PITUITARY AND OVARIAN FUNCTION IN WOMEN
ON CONTINUOUS LOW DOSE PROGESTOGENS; EFFECT OF
CHLORMADINONE ACETATE AND NORETHISTERONE

By
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K.-G. Tillinger and L. Wide

ABSTRACT

Chlormadinone acetate (6-chloro-17α-hydroxyprogesterone-4,6-dione-3,20-dione-17-acetate; hereafter CAP = chloro-acetoxy-progesterone) was administered continuously in daily oral doses of 0.5 mg to three normally menstruating women and the urinary excretion of luteinizing hormone (LH), oestrone (OE1), 17β-oestradiol (OE2), oestriol (OE3) and pregnanediol (5β-pregnane-3α,20α-diol; hereafter P-diol) was studied. Forty-eight hour specimens were analyzed during a pretreatment cycle, the first and fourth CAP cycles and the first posttreatment cycle.

Considerable individual variations were found in all parameters studied. In addition, significant differences were observed in the response of the same subjects to the first and to the fourth course of CAP. A well defined midcycle LH peak was found in all pretreatment cycles. The administration of CAP abolished the midcycle LH peak in 5 of the 6 treatment courses studied; the 

ovulatory

LH peak re-appeared in all subjects during the first posttreatment cycle. The oestrogen and P-diol excretion patterns were often inconsistent with the LH pattern; on several occasions a distinct 

ovulatory

oestrogen or P-diol pattern was found in the absence of any midcycle LH peak. Such a P-diol pattern was accompanied by an elevated basal body temperature.

A similar study was conducted in two normally menstruating women, before and during the continuous administration of a low dose (0.1 mg/d) of oestrogen-free norethisterone (17α-ethynyl-17β-hydroxy-19-norandrost-

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4-en-3-one; hereafter NET). followed by a course of a high dose (2.5 mg/d) of NET. The inhibitory effect of NET on LH excretion was less marked than that of CAP. The oestrogen excretion remained rather high. In only one of the two subjects did the administration of 0.1 mg of NET abolish the «ovulatory» P-diol pattern. Elevated P-diol values were not found during the administration of 2.5 mg of NET. The correlation between «ovulatory» LH- and P-diol patterns was rather poor.

The data indicate that the continuous administration of low doses of CAP interferes only slightly with oestrogen excretion, but has a marked effect on the midcycle LH-peak.

It is concluded that the assertion of ovulation inhibition on the basis of urinary LH- and steroid excretion studies may be associated with major uncertainties. Also, it seems that for a better understanding of the mechanism of contraceptive action of low level progestogens more information is required on the minimal amounts of urinary LH and P-diol, which are still compatible with a normal ovulation and corpus luteum function.

The balance of evidence indicates that combined or sequential oral contraceptives interfere with ovulation (for a review: Diczfalusy 1968). On the other hand, evidence is available indicating that by the continuous administration of low levels of progestogens satisfactory contraception can be achieved without consistent inhibition of ovulation (Martinez-Manautou et al. 1967; Foss et al. 1968; Martinez-Manautou 1969). How the contraceptive effect is achieved is incompletely understood.

The purpose of the present investigation was to study the immediate as well as late effects of the continuous administration of 0.5 mg of chlormadinone acetate (CAP) on the urinary elimination of LH, OE_1, OE_2, OE_3 and P-diol during several cycles. The investigation was also extended to the study of the effects of a low (0.1 mg/day) and a high (2.5 mg/day) dose of oestrogen-free norethisterone (NET).

EXPERIMENTAL

Subjects. – Five normally menstruating healthy women volunteered to participate in this study. All of them exhibited regular cycles of normal length, with 27–29 days of duration. With the exception of M. W., who had two previous pregnancies, they were nulliparous. The age of the subjects at the time of the study was as follows: K. M.: 31, A. S.: 21, G. O.: 34, G. T.: 29 and M. W.: 28 years.

