Bioassay of Estradiol 17-Cyclopentylpropionate

By W. F. BEYER, D. H. EMMONS, and M. L. PABST

The immature mouse uterus has been utilized for the bioassay of a long-acting estrogen, estradiol 17-cyclopentylpropionate. The compound was dissolved in cottonseed oil and injected in a single subcutaneous dose of 0.10 ml. The uteri were removed and weighed 72 hours after the injection. Statistical analysis of uterine weights, using a 2 × 2 balanced bioassay, gave an approximate average standard error of potency of 12%. Similar uterine weights were produced by estradiol 17-cyclopentylpropionate when given in a single subcutaneous injection or when given in divided doses over a 3-day period. In the case of estrone and estradiol, a single dose was considerably less effective than divided daily doses. The similarity in response to one and three doses of estradiol 17-cyclopentylpropionate and its ability to maintain an increase in uterine weight following a single dose has been utilized as the basis of the assay for a long-acting estrogen.

RUBIN, *et al.* (1), reported a bioassay for estrogens using the increase in uterine weight of immature mice as the response. This method involved the use of a divided dose schedule and uterine ratios (100 times the uterine weight in mg. divided by the body weight in Gm.) as the response. Modifications of the method resulted in a shorter assay procedure for estradiol 17-cyclopentylpropionate (ECP).[1] The modified method is described for a single dose schedule and the use of uterine weights of immature mice. Uterine responses to estrone U.S.P. and estradiol U.S.P. are presented along with dose response curves and assay results for ECP.

ANIMALS AND METHOD

Twenty-one to twenty-five-day old immature mice of the CFW or CF No. 1 strain were used. No significant differences were noted in responses of the two strains. All mice utilized for a single test were of the same age group and strain. Commercial mouse diet and tap water were permitted *ad libitum*.

A cottonseed oil solution of ECP was injected subcutaneously in a single dose of 0.10 ml. The mice were sacrificed and the uteri were removed 72 hours after the injection. The uteri were separated from the ovaries, severed at the uterocervical junction, stripped of extraneous tissue, blotted free of contained fluid, and rapidly weighed to the nearest 0.10 mg. In many cases the body weights were obtained at the time the animals were sacrificed. This made it possible to obtain uterine ratios as well as uterine weights.

The log dose-uterine response relationship with a constant ratio of two between successive doses was used to calculate potency. The 2 × 2 assay design of Bliss (2) or U.S.P. XVI (3) was used. When more than one sample was assayed against a single standard, calculations for the joint assay of several preparations (4) were used.

RESULTS AND DISCUSSION

It was necessary to obtain dose response data and to test the duration of response before establishing an assay procedure. The duration of response was tested using both single and multiple dose schedules. By repeated trials it was found that a total dose of 0.32 mcg. of ECP per mouse resulted in a useful uterine weight response. For the single dose schedule, 0.32 mcg. of ECP was contained in a volume of 0.10 ml. of cottonseed oil. For the multiple dose schedule, 0.32 mcg. of the compound was contained in 0.30 ml. of cottonseed oil and 0.10 ml. was administered daily for 3 days. The uteri were removed at varying intervals after the injections to determine the duration of response.

The results in Table I show that the largest uterine weights and ratios occurred during the 48 to 120-hour period after a single injection and approximately 72 to 144 hours after the initial injection using multiple doses. Although the uterine weights remained fairly constant over most of the test period for both dosage schedules, the uterine ratio values decreased as the body weights of the mice increased. The body weights increased as the interval between initial injection and uterine removal was lengthened.

Dose response data for the single and multiple dose schedules were obtained using an interval of 72 hours from beginning of injection to uterine removal. At this time, uterine weights of mice receiving a total dose of 0.32 mcg. per mouse (Table I) were near maximal and were approximately five times that of uninjected controls (Table II). This period was also the earliest time for uterine removal when an injection interval of 24 hours was maintained for the multiple dose schedule.

The mean uterine weights and uterine ratios for the dosage levels tested are shown in Table II. The responses over a dose range of 0.08 to 0.64 mcg. of ECP per mouse exhibited a graded dose response relationship. There was a significant difference between the average responses for successive dosage levels tested, with the dose range suitable for bioassay extending over an eightfold difference in dose.

The data used in Table II were analyzed by linear regression methods yielding slopes (b),

Received November 8, 1960, from the Biological Control Laboratories of The Upjohn Co., Kalamazoo, Mich.
Accepted for publication September 25, 1961.
The authors acknowledge with appreciation the advice of Dr. O. S. Carpenter, Sc. Admn. Staff, The Upjohn Co., on statistical treatments. The assistance of R. J. Cole, Biological Control, The Upjohn Co., for computations using the IBM-610 computer is also gratefully acknowledged.

