RECENT WORK ON THE QUANTITATIVE DETERMINATION OF PITUITARY GONADOTROPHINS IN URINE

By

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The subject will be considered under the following headings:
I. Methods of assay of pituitary gonadotrophins in urine.
II. Extraction of gonadotrophins from human non-pregnant urine.
III. Human Menopausal Gonadotrophin (HMG) as a standard preparation for the assay of pituitary gonadotrophins in urine.
IV. A quantitative method for the routine estimation of gonadotrophins in human non-pregnant urine.
V. Some clinical applications of gonadotrophin assays.

I. METHODS OF ASSAY OF PITUITARY GONADOTROPHINS IN URINE

This topic has been recently reviewed (Loraine, 1956). Assay methods can be divided into 3 main groups.

1. Tests claimed to be specific for FSH.
2. Tests claimed to be specific for ICSH.
3. Non-specific tests.

1. Tests claimed to be specific for FSH.

It is generally agreed that any specific assay method for FSH should employ hypophysectomised rather than intact animals in order that the secretion of gonadotrophins from the animal's own pituitary should not interfere with the response of the end-organs. The test animals employed have generally been rats which are more sensitive to gonadotrophic stimulation than are, for example, rabbits, guineapigs and amphibia. Three tests will be considered.

(a) Follicular growth in the ovaries of hypophysectomized immature female
rats. This method was introduced by Evans, Simpson, Tolksdorf & Jensen (1939) for the assay of FSH in pituitary tissue. The test is highly specific but has a low degree of precision; the procedure as described by its originators is very laborious and is probably too insensitive for use in the clinical field.

(b) Increase in testicular weight in hypophysectomised immature male rats treated with an excess of HCG. This test was described by Paesi, Wijnans & de Jongh (1951) and was used to determine the FSH content of rat pituitaries. The technique is laborious and tedious and is not suitable for clinical application. According to Simpson, Li & Evans (1944) the specificity of this test is doubtful in view of the fact that purified preparations of both FSH and ICSH can cause testicular growth in hypophysectomised rats.

(c) Increase in ovarian weight in intact immature rats treated with HCG. This method, which was proposed by Steelman & Pohley (1953), depends on the observation that HCG will augment the action of FSH on the rat ovary. This test employs intact rather than hypophysectomised animals and for this reason appeared promising as a simpler means of determining FSH activity in human urine. However, in the author’s laboratory the method as described by the originators had a low degree of precision and could not be made the basis of a satisfactory quantitative assay for FSH.

2. Tests claimed to be specific for ICSH.

As in the case of FSH most assays for ICSH must be conducted on hypophysectomised animals. Only two such tests will be mentioned.

(a) Repair of interstitial tissue in the ovaries of hypophysectomised immature female rats. This method was used by Evans et al. (1939) to estimate the ICSH concentration in sheep pituitary extracts. The technique is reasonably specific but is too insensitive and too laborious for routine use in clinical studies.

(b) Enlargement of the ventral lobe of the prostate in hypophysectomised immature male rats. Greep, Van Dyke & Chow (1942) and Simpson, Li & Evans (1943) have used this test to estimate the ICSH activity of pituitary extracts. McArthur (1952) and Loraine & Brown (1954, 1956a) were able to adapt it for studies in human urine. The test is specific, reasonably precise and is sufficiently sensitive to estimate the urinary excretion of ICSH under normal and pathological conditions in man. The technique is too laborious for routine use but may provide valuable information in selected cases.

3. Non-specific tests.

Such tests usually depend on observations in intact rats and mice and can be classified according to whether the effect is primary or secondary. In assays depending on primary effects the gonads themselves are inspected while in the secondary group the effects result from the liberation of oestrogens or androgens from the gonads. Of the many methods proposed for the assay of
gonadotrophins in intact animals only the mouse uterus test will be considered.

