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RADIOIMMUNOASSAY OF PLASMA NORETHISTERONE AFTER ETHYNODIOL DIACETATE ADMINISTRATION

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SUMMARY

A radioimmunoassay for norethisterone was developed and used to measure plasma levels of the compound in women following oral administration of tablets containing 1 mg ethynodiol diacetate and 0.05 mg ethinyloestradiol.

The results showed that the norethisterone assay was adequately specific, sensitive and accurate for application to studies of the absorption and clinical pharmacology of orally administered ethynodiol diacetate. Peak concentrations of norethisterone (between 7.22 and 15.35 ng/ml) were reached between 1½ and 3 h after dosage and norethisterone could be measured in plasma up to 48 h after a single oral dose of ethynodiol diacetate. Comparison of mean area under the concentration–time curve (AUC) data indicated that measurement of plasma norethisterone could be used to monitor the absorption of orally administered ethynodiol diacetate.

INTRODUCTION

Ethynodiol diacetate (SC-11800, 19-nor-17α-pregn-4-en-20-yne-3β,17-diol diacetate, Fig. 1, A) in combination with small amounts of oestrogens has been widely used as an oral contraceptive. Orally administered ethynodiol diacetate is rapidly metabolized in humans and only its metabolites are found in the plasma [1]. There was no evidence for the presence of the administered compound which appeared to be rapidly deacetylated and oxidized to norethisterone (Fig. 1, L), which was present in the blood predominantly in an unconjugated form.

In the absence of the intact drug in plasma we considered that measurement of norethisterone plasma levels could be of value in studying the absorption and clinical pharmacology of orally administered ethynodiol diacetate. The present report describes the development and validation of a radioimmunoassay for norethisterone, and the application of the assay to the measurement of norethisterone in plasma following the administration of ethynodiol diacetate to normal women.

MATERIALS AND METHODS

Ethyl acetate and 40–60 petrol were Analar grade (Hopkin and Williams, Chadwell Heath, Essex). The phosphate buffered saline (PBS) was 0.05 M sodium phosphate (pH 7.0) in 0.15 M sodium chloride. All stock solutions of non-radioactive steroids were stored at a concentration of 1 μg/ml in ethanol, and diluted as required with ethanol. [^{3}H]-Norethisterone (S.A. 20 Ci/mmol) was purchased from NEN GmbH, Frankfurt am Main, Germany. The Dextran-charcoal reagent was prepared as a 1.0% suspension of Norit-A charcoal in a 0.1% solution of Dextran in PBS. Reference steroid samples were supplied by Searle Laboratories, Skokie, Illinois.

Liquid scintillation counting was carried out for 20 min or 10,000 counts in a Nuclear Chicago Mark I scintillation counter, using a 2-ethoxyethanol-toluene scintillant (1:1 v/v) containing 2,5-diphenyloxazole (PPO, 4 g/litre).

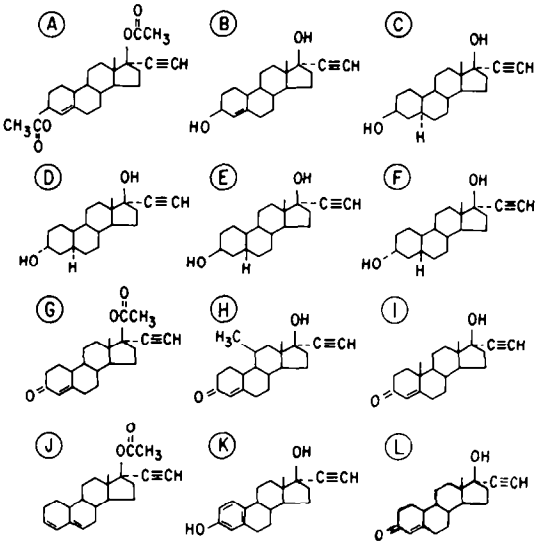

Fig. 1. Compounds tested for cross reactions with norethisterone.

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Antigens and antisera. Norethisterone-3-(O-carboxymethyl) oxime (N-3-CMO) and norethisterone-3-(O-carboxymethyl) oxime-bovine serum albumin conjugate (N-3-CMO-BSA) were prepared by Dr. K. King, Searle Laboratories, Skokie, Illinois, according to the method which Erlanger *et al.* [2] used for testosterone.

