THE BIOLOGICAL ASSAY

of

ESTROGENIC HORMONES

By

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INTRODUCTION

The biological assay of estrogenic substances has long been a subject of debate. Although there are many methods of estimating the activity of such substances, modifications of the vaginal smear method on rodents as described by Allen and Doisy (1), and Kahnt and Doisy (2) are most widely employed.

The potency thus determined was originally expressed in rat or mouse units. This type of standard was shown to be inadequate (18) since the route of administration, the solvent, the number of injections and the interval of injections produced wide variations in the estrus response and consequently in the assigned potency.

In effort to overcome these difficulties two reference standards were established by the Health Organization of the League of Nations (3,4). One of these is a sample of crystalline estrone and the other is a sample of crystalline estradiol benzoate. The second standard was devised because it appeared that the esterified and the free hormone differed qualitatively in their activity.

The unit of each standard is defined as "the specific oestrus-producing activity contained in 0.1 gamma of the standard preparation (3,4)."

The standard of reference is to be treated in every assay in exactly the same manner as the estrogen under test. In this way the effect of variations in the animals can be

overcome.

The above named committee defined the term "specific estrus-producing activity" as "the power of producing, in the adult female animal completely deprived of its ovaries, an accurately recognizable degree of the changes characteristic of the normal oestrus." The only such change regarded as providing a suitable basis for quantitative determination of the activity in comparison with the standard preparation is the series of changes in the cellular contents of the vagina of the rat and mouse (3,4).

In further regard of the assay method it was stated (3,4) that the comparison of an unknown preparation with the standard preparation for such specific activity could be accurately made only if the conditions of administration of both, and of observing the results produced in each case, were completely identical, particularly in the following respects:

(a) The standard and the unknown preparation under test must be administered in identical solvents.

(b) Administration should be by truly subcutaneous and not by intramuscular injection.

(c) The standard prearation and the preparation under test should be administered by a method ensuring slow absorption at identical rates. This condition, could be attained by administering both dissolved in the same oily solvent, in which case single injections could be used, or by preparing both in watery solution, and administering in an equal number

of fractions, not less than three. In the latter case, the injections should be spaced in an identical manner for both the unknown and the standard preparation over a total time of not less than 9 hours.

(d) The vaginal smears should be taken at the same intervals after the injection from the animals injected with the standard and from those injected with the preparation under test, but should be continued at least once daily until the animals under test return to a diestrus condition. On the day in which the maximum response is expected at least 2 smears should be taken. If the maximum in the animals receiving the standard and in those receiving the preparation under test, are attained at intervals differing by more than 24 hours, the comparison cannot be regarded as valid.

(e) In comparing the activity of a preparation with the standard, the procedure should aim at finding a dose of the unknown preparation producing the chosen limiting reaction in the same proportion of animals as that in which it is produced by a known dose of the standard.

This report by the Health Organization of the League of Nations was a definite advance in the estimation of biological activity in estrogenic preparations.

However, since estrogenic preparations are not described in the U. S. P. XI and N. F. VI, no official standard of reference or no official method of assay has been made available with which the preparations of domestic origin can be

compared. The method used at present by the Food and Drug Administration (5) like that used by the Health Organization of the League of Nations (3,4) is based on the Allen and Doisy (2) vaginal smear technique. The test, as conducted by the Washington laboratories employs the international standard assayed under identical circumstances as the preparation under test. The design of the assay is also modified to allow statistical evaluation of the results.

The general acceptance of the reference standard in this assay procedure has resulted in a great improvement in the uniformity of commercial preparations so standardized, and in products and procedures in the process of development and in research in this field.

The variation in results obtained when a reference standard and these procedures are not used can be found in the literature. In the first report of the Health Organization of the League of Nations (3) it was stated that the international unit was approximately 1/3 of the original Allen and Doisy rat unit. Various other laboratories (6-17) have placed this value from 1/5 to 1/38 of the rat unit. That is, various laboratories, in determining the potency of one estrogen (international standard estrone) found it to vary approximately 8 times in terms of rat units. This is a much greater error than would be expected for this particular procedure, since Bulbring and Burn (11) found the error of assay to be not greater than 30 per cent. Therefore, there must be great variation from laboratory to laboratory in determining

the rat unit. This divergency can to a great extent be controlled by assaying the reference standard and test product under the same circumstances.

An objection of this mode of assay concerns the method of administration. This factor is accepted as one of the variables which influences the estrogenic response. Commercial estrogens regardless of their chemical nature are generally standardized against estrone and usually by subcutaneous administration. Investigation by Rowe and Simond (19,20), and Sondern and Sealey (21) have indicated that the ratios of biological activity for these different estrogens, when compared to estrone, are not the same by subcutaneous and oral administration. Therefore, if this condition does exist, a product having a certain estrone value when given subcutaneously may have an entirely different estrone value when given by mouth. Consequently, an oral assay for products intended for oral use should give a truer value of the clinical activity as exerted by this route of administration.

The prime object of this research is to determine the relative potency evaluation of the two methods as applied to commercial products intended for oral therapy.

EXPERIMENTAL

Animals

The animals used in this study were female, albino rats obtained from two sources. One was the Laboratory Supply Corporation, Philadelphia, Pa. These animals were 21 to 30 days of age when purchased. After receipt they were maintained on a uniform diet consisting of "fox blocks" manufactured by Allied Mills Co. of Wayne, Indiana.

