J. Reprod. Fertil. (1965) **10,** 105–113

ANTIFERTILITY ACTIVITY OF VARIOUS STEROIDS IN THE FEMALE RAT

FRED A. KINCL AND RALPH I. DORFMAN

Institutes of Clinical Medicine and Hormone Biology, Syntex Research Center, Palo Alto, California

(*Received* 12*th January* 1965)

Summary. Antifertility activity of fifty-three steroids was studied in the adult, mated female rat. The steroids were given daily for 7 days beginning on the day of pro-oestrus. On Day 9 of the test implantation sites were counted.

In most cases the antifertility activity was correlated with oestrogenic activity. Several tetrahydropyranyl ethers derived from oestradiol and synthetic steroids lacking a 3, or a 17 oxygen function showed a separation on these two parameters; this may indicate a relatively effective antifertility agent with low oestrogenicity.

INTRODUCTION

A bioassay procedure based on the fact that ovulating hormone in the rat is released between 2.00 and 4.00 p.m. (Everett, Sawyer & Markee, 1949) has been developed to detect compounds which interfere with a succession of reproductive processes from ovulation to implantation. This paper reports the relative potencies of various phenolic and neutral steroids when studied in this assay and administered subcutaneously and/or orally.

MATERIALS AND METHODS

The oestrous cycle of the adult white albino rats of the Holzman strain was followed by vaginal cytology. The first dose of the test compound was given during the morning of pro-oestrus. At 4.00 p.m. on the same day, the females were caged with fertile males for 2 days in succession. Treatment was either by subcutaneous injection or by gavage for a total of 7 days. The total dose of steroid was contained in 3·5 ml of an aqueous solution of sodium chloride (0·9%), polysorbate 80 (0·4%), carboxymethylcellulose (0·5%) and benzyl alcohol (0·9%). The daily dose was contained in 0·5 ml of vehicle. Fertilization was confirmed by the presence of sperm in vaginal smears taken on the 1st or 2nd day. At autopsy, 48 hr after the last treatment, the uterine horns were examined and the number of implantation sites recorded.

Oestradiol-17β served as the reference standard for studies of phenolic steroids and their derivatives administered by subcutaneous injection and the relative potencies of these compounds, administered by gavage, are listed in terms of mestranol (3-methoxy-17α-ethynyloestra-1,3,5(10)-trien-17β-ol). 17α-Ethynyl-5α-androst-2-en-17β-ol served as the corresponding reference standard for the neutral steroids administered either by subcutaneous injection or by

gavage. Results are based on the mean number of implantation sites calculated by graphic estimate. For the reference standards the percentage of pregnancies in any given group is also included.

TABLE 1

REFERENCE PHENOLIC STEROID STANDARDS FOR ANTIFERTILITY ASSAYS BY SUBCUTANEOUS AND ORAL ADMINISTRATION TO RATS

Route of administration	*Steroid*	*Daily dose* (μg)	*No. rats*	*Pregnant* (%)	*No. implantation sites*	
					Mean	*Range*
	0	0	6	100	11	9 to 13
Subcutaneous injection	Oestradiol-17β	0·005	3	100	14	12 to 16
		0·01	8	100	12	9 to 14
		0·015	3	100	12	11 to 14
		0·02	7	100	6	4 to 9
		0·05	5	100	13	11 to 14
		0·15	6	83	10	10 to 14
		0·2	6	66	6	2 to 10
		0·3	14	78	4	0 to 10
		0·4	6	66	2	0 to 10
		0·45	3	0	0	0 to 0
Oral	Mestranol	3	3	100	8	6 to 13
		5	14	79	8	0 to 14
		10	13	69	6	0 to 12
		15	6	67	4	0 to 10
		20	13	42	2	0 to 7
		45	3	0	0	0 to 0
		75	3	0	0	0 to 0

TABLE 2

ANTIFERTILITY ACTIVITY OF 17α-ETHYNYL-5α-ANDROST-2-EN-17β-OL BY SUBCUTANEOUS AND ORAL ADMINISTRATION TO RATS

Route of administration	*Total dose* (μg)	*No. rats*	*Pregnant* (%)	*No. implantation sites*	
				Mean	*Range*
Subcutaneous	100	14	100	11	6 to 12
	200	8	100	7	4 to 11
	300	6	33	1	0 to 5
	400	8	12	0·3	0 to 2
	900	3	0	0	0 to 0
	3000	3	0	0	0 to 0
Oral	150	7	100	9	4 to 12
	300	10	100	7	4 to 11
	600	7	14	0·1	0 to 1
	900	3	0	0	0 to 0