The N-3-CMO-BSA (0.5 or 1.0 mg ml) in Freund's complete adjuvant:saline (2:1 v/v) emulsion was used to raise antisera in six half lop rabbits. Test bleeds were taken from a marginal ear vein ten days after the fifth injection and all subsequent injections until a suitable antiserum titre was reached. After separation the antisera were stored at −20 C and were diluted to 1:25,000 in PBS solution containing 0.5% bovine serum albumin for use in the assay.

Assay procedure. Plasma samples were extracted with 5 vol. of ethyl acetate:40–60 petrol (1:1 v/v). In initial studies control plasma samples containing added [^{3}H]-norethisterone (3000 d.p.m.) were extracted and the recovery of added label was measured and found to be generally > 95%.

Norethisterone standards (range 30–1000 pg) in ethanol and extracts of each plasma sample were added to separate assay tubes and taken to dryness at 50 C in a stream of nitrogen. [^{3}H]-Norethisterone (9000 d.p.m.) in PBS solution (200 μl) was added to each assay tube. The tubes were Vortex mixed, incubated for 1 h at 37 C, cooled in ice and 100 μl of Dextran-charcoal suspension were added to each tube. The tubes were Vortex mixed, centrifuged at 4 C for 10 min at 2000 rev. min and a 400 μl aliquot of each supernatant was counted in 10 ml of scintillation fluid. All samples were assayed in triplicate. The binding of [^{3}H]-norethisterone for each norethisterone standard was expressed as a percentage of the binding of the radioligand in the presence of the zero standard (B/B_0%).

The plasma concentrations of norethisterone (ng/ml) were calculated from the standard curve, corrected for the vol. of plasma extracted and the vol. of extract used in the assay. Quality control plasma samples containing known amounts of added norethisterone were analysed in each batch of assays.

The cross-reactions of the antiserum were investigated using 2 and 20 ng of each compound, and the percentage cross-reaction was calculated as $x/y \times 100$, where x was the mass of norethisterone required to produce the same percentage inhibition of binding of [^{3}H]-norethisterone to the antiserum as the mass (y) of heterologous compound. Compounds which showed the highest degree of cross-reaction were also investigated at a wider range of concentrations.

Clinical procedure. Four normal women, who had given informed consent to participate in the study and had not been treated with oral contraceptives for at least three months were administered one Ovulen 50* tablet (1 mg ethynodiol diacetate and 50 μg ethinyl oestradiol) at weekly intervals in a random cross-over study, within four days after the end of menstruation. The tablets were selected on the basis of differences in their *in vitro* dissolution rates. Venous blood samples (10 ml) were collected into lithium heparin tubes at selected times after dosage, plasma was separated by centrifugation and plasma samples were stored frozen at −20 C until analysed. Aliquots of each plasma sample were analysed for norethisterone by the procedure described above.

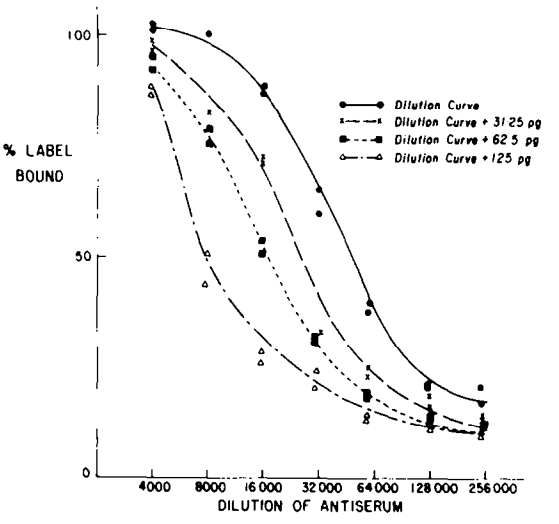

Fig. 2. Antinorethisterone-3-(O-carboxymethyl) Oxime-BSA dilution curves: effect of addition of norethisterone.