Vaginal smears were made daily on these rats to establish the presence of the estrus cycle. Any animal not showing a regular cycle was discarded. Those giving the proper response were ovariectomized under light ether anesthesia according to the technique described by Burn (41). Care was exercised in this operation to remove a good portion of the fallopian tubes and the surrounding fat in order to remove any accessory ovarian tissue. The animals were allowed to completely recover from the operation and again vaginal smears were taken for a period of 2 weeks. Animals not showing a permanent diestrus were then discarded.

The second source of animals was the Research Laboratories of Ayerst, McKenna and Harrison, Montreal, Canada. These rats were received already castrated. Vaginal smears were made for 2 to 3 weeks to confirm the castration. These animals were maintained under the same laboratory conditions as those from the source first mentioned. The average weight of the animals

from both sources during these experiments was 150 gm.

This entire group of animals would simulate the population used in most commercial assays as far as selection, care and diet are concerned.

Materials

Standard Oestrone. International Standard of the Hydroxy-Ketonic Form of the Oestrus-producing Hormone. Obtained from Laboratory of Hygiene Department of Pensions and Health, Ottawa, Canada.

Amniotin. Soluble gelatin capsules manufactured by E. R. Squibb and Sons, labelled to contain 2000 international units per capsule. Further claimed to contain Estrin, The Estrus-Producing Hormone.

Folestrin. 4 minim glanules (soluble gelatin capsules) manufactured by The Armour Laboratories. This product is labelled to contain purified extract of pregnant mares' urine. The potency is expressed as 2000 international estrogenic units per glanule.

Estrogenic Hormones. Tablets manufactured by Reed and Carnick. According to label each tablet contains mixed estrogens from prenatal mares' urine equivalent to 1000 international estrone units.

Progynon-DH. Tablets manufactured by the Shering Corporation and labelled to contain Estrogenic Substance. It is also stated that each contains 1/10 mg. of crystalline a-estradiol or 1200 Rat Units (Allen-Doisy).

Premarin. An experimental sample of tablets of estrone sulfate manufactured by and obtained from Ayerst, McKenna and Harrison of Montreal, Canada.

Diethylstilbestrol (Merck's). An experimental sample of crystalline 4.4'-dihydroxy-alpha-beta-diethylstilbene obtained from Ayerst, McKenna and Harrison, Montreal, Canada.

The problem of bio-assay does not apply to this synthetic estrogen since it is a crystalline product and generally prescribed as a chemical entity on a weight basis. It has been included in this work for comparative purposes. It was assayed by oral and subcutaneous administration in both oil and aqueous mediums. These tests were conducted exactly as those to be described.

In addition the potency of this preparation was determined in aqueous medium using a total of 3 administrations of drug. These doses were spaced at 24 hour intervals.

All of these samples mentioned except the International Standard Oestrone, diethylstilbestrol, and Premarin Tablets were purchased on the open market.

Procedure

Design of Investigation. The design of this investigation is such that commercial preparation of estrogenic substances sold for oral therapy can be compared to the international standard estrone both by oral and subcutaneous assay, and also to determine any differences in ratio of different types of estrogens when compared to international

standard estrone by the two methods of administration. Further, the experiments are arranged for a statistical evaluation of the results.

Arrangement and Preparation of Animals. The animals under test for each product were divided into two groups. One of these groups received the estrogen by subcutaneous injection and the other by oral administration.

These groups were further divided in such a manner to permit at least three dosage levels for each route of medication. The value of such gradients in dosage level has been pointed out by various investigators (5,22,23). This method furnishes the following information:

1. Changes in the absolute sensitivity of the animals.
2. The standard error of the assay.
3. The significance of differences in the response by the two routes of administration.
4. The homogeneity of a group of animals used on any single assay.

The pattern of these tests was such that regression lines could be calculated for each product by oral and subcutaneous administration and compared to those computed for the standard under the same experimental conditions.

One week before a test each animal was "primed" by subcutaneous injection once daily on two successive days with 3 gamma of estrone in 0.2 cc. aqueous solution. This procedure has long been used to increase the uniformity of response (1). Vaginal smears were then taken and any animal

not responding to this priming dose was discarded. Seven days after the first priming dose all responding animals that had returned to a diestrus condition were ready for assay.

Following one assay the animals were rested for one week before priming for another test.

The same rats were used throughout these experiments. Those receiving an estrogen by mouth on one test were injected subcutaneously in the next. Moreover, the animals were shifted from one dosage level to another on succeeding assays. There is evidence (1) that the extent of genital atrophy in castrates influences the amount of estrogen required. For this reason priming and the cross-over technique was employed.

Administration of Dosage. Different opinions are held as to the number of injections to be given in estrogenic assays. These include the single injection method of Coward and Burn (24), the 4 divided doses of Harrison and Parkes (25), and the 12 divided dose method as described by Hain and Robson (16). In the procedure adopted in this work doses were administered twice a day for 3 successive days making a total of 6 doses. This system was followed in both oral and subcutaneous tests in order that the results would be comparable.

