8). In all assays the FSH preparation is 2. IRP-HMG. The relative potency – calculated in terms of assay No. 1 – and the 95% and 99% fiducial limits are shown. The effect of reducing the number of rats per dose from 5 to 3 on the relative potency and the fiducial limits is indicated.

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DISCUSSION

The addition of HCG to the test material in the Steelman-Pohley assay for FSH has 2 purposes. Firstly, it means that the ovaries become maximally stimulated with LH-like material so that the eventual content of LH in the test material plays either a very small or no role at all and thus does not influence
the ovarian response to FSH. Secondly, it sensitizes the ovaries to FSH so that
dose-response curves can be obtained with much smaller amounts of FSH,
making it possible to measure FSH in biological materials.

In their original paper Steelman & Pohley (1953) used 21–22 days old rats
of the Sprague-Dawley strain and the injection schedule included 1 daily in¬
jection for 3 days, each in a volume of 0.5 ml. Autopsy was performed on the
4th day. Three doses of HCG were investigated, using 10, 20 and 40 IU as
the total dose per rat. It was concluded that 20 IU HCG was the most suitable
dose but the difference between 20 and 40 IU was small. Our investigations
show that rats of 26–28 days are preferable and that much steeper curves
are obtained by using 2 daily injections for 3 days. Like Steelman & Pohley
(1953) we find that 20 IU HCG is the most suitable amount for augmentation.

In assays with hypophysectomized rats Steelman & Pohley (1953) found a
greatly decreased sensitivity when augmenting with 20 IU HCG but mentioned
that it might be possible to increase the sensitivity by increasing the amount
of HCG. Using rats 24 hours after the operation we find – with 20 as well as
with 100 IU HCG – quite as good a sensitivity as in identical assays with
intact rats.

Christiansen (1967) has reported that there is a difference in the course of
the curves for human and ovine LH in the ventral prostate weight method
(VPW) for LH, the human curve being much steeper than the ovine curve,
indicating a species difference between human and ovine LH. In the Steelman-
Pohley assay for FSH such a difference is not seen, as the regression lines
for human and ovine FSH do not differ significantly from parallelism. This
is in agreement with the investigations of Parlow & Reichert (1963) who found
parallel curves in the Steelman-Pohley assay for FSH of ovine, equine, rat
and human origin.

The reproducibility of the assay is found to be satisfactory as 6 out of 7
assays with the 2. IRP-HMG had almost identical relative potency. In the
7th assay, performed in the month of May the relative potency was higher.
All the curves were parallel and linear within the dose-range used. In all assays
5 rats per dose have been used. However, decreasing the number of rats per
dose from 5 to 3 only slightly decreased the precision of the assay.

The sensitivity of the assay is about 2 IU of the 2. IRP-HMG. Compared
to the excretion of FSH in normal human subjects it is poor and must be
compensated for by collecting sufficient numbers of 24 hour urine samples.

It is concluded that the Steelman-Pohley method, modified as mentioned
above is suitable in our laboratory for the quantitative determination of FSH.
ACKNOWLEDGMENTS

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