Plan of study. – In the study of the effect of CAP a) the scheme was as follows. Forty-eight hour urine specimens were collected from subjects K. M., A. S. and G. O. continuously during a pretreatment (control) cycle, followed by the first CAP cycle.

a) Chlormadinone acetate and oestrogen-free norethisterone were kindly supplied by the Syntex Institute of Clinical Medicine, Palo Alto, Calif., U. S. A.
The uninterrupted daily administration of 0.5 mg of CAP continued during three more cycles, but urine was only collected and analyzed during the last CAP cycle. The administration of CAP was then discontinued and urine was collected during the first posttreatment cycle (e.g. Fig. 1). Basal body temperature was recorded daily.

The design was somewhat different in subjects G.T. and M.W. and consisted of the analysis of a pretreatment cycle, a cycle with a low dose (0.1 mg/day) of NET, followed by a course with a high dose (2.5 mg/day). In this part of the study no posttreatment cycle was analyzed and the basal body temperature was not recorded. In addition, in subject M.W. oestrogen assays could only be conducted during the administration of the high dose of NET.

Assay methods

Luteinizing hormone (LH). – This was analyzed by a radioimmunoassay procedure based on the radioimmunosorbent technique of Wide & Porath (1966). Antibodies to human chorionic gonadotrophin (HCG) were raised in rabbits and were coupled to CNBr activated Sephadex (Wide et al. 1967). A highly purified preparation (12 000 IU/mg) was labelled with $^{125}$I to a specific activity of approximately 100 mCi/mg by the chloramine T method (Hunter & Greenwood 1962). All samples were assayed twice in duplicate and the results are expressed in IU/48 h in terms of the 2nd IRP of HMC$^c$.

Oestrone (OE$^1$), 17β-oestradiol (OE$^2$) and oestriol (OE$^3$). – The methods of Brown (1955) and Brown et al. (1957) have been modified in order to improve their specificity and accuracy and to estimate properly the losses occurring during hydrolysis and processing of the extracts. The principles of the modified method (Goebelsmann & Diczfalusy, unpublished) are as follows.

A urine specimen of 400 ml is diluted with the same amount of distilled water, to which the following labelled internal standards are added: 11 000 dpm each of oestrone-4-14C-3-sulphate and 17β-oestradiol-4-14C-3-sulphate, 40 000 to 60 000 dpm of both oestrone-6,7-3H-glucosiduronate and 17β-oestradiol-6,7-3H-3-glucosiduronate as well as 30 000 dpm oestril-15-3H-3-glucosiduronate and 14 000 dpm oestriol-16-14C-16-glucosiduronate. The urine specimens are then hydrolysed for 72 h at 37°C with a Helix pomatia enzyme preparation$^e$ in acetate buffer at pH 4.2. To each ml of diluted urine the equivalent of 375 units of β-glucuronidase (corresponding to 3000 units of sulphatase) is added at the start of the hydrolysis and 250 units of

$b$) Supplied by Dr. T. Perklev, AB Leo, Hälsingborg.

c) Because of the incompletely established relationship between biological and immunological gonadotrophic activities (e.g. W. H. O. 1968) these units should be considered as approximate.

d) Labelled oestrone sulphate was synthesized as described by Kirschner et al. (1966) and Bolté et al. (1964). 17β-Oestradiol-3-sulphate was prepared from oestrone sulphate by KBH$_4$-reduction. Oestrone-3-glucosiduronate was prepared according to Zucconi et al. (1967), and 17β-oestradiol-3-glucosiduronate by KBH$_4$-reduction of oestrone glucosiduronate. Oestriol-16-glucosiduronate was synthesized according to the method described by Staunwhite et al. (1964), and oestriol-3-glucosiduronate according to the method of Goebelsmann et al. (1965). The specific activities (dpm/µg) were such that the total mass of labelled oestrogens added to each sample was less than 0.1 µg.