[1] Estradiol 17-cyclopentylpropionate (ECP)—The Upjohn Co.

TABLE I.—COMPARISON OF MOUSE UTERINE RESPONSE TO 0.32 MCG. ECP GIVEN IN A SINGLE DOSE OR IN THREE SUBCUTANEOUS DOSES[a]

	Hours after Beginning of Injection						
	24	48	72	96	120	144	168
One Dose							
Mean uterine weight (mg. ± S.E.)	21.6 ± 1.4	50.3 ± 3.3	45.2 ± 1.6	39.7 ± 1.7	48.0 ± 2.8	37.1 ± 2.5	...
Mean uterine ratio (± S.E.)	154 ± 8	329 ± 16	335 ± 10	271 ± 12	321 ± 19	240 ± 12	...
Mean body weight (Gm.)	14.0	15.2	13.6	14.9	15.1	15.5	...
No. of Mice	8	8	18	18	9	10	...
Three Doses							
Mean uterine weight (mg. ± S.E.)	...	...	47.5 ± 2.6	51.9 ± 2.7	44.7 ± 2.0	46.5 ± 2.6	40.7 ± 2.0
Mean uterine ratio (± S.E.)	...	...	321 ± 20	342 ± 18	279 ± 18	279 ± 17	230 ± 11
Mean body weight (Gm.)	...	...	14.9	15.2	16.1	16.8	17.8
No. of Mice	...	...	10	10	10	10	11

[a] Uteri removed at varying intervals after initial injection.

TABLE II.—COMPARISON OF UTERINE RESPONSE TO ECP GIVEN IN A SINGLE OR IN THREE SUBCUTANEOUS INJECTIONS[a]

Total Dose, mcg.	No. Mice	Mean Uterine Weight, mg. ± S.E.	Mean Uterine Ratio, ±S.E.
One Dose			
0.00	6	9.8 ± 1.1	77 ± 7
0.08	9	18.6 ± 1.6	143 ± 13
0.16	9	22.8 ± 2.7	193 ± 23
0.32	9	41.9 ± 4.0	349 ± 33
0.64	9	50.2 ± 5.1	367 ± 28
Three Doses			
0.00	7	9.5 ± 1.3	69 ± 7
0.08	14	16.3 ± 2.2	117 ± 16
0.16	14	33.0 ± 3.9	240 ± 27
0.32	14	46.1 ± 3.1	318 ± 21
0.64	14	57.1 ± 3.9	397 ± 20

[a] Uteri removed 72 hours after beginning of injection.

TABLE III.—ANALYSIS OF ECP RESPONSE CURVES OF TABLE II

	Uterine Weight	Uterine Ratio
One Dose		
Standard Deviation (s)	10.85	76.22
Slope (b)	37.87	275.42
Index of precision (λ)	0.2866	0.2734
Three Doses		
Standard Deviation (s)	12.49	80.22
Slope (b)	44.98	305.33
Index of precision (λ)	0.2779	0.2620

standard deviations (s), and indexes of precision (λ) shown in Table III. Tests for curvature indicated a linear response over the dose range used. The results in Table III show a similarity in response for single and multiple dose schedules. The standard deviations, slopes, and indexes of precision using either method compare favorably.

Estrone U.S.P. and estradiol U.S.P. were used as examples to compare the effects of ECP to that of more rapidly acting estrogens. Table IV shows that single doses of either estrone or estradiol, with uterine removal 72 hours later, have little effect, whereas multiple doses given over a 3-day period produce significantly larger uteri.

TABLE IV.—COMPARISON IN UTERINE RESPONSE TO ESTRONE U.S.P. AND ESTRADIOL U.S.P. GIVEN IN A SINGLE OR IN THREE SUBCUTANEOUS INJECTIONS[a]

Total Dose, mcg.	No. Mice	Mean Uterine Weight, mg.
Estrone, One Dose		
0.00	9	10.7
0.08	5	10.8
0.16	5	10.8
0.32	22	14.2
0.64	22	14.8
Estrone, Three Doses		
0.00	8	11.7
0.0938	6	26.7
0.1875	6	50.1
0.375	6	61.3
0.75	6	69.7
Estradiol, One Dose		
0.00	5	7.7
0.01	5	7.9
0.03	5	9.4
0.09	5	11.4
0.27	5	11.1
Estradiol, Three Doses		
0.01	5	14.3
0.03	5	26.1
0.09	5	35.2
0.27	5	37.0

[a] Uteri removed 72 hours after beginning of injection.