*Mouse uterus test.* This assay method has become very popular for the estimation of pituitary gonadotrophins in urine in health and disease. Its chief advantage is that it is very sensitive and will give positive results even with extracts from low-titre urines. Most centres have up till now used the technique described by *Klinefelter, Albright and Griswold* (1943) or some modification thereof. In this procedure intact immature mice weighing 8–10 g. are injected subcutaneously once or twice per day for three days and are killed approximately 72 hours after the first injection. Results are expressed in »mouse uterine units« of »FSH« per 24 hours, a unit being defined as that quantity necessary to produce a given effect – usually 100–150 % increase in uterine weight.

It should be emphasised that the technique as described by *Klinefelter et al.* (1943) is a qualitative test rather than a quantitative assay. The numbers of animals used per dose level of urine extract are very small and the expression of results in »animal units« without reference to a standard preparation makes the error of the method very large. Furthermore it must be stated that the mouse uterus test measures a mixture of FSH and ICSH activities and should not be regarded as specific for FSH.

II. EXTRACTION OF GONADOTROPHINS FROM HUMAN NON-PREGNANT URINE

Numerous methods have been proposed for the preparation of gonadotrophins from human urine. Some of these are shown in Table 1.

*Table 1.*

Methods of Extraction of Pituitary Gonadotrophins from Urine.

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol precipitation</td>
<td>Zondek (1931)</td>
</tr>
<tr>
<td>Alcohol precipitation with dialysis</td>
<td>Heller &amp; Heller (1939)</td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td>Frank &amp; Salmon (1935)</td>
</tr>
<tr>
<td>Tannic acid precipitation</td>
<td>Levin &amp; Tyndale (1936)</td>
</tr>
<tr>
<td>Benzoic acid adsorption</td>
<td>Katzman &amp; Doisy (1934)</td>
</tr>
<tr>
<td>Kaolin adsorption with acetone precipitation</td>
<td>Dekanski (1949)</td>
</tr>
<tr>
<td>Kaolin adsorption with acetone precipitation and pH control</td>
<td>Loraine &amp; Brown (1954)</td>
</tr>
<tr>
<td>Kaolin-acetone tricalcium phosphate procedure</td>
<td>Loraine &amp; Brown (1956 a)</td>
</tr>
<tr>
<td>Aluminium hydroxide adsorption</td>
<td>Malburg &amp; Goodman (1954)</td>
</tr>
<tr>
<td>Permutit adsorption</td>
<td>Katzman, Godfrid, Cain &amp; Doisy (1943)</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>Gorbman (1945)</td>
</tr>
</tbody>
</table>
The procedures differ considerably in nature and complexity. Usually, however, they suffer from the common disadvantages that they are laborious and tedious, that considerable loss of gonadotrophic activity may occur during their performance and that the final extracts obtained may be toxic to the experimental animals. For a detailed description of the advantages and limitations of the various procedures the reader is referred to a recent review by *Diczfalusy & Heinrichs* (1956).

Techniques depending on simple alcohol or acetone precipitation give satisfactory results in high-titre urines such as are found at and beyond the menopause and in various types of ovarian and testicular failure. However, in low-titre urines, e.g. in males and in normally menstruating women, crude alcohol or acetone extracts are often too toxic for routine use. Attempts to reduce toxicity, e.g. by dialysis, cause a considerable loss of gonadotrophic activity (*Varney & Koch*, 1942). Extracts prepared by the tannic acid method also tend to be toxic (*Heller & Heller*, 1939) and the technique is too laborious for general use. Methods employing benzoic acid adsorption and permutit adsorption usually give extracts of low toxicity but the yield of gonadotrophins obtained by these procedures is too low to permit of their use in quantitative studies.

Recent work by *Loraine & Brown* (1951, 1956a) has demonstrated that the kaolin-acetone method with accurate pH control is a suitable procedure for the extraction of gonadotrophins from human non-pregnant urine. In this technique the gonadotrophins are adsorbed on to kaolin at pH 4.0 and are eluted from kaolin with water at a pH of 11.3; the hormones are subsequently precipitated with 5 volumes of acetone. In low-titre urines crude kaolin-acetone powders may be toxic to the experimental animals. Treatment of such powders by tricalcium phosphate greatly reduces toxic effects. In this way it is possible to administer to intact mice and to hypophysectomised rats the equivalent of approximately 1 litre of original urine.