RESULTS AND DISCUSSION

The titration curve (Fig. 2) indicated that the antiserum could be used at a dilution of approximately 1:25,000, at which 65% of [^{3}H]-norethisterone radioligand was bound. Figure 3 shows the type of standard curve obtained and the effect of 20 μl of plasma on the standard curve. The effect of 50 μl of added plasma was much greater, showing considerable non-specific binding of the labelled norethisterone, and

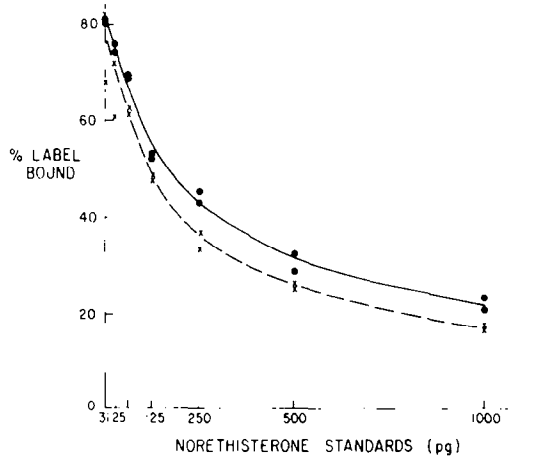

Fig. 3. The standard curve for norethisterone over the range 0–1000 pg (× — ×) and the effect of the addition of 20 μl plasma (● — ●).

Table 1. Compounds shown in Figure 1 and their percentage cross-reaction with the norethisterone antiserum

		% Cross reaction
A	19-nor-17α-pregn-4-en-20-yne-3β,17-diol diacetate (ethynodiol diacetate)	0
B	19-nor-17α-pregn-4-en-20-yne-3β,17-diol (SC-8470)	25
C	19-nor-5α,17α-pregn-20-yne-3β,17-diol (SC-22280)	25
D	19-nor-5α,17α-pregn-20-yne-3α,17-diol (SC-22421)	17
E	19-nor-5β,17α-pregn-20-yne-3β,17-diol (SC-22281)	6
F	19-nor-5β,17α-pregn-20-yne-3α,17-diol (SC-22279)	3
G	17-acetoxy-19-nor-17α-pregn-4-en-20-yn-3-one (norethisterone acetate)	0
H	17-hydroxy-11β-methyl-19-nor-17α-pregn-4-en-20-yn-3-one	25
I	17-hydroxy-17α-pregn-4-en-20-yn-3-one (ethisterone)	100
J	17-acetoxy-19-nor-17α-pregn-3,5-dien-20-yne(diene acetate)	0
K	17-ethinyl-estr-1,3,5(10)-triene-3,17-diol (ethinyl estradiol)	3

Table 2. The agreement between the concentrations of norethisterone added to plasma and the measured concentrations

Norethisterone in plasma ng/ml		25.00	12.50	6.25	3.125	1.55	0.78
Assay results (average of triplicates)		23.77	13.87	6.13	3.32	1.66	0.75
		28.00	11.75	6.13	3.38	1.50	—
		26.00	14.56	6.45	3.19	1.42	0.83
		25.00	12.42	6.13	2.71	1.38	—
		25.85	10.92	6.08	3.13	1.38	0.88
		25.00	12.50	6.33	3.90	2.00	0.83
	Mean	25.59	12.67	6.21	3.27	1.55	0.82
	S.D.	1.42	1.34	0.15	0.39	0.23	0.05
	S.D. %	5.50	10.60	2.40	11.90	14.80	6.10

interferences with displacement of the bound radioligand by cold steroid. These effects would have limited the vol. of plasma that could be used and thus the range of norethisterone concentrations that could be assayed. However, dried solvent extracts from up to 1 ml of norethisterone free plasma had no effect on the standard curve, indicating that endogenous steroids at the levels present in the assay would not cross-react with the antiserum. This extraction method allowed a wide range of norethisterone concentrations to be measured in plasma.

The cross-reactions of the antisera with a number of other metabolites of ethynodiol diacetate, and compounds related to norethisterone are listed in Table 1, and the structures of the compounds tested are shown in Figure 1.

Accuracy, precision and sensitivity

The accuracy and precision of the assay method are shown by the results of six separate determinations of norethisterone in plasma samples containing added norethisterone at concentrations ranging from 0.78 to 25 ng/ml (Table 2). The amount of norethisterone measured showed a high degree of correlation with the amount added to plasma over the range of concentrations, with a correlation coefficient of 0.995. Linear regression analysis of the data (Figure 4) gave an equation of $y = 1.02\ x - 0.04$. The coefficients of variation of the results (Table 3) from the six separate assays did not exceed 15%.

The amount of norethisterone that could be significantly distinguished from zero was calculated to be 20 pg at the 95% confidence level and 35 pg at the 99% confidence level.

