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RADIOIMMUNOASSAY OF SERUM NORETHISTERONE OENANTHATE LEVELS IN WOMEN AFTER INTRAMUSCULAR ADMINISTRATION

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SUMMARY

A radioimmunoassay is described for measuring norethisterone oenanthate directly in samples of plasma after methanol precipitation. The method utilises an antiserum produced against norethisterone oenanthate-3-carboxymethyloxime coupled to bovine serum albumin. The method is highly specific for norethisterone oenanthate, the antiserum showing a cross-reaction of only 6% with the dihydro-metabolite of norethisterone oenanthate and less than 0.01% with norethisterone itself. Norethisterone oenanthate was injected intramuscularly into three women. Plasma levels of the steroid ester were from 1000–2500 pg/ml one week after injection decreasing to 100–200 pg/ml over the next six weeks. The pattern of norethisterone oenanthate concentrations in plasma was similar to that of norethisterone itself.

INTRODUCTION

Norethisterone oenanthate (17α-ethynyl-17β-hydroxy-oestr-4-en-3-one-17β-heptanoate, NET-OEN) is used as a long-acting contraceptive. Intramuscular administration of a dose of 200 mg will provide a contraceptive effect for up to three months [1]. The compound presumably acts by the slow release into the circulation of norethisterone produced by hydrolysis of the ester. Levels of norethisterone (NET) in blood after administration of NET-OEN have already been estimated [2, 3]. Whether hydrolysis of the ester occurs intramuscularly or after release of NET-OEN into the circulation or under both circumstances is not known. This paper describes a sensitive radioimmunoassay for NET-OEN and its application to the measurement of NET-OEN in blood.

METHODOLOGY AND SUBJECTS

Production of antibodies to norethisterone oenanthate. Norethisterone oenanthate-3-O-carboxymethyloxime (NET-OEN-3-CMO) was prepared from norethisterone oenanthate [4]. The oxime was conjugated with bovine serum albumin (BSA) by the mixed anhydride method; spectrophotometric analysis showed that 32–39 residues of NET-OEN-3-CMO were coupled per molecule of BSA.

Antibodies against NET-OEN-3-CMO-BSA were raised in New Zealand White female rabbits. Antigen (1.5 mg in 1 ml Freund's complete adjuvant: 0.15 M sodium chloride (7:3, v/v)) was injected in both flanks intramuscularly at weekly intervals for 1 month and then at fortnightly intervals for 5 months. Assay of the blood for antibodies to the injected antigen was performed monthly. A high titre of antibodies was found in samples taken six months after injection and the rabbit was bled at this time. After clotting, the blood was centrifuged to obtain serum which was stored at −20°C.

Radioimmunoassay of norethisterone oenanthate. Plasma (0.5–1.0 ml) was deproteinised in conical centrifuge tubes by addition of 2 ml methanol (A.R.). The contents were mixed for 15 s on a vortex mixer and then centrifuged at 4°C for 15 min at 500 *g*. Samples (0.5 ml) of the supernatant were pipetted in triplicate into 10 × 75 mm glass tubes for assay. A similar volume of supernatant obtained as described above from blood bank plasma was added to each assay tube to be used for the standard curve. Standard curve tubes were set up in triplicate. The contents of the assay tubes were evaporated in a vacuum oven. Buffer solution (0.5 ml 30% methanol–Tris hydrochloride buffer pH 8.5) was added to each sample assay tube. Buffer (0.5 ml) containing unlabelled NET-OEN (concentration range 5-2000 pg/ml) was pipetted into assay tubes for the standard curve. The contents of the tubes were mixed vigorously on a vortex mixer for 5 s each and kept at room temperature (20°C) for 30 min. Antiserum solution (0.1 ml of a 1:12000 dilution in Tris buffer) and 0.1 ml (10,000 d.p.m.) [^{3}H] NET-OEN (S.A. 40 mCi/mmol) in methanol–Tris buffer (3:7, v/v) were pipetted into the tubes and the contents were mixed thoroughly. After incubation for 18 h at 4°C the tubes were transferred to an ice bath and free and bound NET-OEN were separated by addition of 0.5 ml 2.5% charcoal in 0.25% dextran T-70 solution. Each sample was mixed for 2 s on a vortex mixer and after 10 min, centrifuged at 500 *g* for 15 min at 4°C. The clear supernatant containing bound NET-OEN was decanted directly into counting vials containing 10 ml Triton-based scintillation fluid and the radioactivity estimated. The amount of NET-OEN in unknown samples was calculated from