RESULTS

Antifertility activity of the standard reference compounds administered subcutaneously and/or by gavage are given in Tables 1 and 2. Oestradiol-17β was studied at a daily dose of 0·005 to 1 μg. A daily dose of 0·05 μg did not influence

fertilization and pregnancy, but an increase to 0·15 μg resulted, in six females, in a modest decrease in fertility from 100% to 83%, and a mean of ten implantation sites. At the daily dose of 0·3 μg, fertility decreased to 67% of the pregnancy rate and a mean of four implantation sites. At the daily dose of 0·45 μg, pregnancy was completely inhibited and implantation sites could not be found.

On a weight basis mestranol was a less effective antifertility agent than oestradiol-17β by injection. The average number of implantation sites and frequency of fertility decreased at daily doses between 5 and 20 μg, and at a daily dose of 45 μg pregnancy was completely inhibited in the three animals studied (Table 1).

17α-Ethynyl-5α-androst-2-en-17β-ol demonstrated a significant antifertility effect at the daily dose level of 300 μg, given subcutaneously, as indicated by the fact that only two of the six rats became pregnant and the mean number of implantation sites decreased to one (Table 2). When this compound was given orally, 600 μg/day was needed to inhibit fertility. At the daily dose of 300 μg, inhibition was not observed in either of the two parameters (i.e. percentage of pregnant rats; mean number of implantation sites). Tripling the daily dose to 900 μg resulted in complete inhibition, judged by the same indices.

Our findings accord with the results reported by Guéritée & Savini (1964) who found that the acetate inhibits luteinization in female rats at a daily dose of 5 mg/kg.

Antifertility activity of phenolic steroids by subcutaneous injection is given in Table 3, and by oral route in Table 4. The following information has been included: the daily dose range studied in μg, the total number of rats used, relative antifertility activity, and relative oestrogenic activity determined in immature mice by the method of Rubin, Dorfman, Black & Dorfman (1951). Fifteen steroids, all less active than oestradiol-17β, were given by subcutaneous injection. Three of these were roughly one-tenth as active as oestradiol-17β. Mestranol was found to have 13% of the antifertility and 10% of the oestrogenic activity of oestradiol. A closely related compound, 3-methoxy-17β-cyanethoxy-oestra-1,3,5(10)-trien, showed 9% of the antifertility and 8% of the oestrogenic activity. A three-fold separation between antifertility and oestrogenic activity was found for 17α-ethynyloestra-1(10),5-diene-3β,17β-diol, which had 7% of the antifertility but only 2% of the oestrogenic activity of oestradiol-17β. Although this compound is not a phenolic steroid, it has been included in this group since it may readily aromatize. The corresponding 17-ketone derivative and 17-deoxyoestradiol (oestra-1,3,5(10)-trien-3-ol) were only moderately active, having 1% and 2% of the activity of oestradiol-17β. Three steroids had between 0·3 and 0·1% of the antifertility activity of oestradiol-17β. These include 3-methoxy-17α-fluoroestra-1,3,5(10)-trien (0·3%); oestra-1,3,5(10)-trien-17-one (0·2%); and 3-methoxy-5,10-methylenoestra-1,3-dien-17-one (0·1%). The antifertility activity of the remaining six weakly active steroids could be defined with much less certainty since in several cases the compounds were tested only at one dose level and on only three rats/dose. These compounds include 1-methyl-3-acetoxyoestra-1,3,5(10),6-tetraen-17-one, 2-methyl oestradiol, oestradiol 3-methyl-17-methylformyl bis ether, 4-allyl oestradiol, 2,4-diallyloestradiol and 3-hydroxypregna-1,3,5(10)-trien-20-one.