Plasma concentrations of norethisterone

Peak plasma concentrations of norethisterone were reached between 1½ and 3 h after a single oral administration of ethynodiol diacetate (1 mg) to four normal women. At the peak, concentrations of norethisterone ranged from 7.22 to 12.19 ng/ml for tablet A, and from 7.72 to 15.35 ng/ml for tablet B. The plasma

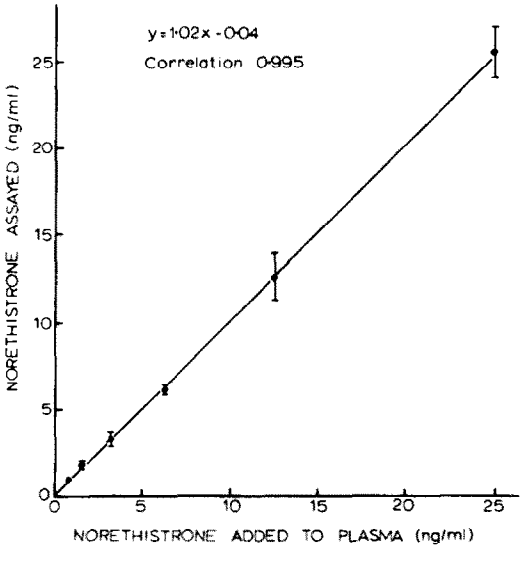

Fig. 4. Correlation plot of measured norethisterone levels and the amount of norethisterone added to the plasma.

concentration time curves shown in Fig. 5 revealed that norethisterone levels decreased rapidly between about 3 and 9 h after each administration, and declined more slowly during the next 15 h. Norethisterone could be detected in plasma up to 48 h after a single administration of ethynodiol diacetate.

Comparison of the mean area under the curve (AUC) obtained for tablet A and tablet B, in the four subjects, provided evidence of differences in the amount of ethynodiol diacetate absorbed from these two tablets.

The antiserum raised to the N-3-CMO-BSA conjugate showed the response to changes in the substituents in the D-ring of norethisterone expected for an antiserum to an antigen linked through the A-ring [3]. A comparison of the cross-reactions with ethynodiol diacetate (A), norethisterone acetate (G), the diene acetate (J) and ethisterone (I) suggest that the nature of the substituents at C-17 were the most important determinants of antiserum specificity. However, the percentage cross-reactivity of the antiserum with ethynodiol (B), the four tetrahydro metabolites (C, D, E and F) of ethynodiol diacetate and 11β-methylnorethisterone (H) indicated that other structural features were also recognized by the antibodies. An important feature of the three compounds (B, C and H) with 25% cross-reactivity was the presence of predominantly planar A-ring similar to that of norethisterone and its 3-(O-carboxymethyl) oxime.

A comparison of the cross-reactivities and structures of ethisterone (I), ethynodiol (B) and the tetrahydro compounds (C, D, E and F) suggests that the presence of a conjugated carbonyl system in the A-ring may also be involved in determining the degree of cross-reaction of the antiserum with heterologous steroids.

The apparent involvement of the conjugated carbonyl group at C-3 and the stereochemistry of the A-ring as determinants of cross-reactivity of heterologous steroids reported here has also been reported previously for antisera to androstenedione and testosterone [4].