e) Purchased from Industrie Biologique Française, Gennevilliers, France.
\(\beta\)-glucuronidase + 2000 units of sulphatase 24 and 48 h later. The urine is extracted with \(1 \times 800\) and \(2 \times 400\) ml of ether. The volume of the combined ether extract is reduced to 800 ml at a reduced pressure and the ether is washed with 160 ml of the carbonate buffer (pH 10.5) of Brown (1955). The buffer is discarded and the ether is shaken with 40 ml of \(2 \times \) NaOH prior and subsequent to the addition of 160 ml of a saturated NaHCO\(_3\) solution. The buffer is discarded. The ether is washed with 40 ml of an 8\% (w/v) NaHCO\(_3\) solution and 20 ml of distilled water and is evaporated almost to dryness. The wet residue is taken up in 50 ml of toluene and the toluene is extracted with \(3 \times 20\) ml of \(\pi\) NaOH. The aqueous phase (containing the oestrogens) is boiled at 100°C for 30 min («saponification» of Brown et al. 1957), cooled, the pH adjusted to 10.3 and the solution extracted with \(3 \times 40\) ml of ether. The ether is evaporated to dryness and the OE\(_1\) + OE\(_2\) fraction is separated from the OE\(_3\) fraction by a »Brown partition« \([2\% (v/v)\) aqueous ethanol, benzene, petroleum ether \((2:1:1)\)].

Methylation and chromatography of the 3-methyl ethers formed is carried out according to Brown (1955), as slightly modified by Diczfalusy & Westman (1956). A 10\% aliquot of the chromatographic oestrone methyl ether (MOE\(_1\)) fraction is used for counting (to correct for losses) and the rest \((90\%)\) is subjected to the Kober reaction (Brown 1955).

The chromatographed \(17\beta\)-oestradiol 3-methyl ether (MOE\(_2\)) is then converted to MOE\(_1\) by enzymic oxidation in a carbonate buffer of pH 9.3, using 10 mg of a bacterial hydroxysteroid dehydrogenase preparation\(^{f}\) (corresponding to 0.5 \(\beta\)-units, with testosterone as substrate) and 8.0 mg of \(\beta\)-nicotinamide adenine nucleotide\(^{f}\) at 37°C overnight. The aqueous solution is then extracted with an excess of ether. The ether is evaporated, the residue is taken up in n-hexane and chromatographed on alumina as before. Again 10\% is used for counting, 90\% for the colour reaction. Following correction for losses, the MOE\(_1\) estimated in this way represents the true OE\(_2\) content of the urine.

The OE\(_3\) fraction obtained following the Brown partition is subjected to paper partition chromatography, using a system of benzene, methanol, water \((2:1:1, \text{ by vol.})\) for 40 h. The OE\(_3\) zone is eluted, methylated and the oestriol 3-methyl ether (MOE\(_3\))

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**Table 1.**

Recovery of labelled oestrogen conjugates in the final chromatographic oestrone-, 17\(\beta\)-oestradiol and oestriol methyl ether fraction. The conjugates were added to the urine specimens prior to enzyme hydrolysis.

<table>
<thead>
<tr>
<th>Conjugate added</th>
<th>No. of exp.</th>
<th>Mean ± so</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestrone-4(\text{-}^{14}\text{C})-3-sulphate</td>
<td>22</td>
<td>66.6 ± 9.2</td>
<td>51 - 83</td>
</tr>
<tr>
<td>Oestrone-6,7(\text{-}^{3}\text{H})-3-glucosiduronate</td>
<td>22</td>
<td>66.2 ± 10.8</td>
<td>49 - 88</td>
</tr>
<tr>
<td>17(\beta)-Oestradiol-4(\text{-}^{14}\text{C})-3-sulphate</td>
<td>22</td>
<td>57.6 ± 9.6</td>
<td>41 - 79</td>
</tr>
<tr>
<td>17(\beta)-Oestradiol-6,7(\text{-}^{3}\text{H})-3-glucosiduronate</td>
<td>22</td>
<td>57.1 ± 8.9</td>
<td>46 - 79</td>
</tr>
<tr>
<td>Oestriol-16(\text{-}^{14}\text{C})-16-glucosiduronate</td>
<td>14</td>
<td>81.8 ± 8.0</td>
<td>70 - 93</td>
</tr>
<tr>
<td>Oestriol-15(\text{-}^{3}\text{H})-3-glucosiduronate</td>
<td>14</td>
<td>76.4 ± 8.7</td>
<td>66 - 98</td>
</tr>
</tbody>
</table>

\(^{f}\) Purchased from Sigma Chemical Co., Saint Louis, Mo., U. S. A.
formed is chromatographed on alumina. Again 10% of the eluate is used for counting, 90% for colour reaction.