TABLE 3

ANTIFERTILITY ACTIVITY OF PHENOLIC STEROIDS (AND DERIVATIVES) IN A SUBCUTANEOUS INJECTION ASSAY

Steroid	*Daily dose range* (μg)	*No. rats*	*Relative antifertility activity*	*Oestrogenic activity (immature mouse uterus)*
Oestradiol-17β			100	100
Mestranol	1 to 25	18	13	10
3-Methoxy-17β-cyanethoxy oestra-1,3,5(10)-trien	0·1 to 100	33	9	2
17α-Ethynyloestra-1(10),5-diene-3β, 17β-diol	0·1 to 90	18	7	2
3-Acetoxy-6ξ, 7ξ-dichlorooestra-1,3,5(10)-trien-17-one	3 to 30	9	3	
Oestra-1,3,5(10)-trien-17β-ol	10 to 1000	24	2	2
3β-Hydroxyoestra-1(10), 5-dien-17-one	10 to 1000	12	1	
3-Methoxy-17α-fluorooestra-1,3,5(10)-triene	300 to 3000	12	0·3	0·05
Oestra-1,3,5(10)-trien-17-one	50 to 1000	17	0·2	0·7
3-Methoxy-5, 10-methylene-oestra-1,3-dien-17-one	200 to 5000	12	0·1	0·003
1-Methyl-3-acetoxyoestra-1,3,5(10), 6-tetraen-17-one	1000	3	0·03	0·001
2-Methyl oestradiol	2000	3	0·02	15
3-Methoxy-17β-methoxyformyl-oestra-1,3,5(10)-triene	100 to 1000	6	0·02	8
4-Allyloestradiol-17β	1000	3	<0·05	
2,4-Diallyloestradiol-17β	3000	3	<0·02	
3-Hydroxy-19-norpregna-1,3,5(10)-trien-20-one	1000 to 10000	6	<0·005	0·04

TABLE 4

ANTIFERTILITY ACTIVITY OF PHENOLIC STEROIDS (AND DERIVATIVES) IN AN ORAL ASSAY

Steroid	*Daily dose range* (μg)	*No. rats*	*Relative antifertility activity*	*Oestrogenic activity (immature mouse uterus)*
Mestranol			100	100
3-Methoxy-17β-cyanethoxyoestra-1,3,5(10)-triene	0·5 to 270	45	600	8
Oestradiol-17β-tetrahydropyranyl ether	10 to 1000	12	200	160
Oestradiol-17β	1 to 25	15	150	300
Oestradiol-3, 17β bis-tetrahydro-pyranyl ether	1 to 450	21	100	3
17α-Ethynyloestradiol-3, 17-bis tetrahydropyranyl ether	10 to 90	9	75	33
17α-Ethynyloestra-1,3,-5(10)-trien-17β-ol	2 to 1000	24	50	5
17β-(2′-hydroxy)-ethoxyoestra-1,3,5(10)-trien-3 methoxy	30 to 300	9	33	6
Oestra-1,3,5(10)-trien-3-ol	100 to 900	9	15	0·2
3β-Hydroxyoestra-1(10)-5-dien-17-one	100 to 1000	9	10	0·2
Oestra-1,3,5(10)-trien-17-one	100 to 1000	9	5	1

Oral activity of ten steroids defined in terms of mestranol is given in Table 4. The most active steroid was 3-methoxy-17β-cyanethoxyoestra-1,3,5(10)-triene. This compound had only 8% of the oestrogenic activity of mestranol but was six times more active in the antifertility test: this gave a separation between antifertility and oestrogenic activity of over sixty-fold. Two steroids, oestradiol 3-tetrahydropyranyl ether (200%) and oestradiol-17β (150%) were more active than mestranol. Oestradiol 3, 17-bis tetrahydropyranyl ether was as active as the standard and ethynyl oestradiol 3,17-bis tetrahydropyranyl ether was somewhat less active. These four compounds showed marked separation between antifertility and oestrogenic activity. 3-Deoxyethynyl oestradiol (17α-ethynyl-oestra-1,3,5(10)-trien-17β-ol) was ten times more active as an antifertility agent