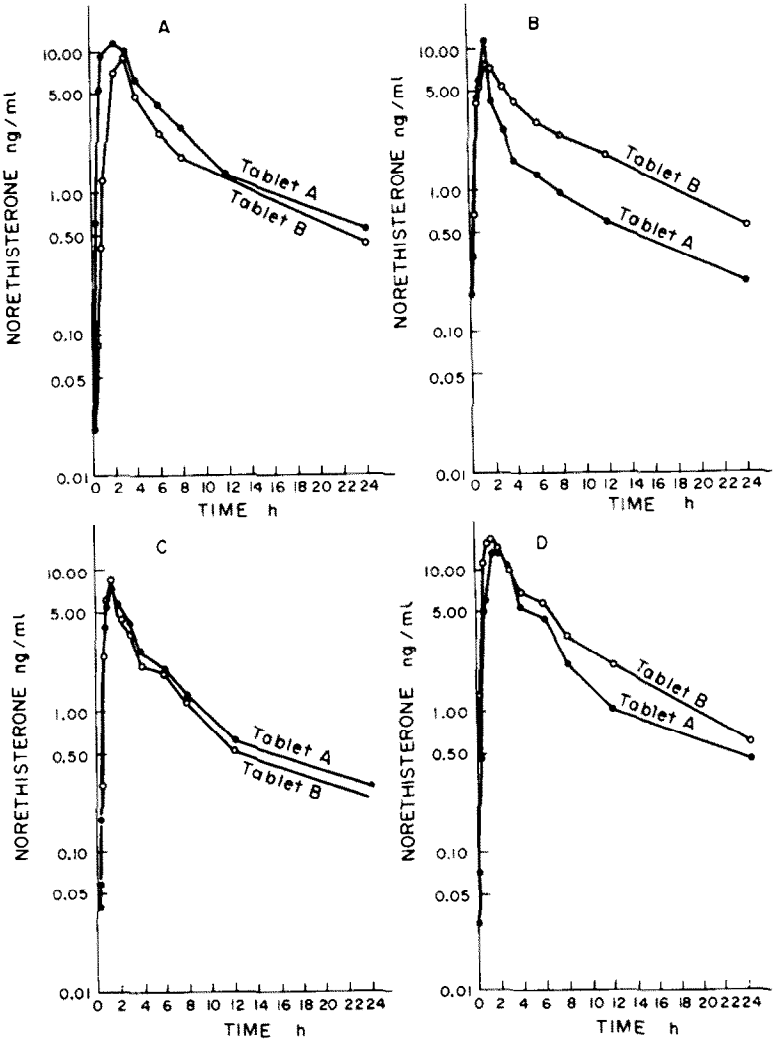

Fig. 5. Plasma norethisterone levels in four normal women following a single oral dose of one tablet from each of 2 batches of Ovulen 50.

The overall cross-reactivity of this anti-N-3-CMO-BSA antiserum was generally similar to that predicted for antisera to C-3 linked steroids by Jeffcoate and James[3]. However, the cross-reaction of the antiserum with 11β-methyl-norethisterone (H) was unexpected, as the axial 11-methyl group confers a unique feature on the structure of this compound. Published reports of other norethisterone antisera [5, 6 and 7] have also revealed cross-reactions of the antisera with norethisterone metabolites and related synthetic steroids. The norethisterone antisera recently described by Morris and Cameron[7] showed lower cross-reactivities with the tetrahydro metabolites (C, D, E and F) but very high cross reactivity with norethisterone acetate, in contrast to the results obtained with the present antiserum.

Although several metabolites of ethynodiol diacetate cross-reacted with the antiserum, these metabolites were either present at low levels in plasma or occurred almost entirely as conjugates [1]. Thus, these metabolites would be unlikely to interfere in the determination of norethisterone in the plasma of women receiving oral doses of ethynodiol diacetate.

The evaluation of the norethisterone radioimmunoassay described above showed that it was adequately specific, sensitive and accurate to measure the compound in plasma. The application of the assay to human clinical material revealed that norethisterone could be detected and measured in plasma up to 48 h after a single oral dose of ethynodiol diacetate (1 mg).

The rapid increase in plasma norethisterone levels observed in the present study, to peak concentrations at 1.5–3 h after dosage confirms the rapid absorption and metabolism of orally administered ethynodiol diacetate previously reported by Cook *et al.*[1]. The disappearance of norethisterone from plasma appears to follow a biphasic mode in the subjects studied, suggesting a two compartment model for the pharmacokinetics of this metabolite after administration of ethynodiol diacetate.

The observed differences in the mean plasma AUC values for the two tablets appear to reflect differences in the amounts of ethynodiol diacetate absorbed, and showed some agreement with predictions made on the basis of *in vitro* dissolution rates for the formulations. Thus the measurement of plasma concentrations of norethisterone may offer a suitable technique to investigate the absorption of ethynodiol diacetate following its oral administration. It is hoped to extend the initial studies reported above to provide further data on the relationship between plasma levels of norethisterone and the absorption of ethynodiol diacetate, and the clinical pharmacology of the progestogen.

CONCLUSIONS

A specific, sensitive and accurate radioimmunoassay has been developed and used to measure plasma levels of norethisterone in women following administration of single oral doses of oral contraceptive formulations containing ethynodiol diacetate. The initial results suggest that application of this technique could be extended to studies of the absorption and clinical pharmacology of ethynodiol diacetate.

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