The prolonged effect of ECP on the mouse uterine weight permits determination of potency by a single injection method. To test the validity and reproducibility of this method, replicate assays using both the uterine weight and uterine ratio methods were done on a preparation containing ECP assayed against a house standard of ECP.

The data of Table V show that results of replicate assays are comparable when either uterine weights or ratios are used. The averages for potency, index of precision, and standard error were similar. Assay No. 4 exceeded the tabular value of 4.03 for the test of parallelism (F) at $P = 0.05$ using uterine weights. However, the same assay using ratios had a relatively high, although acceptable, value of 3.59.

Tests for homogeneity of log potencies of indi-

TABLE V.—COMPARISON IN REPLICATE ASSAYS OF A SINGLE ECP SAMPLE USING UTERINE WEIGHTS, UTERINE RATIOS, AND COVARIANCE

Assay No.	Dosage levels mcg.	Potency, % of House Standard	Slope, b	Standard Deviation, s	Index of Precision, λ	Standard Error, %	Test for Parallelism, F
			Uterine Weight Method				
1	0.16–0.32	86	47.92	11.24	0.2346	15.6	0.87
2	0.16–0.32	90	23.92	6.99	0.2922	19.4	0.96
3	0.12–0.24	126	44.20	10.20	0.2308	15.3	2.42
4	0.12–0.24	114	50.51	9.91	0.1962	13.0	4.24[a]
5	0.12–0.24	97	46.35	5.42	0.1169	7.7	0.00
Averages		103	42.58	8.75	0.2141	14.2	...
			Uterine Ratio Method				
1	0.16–0.32	85	365.41	83.40	0.2282	15.2	2.91
2	0.16–0.32	90	271.70	67.60	0.2488	16.5	0.93
3	0.12–0.24	113	349.36	75.78	0.2169	14.4	3.32
4	0.12–0.24	114	336.48	59.64	0.1772	11.8	3.59
5	0.12–0.24	104	287.07	32.95	0.1148	7.7	0.05
Averages		101	322.0	63.87	0.1972	13.1	...
			Covariance				
1	0.16–0.32	82	45.68	10.72	0.2347	15.6	3.26
2	0.16–0.32	90	24.55	6.40	0.2607	17.3	1.34
3	0.12–0.24	102	42.77	9.15	0.2165	14.4	4.62[a]
4	0.12–0.24	115	54.66	9.56	0.1749	11.6	3.54
5	0.12–0.24	100	46.86	5.41	0.1154	7.7	0.17
Averages		98	42.80	8.25	0.2004	13.3	...

[a] Fail "F" test at $P_{0.05}$.

TABLE VI.—MOUSE UTERINE WEIGHT ASSAYS ON PREPARATIONS CONTAINING ECP

Preparation No.	% of Label Potency	Log Confidence Interval, L	Approx. S.E., %	Slope, b	Standard Deviation, s	Index of Precision, λ
1	118	0.1372	8	55.33	4.88	0.0882
2	101	0.3130	18	35.71	7.21	0.2019
3	97	0.3130	18	35.71	7.21	0.2019
4	92	0.2236	13	47.78	7.14	0.1494
5	91	0.2236	13	47.78	7.14	0.1494
6	98	0.2050	12	38.55	5.33	0.1382
7	94	0.2050	12	38.55	5.33	0.1382
8	95	0.3020	17	49.31	6.33	0.1284
9	91	0.2060	12	49.87	6.90	0.1451
10	99	0.2050	12	49.87	6.90	0.1451
Averages	98	0.2333	14	44.85	6.43	0.1486

vidual assays by both uterine weight and ratio methods were performed; no evidence of heterogeneity was found at $P_{0.05}$ level of significance. Weighted mean potencies, calculated by weighting each log potency inversely as its variance, gave mean potencies of 103 and 101% for uterine weights and ratios, respectively. Since the use of uterine weights and ratios gave very similar results, an analysis of covariance was used to determine whether this would give appreciably better assay precision. Table V shows results using uterine weight as the variate (y) and body weight as the covariate (x) in a covariance analysis. The assumption that body weight had an important influence on uterine weight was not borne out. Although the mean standard error for the covariance method was comparable to that by the ratio method and slightly smaller than that by the weight method, this small increase in precision was not felt to warrant obtaining body weights for subsequent assays.