The recoveries of the 6 internal standards in a number of urine specimens from the 5 subjects of this study are indicated in Table 1.

It appears from the data of Table 1 that the recoveries are satisfactory and that values obtained with different conjugates of the same oestrogen are rather similar. Therefore, for correction for losses, a mean value of the two conjugates of the same oestrogen was employed.

_Pregnanediol_ (P-diol). — The method of Klopper et al. (1955) was used with the exception that the colour correction equation of Allen (1950) was employed with readings at 390, 430 and 470 mµ. In order to assess the specificity of the method under our experimental conditions, an aliquot of each sample of the pregnanediol diacetate obtained after column chromatography on alumina was subjected to gas liquid chromatography on methylsilicone (1% SE-30) at a column temperature of 240°C using a flame ionization detector and cholestane as an internal standard). Appropriate corrections were made for the difference in response between cholestane and pregnanediol diacetate.

The coefficients of correlation obtained from 168 parallel estimations by gas chromatography and the method of Klopper et al. (1955) are presented in Table 2.

In 109 analyses in which the amount of pregnanediol diacetate exceeded 25 µg (corresponding to 1.0 mg/48 h) the coefficient of correlation was as high as r = 0.938, whereas in 54 analyses on P-diol contents of less than 25 µg, the value of r was only 0.460. Since invariably 1/40th aliquots of 48 h urine specimens were analyzed it appears that the estimates obtained by the method of Klopper et al. (1955) were reliable, provided the urine specimens contained more than 1.0 mg/48 h.

**Table 2.**

Coefficients of correlation (r) between gas chromatographic and colorimetric estimates of pregnanediol in the final fractions obtained by the method of Klopper et al. (1955).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>&lt; 25 µg&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>&gt; 25 µg&lt;sup&gt;b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of comparisons</td>
<td>r</td>
</tr>
<tr>
<td>K. M.</td>
<td>19</td>
<td>0.653</td>
</tr>
<tr>
<td>A. S.</td>
<td>16</td>
<td>0.423</td>
</tr>
<tr>
<td>G. U.</td>
<td>19</td>
<td>0.803</td>
</tr>
<tr>
<td>Mean</td>
<td>0.460</td>
<td>0.938</td>
</tr>
</tbody>
</table>

<sup>a</sup> corresponds to < 1.0 mg/48 h.

<sup>b</sup> corresponds to > 1.0 mg/48 h.

We are indebted to Dr. J. Sjövall and Ing. R. Reimendal, Karolinska Institute, Stockholm, for carrying out the gas chromatographic analyses.

These data are in reasonably good agreement with those reported by van der Molen (1968) and Hammerstein & Zielske (1968).
RESULTS


The term »total oestrogen« is used to denote the sum of OE₁ + OE₂ + OE₃. The individual excretion values of these three oestrogens are indicated in Figs. 4–6.

In subject K. M. (Fig. 1) the first course of CAP resulted in the disappearance of the »ovulatory« LH- and P-diol patterns, whereas the oestrogen excretion was not influenced. The fourth CAP course resulted again in a suppression of the LH peak and low P-diol values, accompanied by a diminished oestrogen excretion. It is noteworthy, however, that there was a very slight elevation of LH values during 6 days around »midcycle« and that this was associated

**Fig. 1.**
Urinary excretion of luteinizing hormone (LH), »total oestrogens« (i.e. the sum of oestrone + 17β-oestradiol + oestriol) and pregnanediol in 48 h specimens collected from subject K. M. during a pretreatment (control) cycle, during the first and fourth cycle of continuous chlormadinone acetate administration and during the first post-treatment cycle. Basal body temperature (BBT) and menstrual bleedings (hatched bars) are also indicated.
Urinary excretion of luteinizing hormone, oestrogens and pregnanediol in subject A. S.
For further particulars consult legend to Fig. 1.

with limited increases in oestrogen and P-diol excretion. Indeed, the latter
approached 4.0 mg/48 h. Upon discontinuation of CAP administration, the
»ovulatory« LH- and P-diol patterns reappeared during the first cycle, al¬
though the oestrogen excretion still seemed to be depressed.