TABLE 5

ANTIFERTILITY ACTIVITY OF NEUTRAL STEROIDS IN A SUBCUTANEOUS INJECTION ASSAY

Steroid	*Daily dose range (μg)*	*No. rats*	*Relative antifertility activity*
17α-Ethynyl-5α-androst-2-en-17β-ol			100
2α-Methyl-17β-propionoxy-5α-androstan-3-one	10 to 1000	9	300
17β-Hydroxy-A-homoandrosta-1(10),2,4a-trien-4-one	10 to 1000	9	100
2-Hydroxymethyl-5α-androst-2-en-17β-ol	430 to 1400	9	50
Testosterone	10 to 25000	28	50
2-Hydroxymethyl-17α-methyl-5α-androst-2-en-17β-ol	300 to 1000	6	33
2α,17α-Dimethyl-17β-hydroxy-5α-androstan-3-one	100 to 1000	9	30
6-Chloro-6-dehydro-19-nor progesterone	1000 to 3000	6	13
19-Norprogesterone	100 to 3000	15	12
20β-Hydroxy-19-norpregn-4-en-3-one	1000 to 10000	6	10
2-Carboxy-5α-androst-2-en-17β-ol	1000 to 5000	6	<4

than oestrogen; 3-deoxyoestrone (oestra-1,3,5(10)-trien-17-one) had a ratio of fifty, and 17-deoxyoestrone (oestra-1,3,5(10)-3-ol) a ratio of seventy-five.

Relative antifertility activity of neutral steroids expressed in terms of 17α-ethynyl-5α-androst-2-en-17β-ol is listed in Table 5 (subcutaneous injection) and Table 6 (oral assays). As an antifertility agent, this neutral steroid was itself considerably less active than oestradiol-17β by injection (0·1%), or mestranol given orally (3%). Ten steroids were studied by the subcutaneous route. Two, 2α-methyl-17β-propionoxy-5α-androstan-3-one and 17β-hydroxy-A-homoandrosta-1(10),2,4a-trien-4-one were respectively three times and equally as potent as the standard. The remaining eight compounds were less active. These included three 3-deoxy C_{19} steroids, 2-hydroxymethyl-5α-androst-2-en-17β-ol (50%), the corresponding 17α-methyl derivative (33%) and 2-carboxy-5α-androst-2-en-17β-ol (<4). Testosterone assayed at 50%; 2α,17α-dimethyl-17β-hydroxy-5α-androstan-3-one at 30%, and three pregnan

derivatives at 10 to 13% (6-chloro-6-dehydro-19-norprogesterone at 13%, 19-norprogesterone at 12%; and 20β-hydroxy-19-norprogesterone at 10%).

By the oral test, 2α,3α-difluoromethylene-17α-methyl-5α-androstan-17β-ol

TABLE 6

ANTIFERTILITY ACTIVITY OF NEUTRAL STEROIDS IN AN ORAL ASSAY

Steroid	*Daily dose range (μg)*	*No. rats*	*Relative antifertility activity*
17α-Ethynyl-5α-androst-2-en-17β-ol			100
2α,3α-Difluoromethylene-17α-methyl-5α-androstan-17β-ol	100 to 2000	9	40
19 Norprogesterone	10000	3	2
2-Formyl-17α-ethynyl-5α-androst-2-en-17β-ol	400	3	<50
2-Hydroxymethyl-17α-methyl-5α-androst-2-en-17β-ol	1000 to 5000	6	<5
2-Carboxy-17α-methyl-5α-androst-2-en-17β-ol	1000	3	<20

TABLE 7

COMPOUNDS WHICH DID NOT INHIBIT FERTILITY IN ADULT MATED RATS (SUBCUTANEOUS ADMINISTRATION)

Steroid	*Total No. rats used*	*Daily dose studied (Range in mg)*
Norethindrone*	20	0·15 to 2·4
Chlormadinone acetate*	6	1 to 5
Progesterone	6	5 to 15
5α-Dihydro-19-norprogesterone	6	0·1 to 1
17α-Hydroxy-19-norprogesterone	6	0·1 to 1
3β,17β-Diacetoxypregn-5-en-20-one	6	1 to 5
3β-Hydroxy-17α-acetoxypregn-5-en-20-one	6	0·1 to 1
20β-Hydroxypregn-4-en-3-one	6	0·1 to 1
5β-Pregnan-3,11,20-trione	6	1 to 5
5β-Pregnan-3β,20β-diol	6	1 to 5
3β-Acetoxy-5β-pregnan-20-one	6	1 to 5
3α-Hydroxy-5β-pregnan-20-one	6	1 to 5
3α-Acetoxy-5β-pregnan-20-one	6	1 to 5
3α-Hydroxy-5β-pregn-16-en-20-one	6	1 to 5
3β, 11β, 20ξ-Trihydroxy-5β-pregnan-3,20 diacetate	6	1 to 5
3α,11β,20α-Trihydroxy-5β-pregnan-3,20 diacetate	6	1 to 5
16α-Cyano-3β-hydroxy-5α-pregnan-20-one	6	1 to 5
6α-Fluoro-16α-methylpregna-1,4-diene-3,20-dione	12	0·3 to 3