Dilutions for subcutaneous administration were either in cottonseed oil or in water according to the nature of the product being tested. Solutions in oil were prepared on the

day of the first injection and kept in a refrigerator during the remainder of the injection period. Dilutions in water were prepared each day just previous to injection. The sample under test was always compared to the standard given in the same solvent. One-sixth the total dose was contained in 0.2 cc. and this volume was administered to each rat regardless of body weight. The injections were made from a 1.0 cc. Luer syringe through a 1/2 inch, 24 gauge needle (Becton, Dickinson & Co.).

Solutions for oral administration were prepared at the same time and in the same manner as those used for subcutaneous administration. These dilutions were also prepared in cottonseed oil or water according to the nature of the product. Again, the product was compared to the standard administered in the same solvent and in the same volume per dose. Preparations were diluted in oil to contain 1/6 the total dose in 0.3 cc. Those made in aqueous medium were diluted to contain 1/6 the total dose in 1.0 cc. These volumes were given per rat irrespective of body weight.

The oral doses were placed directly into the stomach to prevent the loss of any of the estrogen. This was accomplished in the following manner. A 2 inch, 24 gauge needle (Becton, Dickinson & Co.) was blunted and slightly curved. The entire shaft was covered by just enough very small catheter tubing to slightly overlap the tip of the needle. This needle when attached to a 1.0 cc. Luer Syringe was very easily inserted directly into the rat's stomach.

Vaginal Smears. The basis of the vaginal smear as a criterion for estimation of estrogenic activity grew out of the work of Stockard and Papanicolaou (26) who showed that different stages of the estrus cycle of the guinea pig are characterized by clear-cut changes in the cellular contents of the vagina. They defined the periods in the guinea pig estrus cycle according to the terminology of Heape (27) and correlated these periods with the activity of the ovaries and the corpus luteum.

In 1922 similar vaginal changes corresponding to the changes in the estral cycle were demonstrated in the rat by Long and Evans (28) and in the mouse by Allen (29). The former authors showed that the length of this cycle in the rat is about 4 to 5 days and that cycles occur in constant succession. They defined the periods in the estrus cycle of a rat as follows:--

First Stage (Proestrus). The first part of the sexual cycle. The ovarian follicles are beginning to develop. Only nucleated epithelial cells appear in the vaginal smear at this time. This stage lasts about 12 hours.

Second Stage (Estrus). The period of heat wherein the ovaries exhibit large mature follicles.

Third Stage (Ovulation). The stage during which the follicles rupture and release the ova. This stage together with stage 2 lasts about 27 to 30 hours and the vaginal smear contains only cornified cells.

Fourth Stage (Metaestrus). Ova are in the fallopian tube

and young corpora lutea are to be found. The smear during this period shows leucocytes and cornified cells and this stage lasts about 6 hours.

Fifth Stage (Diestrus). This is the resting stage lasting about 57 hours. Herein the smear exhibits predominately leucocytes and occasionally epithelial cells.

These rhythmic changes in the rat occur spontaneously under normal conditions but disappear immediately on castration. When the ovaries are removed, a permanent diestrus results. The action of estrogenic substances in the castrate brings the animal into estrus.

After the introduction of the vaginal smear as a method of estimating the activity of estrogenic materials, discussion began to appear in the literature on the method of making smears and on what constitutes a positive reaction (30,31,32). Allen and Doisy (33) considered positive only smears showing considerable number of cornified cells and no leucocytes. Laqueur (30), Loewe (31), and Lipschutz (34) accepted the proestrus smear (small nucleated epithelial cells) as an end-point. Kahnt and Doisy (2) adopted for their positive reaction "a predominance of cornified cells with some nucleated epithelial cells and a very few leucocytes." Laqueur and DeJongh (35) considered the disappearance of leucocytes as a suitable end-point while Burn (36) states that this criterion is too easily confused with variantz of diestrus smears. Kennedy (37) declared complete disappearance of leucocytes and cornification of at least half of the

epithelial cells to be a positive response. The replacement of leucocytes and predominance of cornified cells is also accepted by D'Amour and Gustavson (17) and Sondern and Sealey (21). Marrian (38) stated that faulty interpretation of smears constituted one of the most frequent sources of error in the biological assay of the estrogens.

The frequency of taking the vaginal smears is another point of discussion among bio-assayist. Allen and Doisy (33) took their readings 45, 56 and 72 hours from the beginning of the experiment. Allen, Dickens and Dodds (39) recommend smearing once on the first day, second day, third day and fourth day; and 3 readings on the fifth day. Among other suggestions are 30, 42, and 54 hour intervals (40); 3 smears daily (36); 54, 60 and 70 hours after the original injection (21); and 8 hour intervals starting 48 hours after the first injection (17).

The frequency of smearing in this particular study meets the requirements set forth by the Health Organization of the League of Nations (3,4). As previously mentioned it was stated in this report that the vaginal smears should be taken at the same intervals from the animals used for the standard preparation and for those used for the preparation under test. It was further specified that these smears should be taken at least once daily and twice on the day when the maximum response is expected.

In order to follow these specifications and at the same time to simulate general commercial procedures, the

following smear interval was adopted. Smears were taken starting 8 hours after the last injection. A second smear was taken on the same day 18 hours after last injection. One smear was taken on each of the two succeeding days at intervals of 12 hours each. It had been found by preliminary work that the greatest response occurred on the day following the last injection. Moreover, after 3 days the animals had returned to a diestrus condition.