Numerous preparations containing ECP were assayed by the single dose-mouse uterine weight method. The assays were calculated using statistical methods as outlined in U.S.P. XVI (3) for 2 × 2 balanced assay designs. Table VI shows the results of representative assays of 10 preparations containing ECP assayed at dosage levels of 0.12 and 0.24 mcg., with eight mice at each dosage level. When more than one sample was assayed against a common standard, calculations for the joint assay of several preparations were used (4).

An average log confidence interval (L) of 0.2333 was obtained for the 10 preparations shown in Table VI. Using the value of 0.1486 for the average index of precision (λ), and eight mice per dose, the average standard error of potency was calculated as 12%.

No heterogeneity of assay variance (s^2) was found at $P_{0.05}$ for the independent assays of Table VI when analyzed using Bartlett's test (5). There was also no evidence of heterogeneity of assay slopes for the same group of assays, as determined using the chi square test at the same level of significance.

SUMMARY AND CONCLUSIONS

The method of Rubin, *et al.* (1), using the immature mouse for the bioassay of estrogens has been modified for the assay of a long acting estrogen, estradiol 17-cyclopentylpropionate (ECP). The modifications consist of administering the hormone in a single subcutaneous dose, removing the uteri 72 hours later, and using uterine weights as responses. The results were analyzed according to U.S.P. XVI (3) for a 2×2 balanced assay. When eight mice were used on each dose, the average log confidence interval was 0.2333.

Similar results were obtained by injecting ECP in single or divided doses. This was not the case with estrone or estradiol which were much more effective when multiple injections were given.

It is suggested that the method described for the assay of ECP may be utilized as a rapid bioassay for other long acting estrogens. It may also be used for identification of long acting estrogens and to differentiate between those that are either long or short acting.

REFERENCES

(1) Rubin, B. L., Dorfman, A. S., Black, L., and Dorfman, R. I., *Endocrinology*, **49**, 429(1951).
(2) Bliss, C. I., "The Statistics of Bioassay with Special Reference to Vitamins," Academic Press, Inc., New York, N. Y., 1952.
(3) "United States Pharmacopeia," 16th rev., Mack Publishing Co., Easton, Pa., 1960, pp. 873–885.
(4) Bliss, C. I., *Drug Standards*, **24**, 61(1956).
(5) Snedecor, G. W., "Statistical Methods Applied to Experiments in Agriculture and Biology," 5th ed., Iowa State College Press, Ames, Iowa, 1956, pp. 285–289.

Liquefaction Time of Rectal Suppositories

By IVO SETNIKAR and SERGIO FANTELLI

An apparatus reproducing the conditions of the environment of the rectum is described. With this apparatus, the liquefaction time of rectal suppositories, either with fatty or water-soluble bases, can be measured. The liquefaction times found for a series of suppositories with fatty bases and with so-called water-soluble bases are reported. It is proposed that a maximum limit should be established for the liquefaction time of rectal suppositories carrying drugs with general action.

The United States Pharmacopeia XVI defines suppositories as "solid bodies of various weights and shapes, adapted for introduction into orifices of the human body and usually melting, softening, or dissolving at body temperature."

However, no method is described for testing these characteristics on the whole suppository. The method for the determination of the melting temperature of fatty (class II) substances is useful for suppository bases but not always applicable to the nonhomogeneous drug-base mixtures of which suppositories are often made. Since the drugs present may alter the melting point (m. p.) of the bases and since the m. p. of the drug-base mixtures may change considerably over a period of time (1), the determination of the m. p. of the suppository base alone is clearly not sufficient for the control of the characteristics mentioned in the U.S.P. definition of suppositories.

Several researchers have felt the need and proposed methods by which these characteristics, and particularly the m. p. can be controlled on the whole suppository. These methods can be classified into two groups: (*a*) m.p. determinations in a dry environment (2, 3) and (*b*) m.p. determinations on suppositories in a water bath (4–7).

With both kinds of methods the m.p. is measured in an environment very different from that of the rectum, for the latter is neither anhydrous nor aqueous. Both give useful information on fatty bases only, because almost all of the so-called water-soluble bases melt at a temperature above body temperature with the first technique, while they dissolve independently of the temperature with the second. Further, with neither method is it possible to record a very important datum, i. e., a representative time of melting, softening, or dissolving at body temperature.

The knowledge of this time is essential in suppositories which include a drug for a general action and must, therefore, be absorbed. Indeed, a suppository which takes too long to liquefy may be expelled before liquefaction, together with the drug it includes. Beside this, it may exert a mechanical irritant action on the rectal ampulla even if the base and the drug, *per se*, are not irritant. The U. S. P. should,

Received May 8, 1961, from Recordati S.p.A., Research Dept., Milano, Italy.
Accepted for publication July 27, 1961.