In subject A. S. the first course of CAP resulted in a diminished, but not
completely suppressed midcycle LH peak, accompanied by oestrogen and P-
diol excretion curves lower than in the preceding control cycle. However,
P-diol values higher than 5.0 mg/48 h were recorded. During the fourth course
of CAP »ovulatory-type« oestrogen and P-diol patterns were found in the
absence of any midcycle LH peak. The elevated P-diol values were associated
with a corresponding increase in basal body temperature. Discontinuation
of CAP administration resulted in a typical »ovulatory« pattern of LH, oestrogens
and P-diol.

In subject G. Ö. urine collection had to be interrupted at the end of the first
control cycle, because of an unexpected travel obligation. In this case – like in
subject K. M. – the first course of CAP abolished the »ovulatory« LH- and
P-diol patterns, but not that of oestrogens. Even in the fourth course there
CHLORMADINONE ACETATE 0.5mg/d

Fig. 3.
Urinary excretion of luteinizing hormone, oestrogens and pregnanediol in subject G. O. For further particulars consult legend to Fig. 1.

was a typical cyclic oestrogen pattern in the absence of any elevated LH- or P-diol values. Discontinuation of CAP administration resulted in the reappearance of the »ovulatory« LH- and P-diol patterns during the first post-treatment cycle.

Studies with norethisterone (NET). – The results obtained in subjects G. T. and M. W. are presented in Figs. 7 and 8.

In subject G. T. (Fig. 7) the administration of 0.1 mg of NET abolished the »ovulatory« LH- and P-diol patterns, whereas the oestrogen excretion remained at a very high level. The subsequent administration of 2.5 mg of NET suppressed all three parameters. Discontinuation of the administration of NET resulted in the re-establishment of the LH peak, which was accompanied by limited, but distinct increases in both oestrogen and P-diol excretion.

Due to a laboratory accident, in subject M. W. (Fig. 8) oestrogen assays could only be carried out during the last phase of the experiment (2.5 mg/day of NET).

The administration of 0.1 mg of NET resulted in a diminished and considerably broadened LH excretion, which was associated with an »ovulatory«
Urinary excretion of oestrone, 17β-oestradiol and oestriol in subject K.M. (cf. Fig. 1).

P-diol pattern. When the dose of NET was increased twenty-fivefold, the LH excretion pattern was undistinguishable from that seen during the administration of 0.1 mg of NET; however, this time the P-diol pattern became «anovulatory». The low P-diol values were accompanied by high urinary oestrogen values.

**DISCUSSION**

It is established that the daily administration of 0.5 mg of CAP offers a reliable means of fertility control. In a recent summary of the Mexican studies, Martinez-Manautou (1969) reported a pregnancy rate (Pearl’s index) of 1.0, a figure based on 28 158 cycles studied in 1769 women. As a part of this study, Martinez-Manautou and co-workers studied 200 endometrial biopsies and found that 75% of them were normal secretory, or irregular secretory. Furthermore, culdoscopic examinations in 50 women of this group revealed the presence of a corpus luteum in 37 of them. In view of these data, it was concluded that
inhibition of ovulation is not the main mechanism of contraceptive action of the low level CAP administration (Martinez-Manautou 1969).

How then is the effect of CAP reflected by urinary hormone assays? No study seems to be on record in which LH, oestrogen and P-diol were estimated in the same subjects. However, the effect of CAP on LH excretion was studied by Stevens & Vorys (1967), who found that the administration of 2.0 mg/day abolished the midcycle peak. Elstein (1969) studied the effect of 0.5 mg/day of CAP in 9 cycles in four women and found an »ovulatory« LH peak in 7 of 9 cycles. However, the height of these LH peaks was considerably lower than that found in the untreated cycles in the same women. Since the diminished LH peaks were associated with biphasic basal body temperature charts, it was concluded that the LH peaks were apparently high enough to be associated with ovulation. In the studies reported by Jaffe & Midgley (1969) the administration of 0.5 mg/day of CAP either abolished the LH peak, or »broad, low, abortive peaks« of LH were occasionally seen at midcycle«. Ovulation appeared to occur in one of five subjects.