The method of taking, reading and recording the smears is as follows: The vaginal contents of an animal was removed by flushing several times with about 0.2 cc. of normal saline solution by means of a pipette (medicine-dropper). This pipette was used throughout the tests. Any residue from a preceding rat was removed before each succeeding smear by rinsing in clean tap water, then in 70 per cent alcohol and finally in the saline. The suspension of cells was examined unstained and while still wet under the low power of the microscope.

The cells observed in these smears are leucocytes, nucleated epithelial cells and squamous or cornified cells. For the purpose of this study a smear showing a predominance of squamous cells and no more than a few leucocytes and/or nucleated epithelial cells was judged positive.

In recording the data the following designation for the different types of smears observed were used:

— = no cells present

0 = occasional: 1 to 10 per field

2 = fairly numerous: about half the number of cells present.

3 = numerous: this type comprised most of the cells present.

4 = the smear consisted of a large mass of this particular type of cell.

Mathematical Treatment of Results

Pharmacological studies upon a large variety of organisms by many biologists have established the sigmoid character of the typical dosage-response curve, especially in multicellular forms. It has been shown that such curves can be easily plotted as straight lines and their later analysis thereby facilitated (42,43,44,45). These straight lines are given the name "regression lines."

The fitting of a dosage-response curve to a series of experimental observations, however crude or refined, the technique is an attempt to infer, from a limited number of individuals, the empirical relationship of dosage and response for a given agent in an infinitely larger population from which they represent only a sample.

The regression line may be constructed graphically or calculated mathematically. Sometimes the graphic line will be very near the calculated one. However, the calculated line will often represent an important correction to a graphic line.

In these experiments the arithmetical procedure of fitting has been adopted and the methods and symbols employed by Fisher (46) and described by Bliss (42) have been used.

The formula for the regression line may be expressed as:

$$Y = a - b\,(X - \bar{x}).$$

where the symbols have the following designations:

Y = response in probits on the regression line.

X = any given dosage (in logarithms) corresponding to the response probit Y.

a = $\bar{y}$ = numerically the average probit for all determinations in that part of the experiment which is being fitted.

$\bar{x}$ = averages of the dosages administered (in logarithms) for the same section of data.

b = the regression coefficient or the slope of the line.

It is, therefore, necessary to calculate from the experimental data the quantities $\bar{x}$, $\bar{y}$, and b. The formulae are as follows:

$$\bar{x} = \frac{S(wx)}{S(w)},$$

$$\bar{y} = \frac{S(wy)}{S(w)},$$

$$b = \frac{S(wxy) - \bar{x}\,S(wy)}{A},$$

$$A = S(wx^2) - \bar{x}\,S(wx)$$

These symbols are defined as:

S = "the sum of"

w = weight of a given observation, the product of the weighting coefficient multiplied by the number of animals used.

x = a function of the dose administered experimentally, usually its logarithm.

y = the probit corresponding to the observed percentage response.

The basis for this method of calculation is the normal frequency distribution curve (42,45,47). The probit values corresponding to each percentage response and the weighting coefficients are derived from this curve mathematically. These values can be found in tables proposed by Bliss (42).

Example of Calculation of

the regression line

International Standard Estrone in Water--Oral Assay

Total Dose in gamma	Number of Animals	Per cent Response	X	X^2	Y
101	22	36.6	2.0043	4.016	4.6575
126	22	66.6	2.1004	4.41	5.4289
157	22	59,0	2.1959	4.8220	5.2275
196	22	86.36	2.2923	5.2446	6.0939

Weighting Coefficient	W	WX	WX^2	WY	WXY
.609	13.40	26.8	53.8	62.5	124.9
.615	13.50	28.4	59.6	73.3	154.0
.623	13.70	30.1	66.0	71.6	157.0
.412	9.05	20.7	47.4	55.2	126.0
Sums	49.65	106.0	226.8	262,6	561.9

Example of Calculation of
the Regression Line (continued)
International Standard Estrone in Water--Oral Assay

$$\bar{x} = \frac{S(wx)}{S(w)} = \frac{106.0}{49.65} = 2.13$$

$$\bar{y} = \frac{S(wy)}{S(w)} = \frac{262.6}{49.65} = 5.225$$

$$\bar{x}S(wx) = 2.13 \times 106.0 = 226.0$$

$$\bar{x}S(wy) = 2.13 \times 262.6 = 558.0$$

$$A = S(wx^2) - \bar{x}S(wx) = 226.8 - 226.0 = 0.8$$

$$b = \frac{S(wxy - \bar{x}S(wy)}{A} = \frac{561.9 - 558.0}{0.8} = 4.88$$

Example of Calculation of RD 50

International Standard Estrone in Water--Oral Assay

$$Y = \bar{y} + b\,(X - \bar{x})$$

$$5 = 5.225 + 4.88\,(X - 2.13)$$

$$X = 2.082 \text{ (logarithm)}$$

$$\text{RD } 50 = 120.3$$

RESULTS

Assay of International Standard Estrone in Water

Total Dose in gamma	Number of Animals	Per cent Response
0.50	10	30.0
0.625	10	20.0
0.78	22	40.9
0.97	22	68.18
1.21	22	68.18
1.51	22	90.9