### III. HUMAN MENOPAUSAL GONADOTROPHIN (HMG) AS A STANDARD PREPARATION FOR THE ASSAY OF PITUITARY GONADOTROPHINS IN URINE

*Loraine & Brown* (1956a) have shown that it is possible to use a gonadotrophin extract of human menopausal and post-menopausal urine, prepared by the kaolin-acetone method, as a standard preparation for the assay of gonadotrophin extracts of male, cyclic female and post-menopausal urine prepared in the same way. In these studies two assay methods were used, one depending on the enlargement of the uterus in intact immature mice and the other on the enlargement of the ventral lobe of the prostate in hypophysectomised immature
male rats. The standard was made by Messrs. Organon (Newhouse, Scotland) and was called HMG-20. Part of the batch prepared was subjected to drying and the dried material was termed HMG-20A. In terms of biological activity HMG-20 and HMG-20A are approximately equipotent. At a recent meeting of the Gonadotrophin Club (Birmingham, 1955) at which the establishment of a provisional standard for pituitary gonadotrophins in urine was discussed, the suggestion was made that one unit of the proposed standard should be defined as the activity contained in 1 mg. of HMG-20A.

The introduction of a preparation such as HMG-20A as a universal standard should greatly increase the reliability of pituitary gonadotrophin estimations in urine. It should also make it possible to compare directly results from different laboratories and to eliminate the necessity for expressing results in »animal units«.

IV. A QUANTITATIVE METHOD FOR THE ROUTINE DETERMINATION OF GONADOTROPHINS IN HUMAN NON-PREGNANT URINE

Such a technique has recently been described by Loraine & Brown (1956 b). The main steps are as follows:

1. Extraction of the urine by the kaolin-acetone method with accurate control of the pH at all stages of the procedure.
2. In low-titre urines treatment of the crude kaolin-acetone powders with tricalcium phosphate in order to reduce the toxicity of the extracts.
3. Bioassay by the mouse uterus test.
4. Expression of results in terms of HMG as »HMG units per 24 hours«.

This technique is sufficiently sensitive to measure the gonadotrophin excretion in normal and pathological conditions in man. The endpoint of the assay is not specific for either FSH or ICSH and gives a measure of what may be conveniently termed »total gonadotrophic activity«. When a 3 or 4 point design* is used with 4 animals per dose level of the standard and unknown preparations the index of precision (\( \lambda \)) is generally less than 0.2. This would appear to be a satisfactory degree of precision for quantitative work.

In Fig. 1 are shown dose-effect curves for HMG-20 on the mouse uterus over an 18 month period.

It will be noted that the sensitivity of the animal colony did not vary greatly during that time. In earlier experiments the standard preparation was administered in every assay; subsequently it was felt that conditions within the colony

* 3 point design = 2 doses of the standard preparation and 1 dose of the unknown preparation.
4 point design = 2 doses of both the standard and unknown preparations.
could be kept so constant that it would be justifiable to use the standard at intervals of approximately 2 weeks.

The accuracy of the method has been tested by a series of recovery experiments in which HMG-20 was added to pooled male and cyclic female urine and extracted and assayed as described above. The results are shown in Table 2.

Table 2.
Recovery Experiments with HMG-20.
20 mg. HMG-20 was added to half of a 48-hour specimen of urine and was extracted by the kaolin-acetone tricalcium phosphate procedure. Recoveries are corrected for endogenous blank levels. Bioassays were conducted by the mouse uterus test.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Type of urine</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>Proliferative phase female</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>92</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>70</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>52</td>
</tr>
</tbody>
</table>

Mean recovery % 76.3; Standard Deviation 12.7; No. of experiments 7.
It will be noted that the recoveries varied from 52 to 92% and that the mean figure was 76%. This figure was considered to be reasonably satisfactory considering the many steps involved in the extraction procedure and the additional inherent error of the bioassay.