* Administered orally.

was 40% as active as the standard. The other steroids listed in Table 6 were not highly active.

Table 7 lists various steroids which were inactive in the antifertility test, together with the daily doses and the total numbers of rats used. Except for two

steroids, norethindrone (17α-ethynyl-17β-hydroxyoestr-4-en-3-one) and chlormadinone acetate (6-chloro-17α-acetoxypregna-4,6-diene-3,20-dione) which were given by gavage, all the other compounds were injected. These include progesterone (which was not active at a dose of 15 mg/day) and two derivatives of 19-norprogesterone (5α-dihydro-19-norprogesterone and 17α-hydroxy-19-norprogesterone, both inactive at a dose of 1 mg/day). The remaining compounds represent a variety of C_{21} steroids, many of which are possible progesterone metabolites.

The relation between the subcutaneous and oral activity of phenolic steroids is illustrated in Table 8. Oestradiol-17β was about six times more active by

TABLE 8

RELATIVE ANTIFERTILITY ACTIVITY OF VARIOUS PHENOLIC STEROIDS BY SUBCUTANEOUS INJECTION AND BY GAVAGE

Steroid	*Subcutaneous injection A*	*Gavage B*	*Ratio* $\frac{B}{A}$
Oestra-1,3,5(10)-trien-3β-ol	13	15	1·2
Oestra-1,3,5(10)-trien-17-one	1·6	5	3·1
3-Methoxy-17β-cyanoethoxyoestra-1,3,5(10)-trien	70	600	8·6
Oestradiol-17β	700	150	0·2

(Mestranol = 100.)

TABLE 9

RELATIVE ANTIFERTILITY ACTIVITY OF VARIOUS NEUTRAL STEROIDS BY SUBCUTANEOUS INJECTION AND BY GAVAGE

Steroid	*Subcutaneous injection A*	*Gavage B*	*Ratio* $\frac{B}{A}$
2-Hydroxymethyl-17α-methyl-5α-androst-2-en-17β-ol	33	<5	<0·15
19 Norprogesterone	12	2	0·17

injection than orally, but the other three steroids which were studied by both routes were all more active orally. The oral/subcutaneous ratio was 8·6 for 3-methoxy-17β-cyanoethoxyoestra-1,3,5(10)-trien, 3·1 for 3-deoxyoestrone and 1·2 for 3-deoxyoestradiol.

Two neutral steroids, 2-hydroxymethyl-17α-methyl-5α-androst-2-en-17β-ol and 19-norprogesterone were both more active by injection than by gavage (Table 9).

DISCUSSION

Including the three steroids which were used as primary standards, oestradiol-17β, mestranol and 17α-ethynyl-17β-hydroxy-5α-androst-2-en-17β-ol, fifty steroids were studied for antifertility activity in the adult mated rat by injection

and/or oral routes. The first dose of the test compounds was given on the morning of pro-oestrus several hours before the beginning of the chain of events which leads to ovulation. It could be expected, therefore, that this method might interfere in the overall effect of fertility, including ovulation. Detailed study with two compounds, oestradiol-17β and 3-methoxy-cyanethoxyoestra-1,3,5(10)-trien showed, however, that transport of the zygote is involved. The results of this study will be published separately. It appears that this assay measured mainly the influence of steroids on ovum development and/or transport in the oviduct, or on nidation.