TABLE I. Two subcutaneous doses per day for 3 days

RD 50 = 0.9068 gamma

b value of regression line = 9.08

Total Dose in gamma	Number of Animals	Per cent Response
101	22	36.6
126	22	66.6
157	22	59.0
196	22	86.36

TABLE II. Two oral doses per day for 3 days

RD 50 = 120.3 gamma

b value of regression line = 4.88

Assay of International Standard Estrone in Oil

Total Dose in gamma	Number of Animals	Per cent Response
0.78	15	6.6
0.97	25	36.0
1.21	35	31.1
1.51	30	33.3
1.84	20	45.0
2.25	20	55.0

TABLE III. Two subcutaneous doses per day for 3 days

RD 50 = 2.178 gamma

b value of regression line = 1.795

Total Dose in gamma	Number of Animals	Per cent Response
101	15	33.3
126	15	60.0
157	15	80.0
224	15	93.3

TABLE IV. Two oral doses per day for 3 days

RD 50 = 113.7 gamma

b value of regression line = 4.986

Potency of International Standard Estrone

Subcutaneous RD 50 (water) = 0.9068 gamma = 9.068 I.U.

Oral RD 50 (water) = 120.3 gamma = 1203 I.U.

Subcutaneous RD 50 (oil) = 2.178 gamma = 21.78 I.U.

Oral RD 50 (oil) = 113.7 gamma = 1137 I.U.

Ratio between amount of standard estrone necessary to produce a 50% response by oral and subcutaneous method

$$\frac{\text{Oral (water)}}{\text{S.C. (water)}} = \frac{1203}{9.068} = \frac{132.7}{1}$$

$$\frac{\text{Oral (oil)}}{\text{S.C. (oil)}} = \frac{113.7}{2.178} = \frac{52.3}{1}$$

$$\frac{\text{Oral (water)}}{\text{Oral (oil)}} = \frac{1203}{1137} = \frac{1.06}{1}$$

$$\frac{\text{S.C. (water)}}{\text{S.C. (oil)}} = \frac{9.068}{21.78} = \frac{.417}{1}$$

Assay of Estrogenic Hormone Tablets in Water

TABLE V. Two subcutaneous doses per day for 3 days

Total Dose in parts of tablets	Number of Animals	Per cent Response
0.0078	20	25
0.0097	20	45
0.0121	20	85

RD 50 = 0.00956 tablets

b value of regression line = 9.475

TABLE VI. Two oral doses per day for 3 days

Total Dose in parts of tablets	Number of Animals	Per cent Response
0.54	10	20
1.10	20	55
1.57	20	80

RD 50 = 0.98 tablets

b value of regression line = 4.9

Potency of Estrogenic Hormone Tablets

Subcutaneous RD 50 Estrogenic Hormone Tablets = 0.00956 tablets

Subcutaneous RD 50 Estrone in water = 0.9068 gamma

Oral RD 50 Estrogenic Hormone Tablets = 0.98 tablets

Oral RD 50 Estrone in water = 120.3 gamma

Labelled Potency	1 Tablet = 1000 I.U.
By S.C. Assay	1 Tablet = 95.0 gamma estrone = 950 I.U.
By Oral Assay	1 Tablet = 125.4 gamma estrone = 1254 I.U.

Oral:Subcutaneous ratio expressed in I.U.

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{1254}{950} = \frac{1.32}{1}$$

Oral:Subcutaneous ratio expressed in parts of tablets

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{0.98}{0.00956} = \frac{102.5}{1}$$

Assay of Progynon-DH Tablets in Water

TABLE VII. Two subcutaneous doses per day for 3 days

Total Dose in parts of tablets	Number of Animals	Per cent Response
0.00321	10	50
0.0050	20	65
0.0078	20	85

RD 50 = 0.00361 tablets

b value of regression line = 3.21

TABLE VIII. Two oral doses per day for 3 days

Total Dose in parts of tablets	Number of Animals	Per cent Response
0.379	10	40.0
0.54	19	63.15
0.77	20	80.0
1.10	10	100.0

RD 50 = 0.46 tablets

b value of regression line = 4.58

Potency of Progynon-DH Tablets

Subcutaneous RD 50 Progynon-DH Tablets = 0.00361 tablets

Subcutaneous RD 50 Estrone in water = 0.9068 gamma

Oral RD 50 Progynon-DH Tablets = 0.46 tablets

Oral RD 50 Estrone in water = 120.3 gamma

Labelled Potency	1 Tablet = 0.1 mg. a estradiol	
By S.C. Assay	1 Tablet = 251.0 gamma estrone	= 2,510 I.U.
By Oral Assay	1 Tablet = 262.0 gamma estrone	= 2,620 I.U.