The effect of 0.5 mg CAP on urinary P-diol levels was studied by Martinez-
Fig. 6.
Urinary excretion of oestrone, 17β-oestradiol and oestriol in subject G. O. (cf. Fig. 3).

Manautou et al. (1967). They found »ovulatory« values in approximately 1/3rd of the subjects studied. A complicating factor in the assessment of these values is that under their conditions, P-diol values higher than 1.3 mg/24 h are considered to be consistent with ovulation. Furthermore, Geller (1969) studied the oestrogen and P-diol excretion in 14 women before and during the administration of 0.5 mg of CAP; in the untreated cycles the mean luteal P-diol value was 6.2 mg/24 h, whereas during CAP administration, 0.7 mg/24 h was found in the supposed follicular phase, and 1.1 mg/24 h in the supposed luteal phase. The oestrogen excretion was much higher in the »luteal« than in the »follicular« phase, but also the former was lower than that found in untreated cycles. In view of these data, Geller (1969) suggested that the continuous administration of 0.5 mg/day of CAP brings about an incomplete suppression of the midcycle LH release; thus ovulation would not be invariably inhibited, but the corpus luteum formed would be an insufficient one. A similar theory was advanced earlier by Østergaard & Starup (1968), who observed that during treatment with megestrol acetate the presence of apparently normal corpora lutea was often associated with low P-diol values.
Urinary excretion of luteinizing hormone, «total oestrogens» and pregnanediol in 48 h specimens collected from subject G. T. during a control cycle, a cycle with 0.1 mg/day norethisterone and a cycle with 2.5 mg/day of norethisterone.

On the other hand, Hammerstein (1969), who has carefully followed the oestrogen and P-diol excretion in three women during 4 courses of 0.5 mg CAP per day, reported that the oestrogen and P-diol excretion values during CAP administration could not be distinguished from those seen in the normal ovulatory cycles. Also Gueguen (1965) reported that the urinary oestrogen excretion was not diminished in 9 women when as much as 10.0 mg/day of CAP was administered to them from the 5th to the 26th day of the cycle.

It is indeed difficult to assess properly the effect of contraceptive steroids on the urinary LH- and steroid excretion, unless such assays are carried out continuously throughout several cycles. Therefore, in the present study, the immediate and late effects of the administration of 0.5 mg/day CAP were compared with excretion values in a preceding and a subsequent control cycle. Furthermore, special efforts have been made to ensure that the analytical procedures employed fulfil the recognized criteria of reliability when applied to the urine specimens investigated in this study. By the use of specially controlled techniques, major individual differences were found in the response of
Urinary excretion of luteinizing hormone, »total oestrogens« and pregnanediol in subject M. W. For further particulars consult legend to Fig. 7.

the three subjects to low levels of CAP. Furthermore, significant differences were found in the response of one and the same subject to CAP during the first and fourth courses of administration. The continuous low level CAP seemed to interfere only slightly with oestrogen excretion, but abolished the midcycle LH peak in 5 of 6 cycles studied. Moreover, on two occasions bi-phasic P-diol excretion curves were found in the absence of any midcycle LH peak. At least one of them was associated with an elevated basal body temperature.

Marked individual variations were found also in the response of two subjects to the administration of 0.1 mg/day of NET, which abolished the »ovulatory« P-diol pattern in one subject, but had no effect whatsoever on the P-diol excretion in the other. Furthermore, when in the latter subject the dose of NET was increased to 2.5 mg/day, the »ovulatory« P-diol pattern was completely abolished, although the LH excretion curve was undistinguishable from that seen in the previous cycle (with 0.1 mg/day of NET), in which a normal menstruation and an »ovulatory« P-diol excretion were observed.

Hence, for the time being, the assessment of the complete, or incomplete,
inhibition of ovulation on the basis of urinary LH, oestrogen and P-diol excretion values appears to be associated with major uncertainties. Especially the significance of »diminished« LH- and P-diol excretion values requires clarification. It is felt that, for a better understanding of the mechanism of contraceptive action of low level progestogens, more information will be needed on the minimal amounts of urinary LH- and P-diol which are compatible with a normal ovulation and corpus luteum function.

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