Additional evidence that mechanisms other than ovulation inhibition may be involved is provided by our findings that there was no apparent correlation between the activity in this test and several other parameters of biological activity, i.e. the uterotrophic activity, anti-ovulatory activity measured in the adult oestrous rabbit (Kincl & Dorfman, 1963), anti-oestrogenic activity (Dorfman, Kincl & Ringold, 1961; Dorfman & Kincl, 1963), and inhibition

TABLE 10

MINIMUM EFFECTIVE DAILY DOSES (IN μG) OF SUBCUTANEOUSLY INJECTED STEROIDS NEEDED TO PRODUCE SIGNIFICANT BIOLOGICAL EFFECT

Steroids	*Anti-fertility*	*Oestrogenic*	*Anti-ovulatory*	*Anti-oestrogenic*	*Parabiotic rat*
Oestradiol-17β	0·3	0·01			0·1
Mestranol	15	0·4	100		1*
Progesterone	>15000		100	80	1000
19-Norprogesterone	3000		10	66	1000
Norethindrone	>2400	5	150	1	400*
Chlormadinone acetate	>5000		10	17	>1000

* Administered orally.

of gonadotrophin assayed in the parabiotic rat (Kincl & Dorfman, 1964; Kincl, Birch & Dorfman, 1964). This is more apparent from Table 10 which lists the minimum daily doses needed to produce a significant inhibitory effect.

The amounts of ring A phenolic steroids, oestradiol-17β and of mestranol needed for antifertility effect in the rat or the parabiotic rat were a hundred times less than those needed for anti-ovulatory effect in the rabbit. Compounds with progestational activity, namely progesterone, 19-norprogesterone, norethindrone and chlormadinone acetate were, on the other hand, considerably more active in the anti-ovulation test on the rabbit and only weakly active, if active at all in the antifertility test on the intact and parabiotic rat. Although it may not be permissible to compare different species and under varying conditions, these data indicate that in the present antifertility test on the rat, other factors besides inhibition of ovulation are involved.

A clear dissociation of oestrogenicity, as measured by the uterotrophic assay and the present antifertility test, was observed for the tetrahydropyranyl ethers. This was true for the 3,17-di substituted ether of oestradiol-17β where the separation factor (ratio of relative antifertility to oestrogenic activity) was thirty-three, the three substituted ether of oestradiol-17β had a separation

factor of eight and finally that for 17α-ethynyl oestradiol-3,17-bis tetrahydropyranyl ether was 2·3. This may indicate a relatively effective antifertility activity of these ethers with relatively low oestrogenicity.

ACKNOWLEDGMENTS

These assays were performed at the Endocrine Laboratories, Madison, Wisconsin, under the supervision of Dr Elva G. Shipley.

REFERENCES

Dorfman, R. I. & Kincl, F. A. (1963) Steroid anti-estrogens. *Steroids*, **1**, 185.

Dorfman, R. I., Kincl, F. A. & Ringold, H. J. (1961) Anti-estrogen assay of neutral steroids administered by gavage. *Endocrinology*, **68**, 43.

Everett, J. W., Sawyer, C. H. & Markee, J. E. (1949) Neurogenic timing factor in control of the ovulatory discharge of luteinizing hormone in the cyclic rat. *Endocrinology*, **44**, 234.

Guéritée, N. & Savini, E. C. (1964) Involution gonadique réversible, chez le rat des deux sexes, produite par voie buccale, par un stéroide dépourvu d'actions hormonales et anabolisantes: l'acétate de 17α-éthinyl 2-androstène 17β-ol. *Ann. Endocr.* (*Paris*), **25**, 544.

Kincl, F. A., Birch, A. J. & Dorfman, R. I. (1964) Pituitary gonadotropic inhibitory activity of various steroids in ovariectomized-intact female rats in parabiosis. *Proc. Soc. exp. Biol., N.Y.* **117**, 549.

Kincl, F. A. & Dorfman, R. I. (1963) Anti-ovulatory activity of steroids in the adult oestrous rabbit. *Acta endocr.* (*Kbh.*), **42**, Suppl. 73.

Kincl, F. A. & Dorfman, R. I. (1964) Pituitary gonadotrophin inhibitory action of neutral steroids. *Acta endocr.* (*Kbh.*), **46**, 300.

Rubin, B. L., Dorfman, A. S., Black, L. & Dorfman, R. I. (1951) Bioassay of estrogens using the mouse uterine response. *Endocrinology*, **49**, 429.