Oral:Subcutaneous ratio expressed in I.U.

$$\frac{\text{Oral}}{\text{Subcutaneous}} = \frac{2620}{2510} = \frac{1.042}{1}$$

Oral:Subcutaneous ratio expressed in parts of tablets

$$\frac{\text{Oral}}{\text{Subcutaneous}} = \frac{0.46}{0.00361} = \frac{127.3}{1}$$

Assay of Premarin Tablets in Water

TABLE IX. Two subcutaneous doses per day for 3 days

Total Dose in parts of tablets	Number of Animals	Per cent Response
0.00168	41	14.6
0.00252	41	36.7
0.00378	44	84.2

RD 50 = 0.00276 tablets

b value of regression line = 4.69

TABLE X. Two oral doses per day for 3 days

Total Dose in parts of tablets	Number of Animals	Per cent Response
0.0072	43	16.2
0.0208	44	36.5
0.030	45	69.0

RD 50 = 0.023 tablets

b value of regression line = 4.8

Potency of Premarin Tablets

Subcutaneous RD 50 Premarin Tablets = 0.00276 tablets

Subcutaneous RD 50 standard estrone in water = 0.9068 gamma

Oral RD 50 Premarin Tablets = 0.023 tablets

Oral RD 50 standard estrone in water = 120.3 gamma

Labelled Potency - unknown

By S.C. Assay - 1 Tablet = 328.9 gamma estrone = 3.289 I.U.

By Oral Assay - 1 Tablet = 5230.0 gamma estrone = 52300 I.U.

Oral:Subcutaneous ratio expressed in I.U.

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{5230}{328.9} = \frac{15.9}{1}$$

Oral:Subcutaneous ratio expressed in parts of tablets

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{0.023}{0.00276} = \frac{8.34}{1}$$

Assay of Amniotin Capsules in Oil

Total Dose in parts of capsules	Number of Animals	Per cent Response
0.003125	15	33.3
0.0039	25	24.0
0.00485	25	24.0
0.00605	15	66.6

TABLE XI. Two subcutaneous doses per day for 3 days

RD 50 = 0.00634 capsules

b value of regression line = 2.593

Total Dose in parts of capsules	Number of Animals	Per cent Response
0.505	10	20.0
0.55	14	7.14
0.63	10	30.0
0.785	24	41.7
1.12	15	73.3

TABLE XII. Two oral doses per day for 3 days

RD 50 = 1.05 capsules

b value of regression line = 1.82

Potency of Amniotin Capsules

Subcutaneous RD 50 Amniotin Capsules = 0.00634 capsules

Subcutaneous RD 50 standard estrone in oil = 2.178 gamma

Oral RD 50 Amniotin Capsules = 1.15 capsules

Oral RD 50 standard estrone = 113.7 gamma

Labelled Potency	1 capsule = 2000 I.U.
By S.C. Assay	1 capsule = 343 gamma estrone = 3430 I.U.
By Oral Assay	1 capsule = 98.9 gamma estrone = 989 I.U.

Oral:Subcutaneous ratio expressed in I.U.

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{98.9}{343} = \frac{0.288}{1}$$

Oral:Subcutaneous ratio expressed in parts of capsules

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{1.15}{0.00634} = \frac{181.5}{1}$$

Assay of Folestrin Glanules in Oil

Total Dose in parts of glanules	Number of Animals	Per cent Response
0.003125	15	6.6
0.0039	24	33.3
0.00485	24	20.8
0.00605	15	40.0

TABLE XIII. Two subcutaneous doses per day for 3 days

RD 50 = 0.00877 glanules

b value of regression line = 2.13

Total Dose in parts of glanules	Number of Animals	Per cent Response
0.385	5	40.0
0.505	10	20.0
0.55	31	27.3
0.63	10	50.0
0.785	25	32.0
1.12	15	80.0

TABLE XIV. Two oral doses per day for 3 days

RD 50 = 0.63 glanules

b value of regression line = 1.79

Potency of Folestrin Glanules

Subcutaneous RD 50 Folestrin Glanules = 0.00877 glanules

Subcutaneous RD 50 standard estrone in oil = 2.178 gamma

Oral RD 50 Folestrin Glanules = 0.63 glanules

Oral RD 50 standard estrone in oil = 113.7 gamma

Labelled Potency	1 glanule = 2000 I.U.
By S.C. Assay	1 glanule = 259.0 gamma estrone = 2590 I.U.
By Oral Assay	1 glanule = 173.6 gamma estrone = 1736 I.U.

Oral:Subcutaneous ratio expressed in I.U.

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{1736}{2590} = \frac{0.678}{1}$$

Oral:Subcutaneous ratio expressed in parts of glanules

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{0.63}{0.00877} = \frac{71.8}{1}$$

Assay of Diethylstilbestrol in Water

Total Dose in gamma	Number of Animals	Per cent Response
0.060	34	97.1
0.075	35	91.4
0.171	34	52.9
0.240	13	46.2
0.420	25	28.0
0.600	10	10.0

TABLE XV. One subcutaneous dose per day for 3 days

RD 50 = 0.1544 gamma

b value of regression line = 1.72

Total Dose in gamma	Number of Animals	Per cent Response
0.60	11	18.2
0.90	13	53.8
1.20	24	41.7
1.80	37	62.2
2.70	13	84.6

TABLE XVI. One oral dose per day for 3 days

RD 50 = 1.2594 gamma

b value of regression line = 2.58

Assay of Diethylstilbestrol in Water

TABLE XVII. Two subcutaneous doses per day for 3 days

Total Dose in gamma	Number of Animals	Per cent Response
0.075	12	16.7
0.24	12	33.3
0.42	12	75.0