V. SOME CLINICAL APPLICATIONS OF GONADOTROPHIN ASSAYS

Results in some illustrative cases are presented in Figs. 2, 3 and 4. Estimations were made on pooled urine specimens collected over 48 hour and 72 hour periods. Aliquots were removed for determination of oestrogens and pregnanediol and the remaining urine was used for gonadotrophin assays. Points represented by open circles indicate that the excretion was actually «less than» the value quoted.

In Fig. 2 are shown the effects of bilateral orchidectomy and bilateral adrenalectomy on the gonadotrophin excretion in a patient with mammary carcinoma. It will be noted that the levels began to rise approximately one month after orchidectomy and that after two months a three-fold increase in excretion

![Graph showing effects of bilateral orchidectomy and bilateral adrenalectomy on urinary gonadotrophin excretion](image)

*Fig. 2.*

The effect of bilateral orchidectomy and bilateral adrenalectomy on the urinary excretion of gonadotrophins in a male patient with mammary carcinoma.

(Mr. J. C. aet. 70).
values had occurred over those in the pre-operative period. Bilateral adrenalectomy in this subject produced a slight rise in gonadotrophin output.

Fig. 3 shows the effect of stilboestrol, testosterone and bilateral adrenalectomy on the gonadotrophin excretion in a post-menopausal subject with mammary carcinoma. It will be noted that oral stilboestrol in a dosage of 5 mg. b.i.d. for six weeks produced a rapid fall in the gonadotrophin output and that this fall persisted as long as the drug was being administered. Subsequent treatment by 100 mg. of testosterone phenyl propionate by intramuscular injection once per week for six weeks did not appear to inhibit the secretion of pituitary gonadotrophins. During this time the urinary output rose to levels in the same range as those encountered in the period prior to stilboestrol therapy. In this patient bilateral adrenalectomy did not affect the excretion of pituitary gonadotrophins in urine.

In Fig. 4 is shown the effect of ovarian irradiation on the gonadotrophin excretion in a pre-menopausal patient with mammary carcinoma. In this subject the excretion was studied first during a normal menstrual cycle and

![Graph](image-url)
The effect of ovarian irradiation on the urinary excretion of gonadotrophins in a pre-menopausal subject with mammary carcinoma. (Mrs. A. R. act. 41 : M. P. = menstrual period).

secondly subsequent to ovarian irradiation. It will be noted that readings in the follicular and luteal phases of the cycle were very low and that a peak of excretion occurred at approximately mid-cycle. Immediately after ovarian irradiation the excretion values rose steeply and approximately one month after radiotherapy were some twenty times higher than those found in the follicular and luteal phases of the menstrual cycle.

SUMMARY

1. Urinary gonadotrophins from normal men and from normally menstruating and post-menopausal women can be assayed in terms of Human Menopausal Gonadotrophin (HMG). It is proposed that a preparation of HMG be used as a standard for the assay of human non-pregnant urinary gonadotrophins.

2. For the routine determination of pituitary gonadotrophins in urine in clinical practice the mouse uterus test is the bioassay method of choice. Results of the assay should be expressed in terms of HMG as »units per 24 hours«.

3. The kaolin-acetone method with accurate pH control is a suitable procedure for the extraction of gonadotrophins from human non-pregnant urine. Treatment of crude kaolin-acetone powders with tricalcium phosphate makes it possible to administer without toxic effects the equivalent of 1 litre of the original urine to intact mice and to hypophysectomised rats.

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4. Based on these observations a method is proposed for the quantitative determination of gonadotrophins in the urine of non-pregnant subjects.

5. Some clinical applications of the assay method are described.

REFERENCES

Gorbman, A.: Endocrinology 37, 177, 1945.