RD 50 = 0.24 gamma

b value of regression line = 2.58

TABLE XVIII. Two oral doses per day for 3 days

Total Dose in gamma	Number of Animals	Per cent Response
0.52	12	25.0
1.20	13	61.5
2.70	12	91.6

RD 50 = 1.0330 gamma

b value of regression line = 3.92

Assay of Diethylstilbestrol in Oil

TABLE XIX. Two subcutaneous doses per day for 3 days

Total Dose in gamma	Number of Animals	Per cent Response
0.075	12	8.3
0.24	12	25.0
0.42	11	63.6

RD 50 = 0.339 gamma

b value of regression line = 2.42

TABLE XX. Two oral doses per day for 3 days

Total Dose in gamma	Number of Animals	Per cent Response
0.52	12	8.3
1.20	12	25.0
2.70	12	75.0

RD 50 = 1.7544 gamma

b value of regression line = 4.47

Potency of Diethylstilbestrol

Assay by six dose method in water

Subcutaneous RD 50 diethylstilbestrol = 0.24 gamma

Subcutaneous RD 50 standard estrone = 0.9068 gamma

Oral RD 50 diethylstilbestrol = 1.033 gamma

Oral RD 50 standard estrone = 120.3 gamma

Potency by subcutaneous assay

0.1 gamma diethylstilbestrol = 0.378 gamma estrone = 3.78 I.U.

Potency by oral assay

0.1 gamma diethylstilbestrol = 11.93 gamma estrone = 119.3 I.U.

Oral:Subcutaneous expressed in I.U.

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{119.3}{3.78} = \frac{31.60}{1}$$

Oral:Subcutaneous ratio expressed in gamma

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{1.033}{0.24} = \frac{4.3}{1}$$

Potency of Diethylstilbestrol

Assay by six dose method in oil

Subcutaneous RD 50 diethylstilbestrol = 0.339 gamma

Subcutaneous RD 50 standard estrone = 2.178 gamma

Oral RD 50 diethylstilbestrol = 1.7544 gamma

Oral RD 50 standard estrone = 113.7 gamma

Potency by subcutaneous assay

0.1 gamma diethylstilbestrol = 0.641 gamma estrone = 6.41 I.U.

Potency by oral assay

0.1 gamma diethylstilbestrol = 6.47 gamma estrone = 64.7 I.U.

Oral:Subcutaneous ratio expressed in I.U.

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{64.7}{6.41} = \frac{10.09}{1}$$

Oral:Subcutaneous ratio expressed in gamma

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{1.7544}{0.339} = \frac{5.17}{1}$$

Potency of Diethylstilbestrol

Assay by three dose method in water

Subcutaneous RD 50 diethylstilbestrol = 0.1544 gamma

Oral RD 50 diethylstilbestrol = 1.2594 gamma

Oral:Subcutaneous ratio expressed in gamma of diethylstilbestrol

$$\frac{\text{Oral}}{\text{S.C.}} = \frac{1.2594}{0.1544} = \frac{8.24}{1}$$

S U M M A R Y O F R E S U L T S

Table 21

Product	RD 50 in parts of a unit (caps., tab., etc.)		Potency per unit in I.U.		Oral / S.C. Ratio in I.U.	Oral / S.C. Ratio in parts of unit	b values	
	S.C.	Oral	S.C.	Oral			S.C.	Oral
Estrone (in water)	0.9068 "	120.3 "			132.7 / 1		9.08	4.88
Estrogenic Hormone Tablets	0.00956	0.98	950	1,254	1.32 / 1	102.5 / 1	9.475	4.9
Premarin Tablets	0.00276	0.023	3,289	52,300	15.9 / 1	8.34 / 1	4.69	4.8
Progynon-DH Tablets	0.00361	0.46	2,510	2,620	1.042 / 1	127.3 / 1	3.21	4.58
Estrone (in oil)	2.178 "	113.7 "			52.3 / 1		1.795	4.986
Amniotin Capsules	0.00634	1.05	3,430	989	0.288 / 1	181.5 / 1	2.593	1.82
Folestrin Glanules	0.00877	0.63	2,950	1,736	0.678 / 1	71.8 / 1	2.13	1.79
Diethylstilbestrol (in water)	0.24 "	1.033"	0.1 gamma = 3.78 I.U.	0.1 gamma = 119.3 I.U.	31.60 / 1	4.3 / 1	2.58	3.92
Diethylstilbestrol (in oil)	0.339 "	1.7544"	0.1 gamma = 6.41 I.U.	0.1 gamma = 64.7 I.U.	10.09 / 1	5.17 / 1	2.42	4.47
Diethylstilbestrol (in water)'	0.1544 "	1.2594"				8.24 / 1	1.72	2.58

" = gamma
' = 3 dose method

DISCUSSION

Many commercial estrogens in general use today are of variable and unknown composition. The U.S.P. XI or N.F. VI do not set forth any official assay method, but the majority of such estrogenic products are assayed subcutaneously against international standard estrone, and the potency is expressed in international units.

When an estrogenic product is assayed against a standard, the potency found should always be the same when expressed in terms of the standard regardless of the method of assay. This is based on the assumption that if both the sample and the standard are handled in the same way the test animals will vary to both in the same manner. Consequently, the same potency should be obtained regardless of the assay method.

Since many of the commercial estrogens are intended for oral therapy and assayed by the subcutaneous method this investigation was performed to test the above mentioned concept.

The data set forth under "Results" in this paper show that this theory does not hold true even for crystalline standard estrone itself.

When standard estrone is tested in an aqueous medium the subcutaneous RD 50 is 0.9068 gamma while the oral RD 50 in the same medium is 120.3 gamma (see table I and II).

The ratio between these two doses, therefore, is 1:132.7. When this same comparison is made using an oily solvent the same ratio should hold if the previous assumption is true. However, the subcutaneous RD 50 found in this case is 2.178 gamma while the oral RD 50 remains practically the same (113.7 gamma) and the ratio existing between these two doses is now 1:52.3 (see table III and IV).

This is an indication that an estrogenic reference standard alone is not adequate to insure uniformity of potency. It suggests that a standard assay method is also necessary if uniformity of results are to be obtained.

Further examination of the data bears out this statement. The products tested show great variation in potency by subcutaneous and oral assay when compared to themselves by these two procedures or when compared to the international standard.

The dosage necessary to produce a 50% response was greater by the oral than by the subcutaneous assay for every product tested (see table XXI). The oral-subcutaneous ratio between these two doses varied for the different products and the variation still existed when the RD 50 values were compared to the corresponding values for standard estrone and expressed in international units. The extremes of variation were shown by diethylstilbestrol and Amniotin (see table XX). The former required 4.3 times the corresponding subcutaneous dose to produce a 50% oral response and the latter required 181.5 times its corresponding sub-

cutaneous dose to produce a 50% oral response. When expressed in international units, the oral potencies varied from 0.288 (Amniotin) to 51.6 (diethylstilbestrol) times their corresponding subcutaneous potencies.

Two of the materials included (Tablets of Progynon-DH and diethylstilbestrol) were crystalline estrogens whose potency is expressed by weight. Neither is assayed commercially against standard estrone but they were included to show what variation is possible when two different assay methods are used.

The products assayed in oily medium gave lower values by oral administration than by the subcutaneous route when expressed in international units (see table XXI). On the other hand products tested in water had higher estrone values by the oral method than by the subcutaneous technique. Whether or not this was a function of the solvent cannot be stated since all except diethylstilbestrol were commercial products and the true nature of the active principles could not be ascertained.

The potency of each of these estrogens which was stated on the label in terms of estrone were confirmed by the subcutaneous method of assay. Conversely, when these same products were assayed orally some were below label potency and others were considerably stronger than depicted (see table XXI).

Progynon-DH Tablets and Estrogenic Hormone Tablets (R & C)

whose oral:subcutaneous ratio approaches that of standard estrone gave similar values by both methods, the subcutaneous value being slightly lower in each case.

Amniotin Capsules and Folestrin Glanules were well within the advertised potency by subcutaneous assay and below the labelled value when tested orally (see page 32).

The potency of Premarin Tablets was not expressed since this was an experimental sample. However, its subcutaneous potency was good in comparison with the other like products and its oral potency far exceeded the subcutaneous value (see table XXI).

A difference between the two assay methods is also shown by the relative b values obtained from the regression lines (see table XXI). The values obtained by subcutaneous assays range from 1.795 to 9.475 for similar assays. Generally, the figures obtained when an oily solvent was employed was lower than the values for watery dilutions.

The b values for the oral assays ranged from 1.79 to 4.986 for the six dose technique with only two values lower than 3.92. Both of these low values were obtained from tests conducted using oily solvents.

In the test of diethylstilbestrol when 3 doses were administered the subcutaneous and oral b values were 1.72 and 2.58 respectively.

The difference in range of these two series of b values shows that the uniformity of response is greater for the

oral method of assay. Moreover, the lower values obtained when oily solvents are used indicates a greater animal variation to this medium than to water.

The relatively low values obtained for diethylstilbestrol when tested by the three dose method points out that this method is less uniform and sensitive than a method involving six doses.

From the foregoing, it is concluded that the international standard estrone is not enough in itself to insure uniform potency of estrogenic preparations. It seems advisable that a standard assay technique should also be officially adopted. In this regard, an oral assay should give a better evaluation of the potency as exerted clinically by oral therapy.

SUMMARY

A study of the potency of estrogenic preparations, as expressed in international units, when ~~the~~ assayed by oral and subcutaneous administration, shows wide variation in the potencies obtained by the two methods. The ratios between these two potencies as well as the oral:subcutaneous ratio expressed in parts of the preparations themselves differ markedly from one product to another.

Commercial preparations purchased on the open market were found to conform with the strength as expressed on the label when tested by subcutaneous assay. When these same products were tested orally the potencies as expressed by international units varied considerably from those advertised.

The results obtained by the oral assay method were more uniform than those produced by subcutaneous tests. In this respect the response from preparations administered in aqueous medium were more predictable than the response from products administered in oil. Comparative tests on diethylstilbestrol in water resulted in better uniformity of response when the estrogen was administered in six doses than when three dosages were given.

The oral assay procedure is recommended as superior to the subcutaneous technique for standardizing estrogenic preparations intended for oral therapy. Although there is no evidence that results obtained on laboratory animals

will agree with clinical results, it is reasonable to assume that oral assay procedure on laboratory animals will more nearly parallel the clinical response evoked by oral therapy than will subcutaneous assay methods.

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