Table 1. Recovery of norethisterone oenanthate (NET-OEN) from plasma

NET-OEN added (pg/ml)	No. of determinations	% NET-OEN recovered (Mean ± S.D.)	Coeff. of variation %
0	10	—	—
100	5	94 ± 13.5	14.4
200	10	98 ± 4.9	5.0
500	10	97 ± 8.5	8.8
2000	10	104.5 ± 10.9	10.4
4000	10	104.9 ± 14.1	13.5
8000	10	104.2 ± 6.6	6.4

the standard curve. Duplicate samples of a pool of blood bank human plasma not containing NET-OEN as well as samples from this pool containing known amounts of NET-OEN (ranging from 100 pg/ml to 2000 pg/ml) were analyzed in each assay.

Subjects. Three healthy women (G, B and H) were given an intramuscular injection of 200 mg NET-OEN in 1 ml vehicle. Details of these subjects and the procedure for blood sampling were described previously [2]. Some of the serum samples from these subjects were stored at −20°C and utilised for the assay of NET-OEN in the present study.

RESULTS

Reliability criteria for the assay

Specificity. The antiserum appeared to be very specific for NET-OEN. Of the various steroids tested for their interaction with the antiserum only dihydronorethisterone oenanthate, a possible metabolite of NET-OEN [5], showed a significant cross-reaction, percentage relative activity, 6% [7]. The degree of cross-reaction of both norethisterone (<0.01%) and norethisterone acetate (<0.1%) was not significant. A number of other steroids (progesterone, 17α-hydroxyprogesterone, 16α-hydroxyprogesterone, oestrone, oestradiol, cortisol, testosterone, ethynyloestradiol, norgestrel and medroxyprogesterone acetate) were also tested and the relative activities were all less than 0.01%.

When plasma samples were chromatographed on Sephadex LH20 using the solvent system cyclohexane, toluene, methanol (80:15:5, by vol.) no NET-OEN was detected in any of the eluates or the methanol wash of the column except in the fraction where standard NET-OEN was eluted. There was good agreement between the value for NET-OEN obtained from the column eluate and that obtained by direct assay.

Recoveries and precision. Values obtained for the recovery of NET-OEN added to plasma and assayed ranged from 85% to 110%. Mean values for recoveries at various levels of steroid added to plasma are shown in Table 1. No NET-OEN was detected on the proteins precipitated by addition of methanol to plasma.

The within assay variation also seemed satisfactory, analysis in quadruplicate of three samples of pooled plasma containing concentrations of NET-OEN of 100 pg/ml, 500 pg/ml and 8000 pg/ml gave the following percentage recoveries (mean ± S.D.) 92.5 ± 8.7, 99.3 ± 4.1 and 98.8 ± 3.2. Thus the within assay coefficient of variation was less than 9%. As shown in Table 1, between assay variation was less than 15%. This was confirmed by duplicate analyses carried out on different days, the coefficient of variation for duplicate analyses of 16 samples, varying in concentration from 110–2200 pg NET-OEN per ml, was 11%.

Sensitivity. Standard curves plotted as logit B/BO against log dose were linear between 15 and 1000 pg NET-OEN. Usually about 40% of labelled NET-OEN was bound when no unlabelled NET-OEN was added to the assay tubes. The relative binding was reduced to 90% in the presence of 15 pg unlabelled NET-OEN per assay tube. The minimum detection limit of the assay was regarded therefore as 15 pg/tube.

Application of the method

Plasma concentrations of NET-OEN and norethisterone at various times after intramuscular injection of 200 mg NET-OEN to three women are shown in Fig. 1. One week after injection plasma levels of NET-OEN ranged from 1000–2500 pg/ml and then fell rapidly reaching low levels (less than 200 pg/ml) by eight weeks after injection. At all times plasma levels of NET were 2–4 times higher than those of NET-OEN.

DISCUSSION

No previous method has been described for the estimation of norethisterone oenanthate. The simple method described here in which the level of norethisterone oenanthate is assayed directly in plasma after precipitation of the proteins with methanol obviates the need of addition of internal standards and correction for extraction losses associated with the usual solvent extraction procedures. The results of the reliability criteria indicate that the method has adequate specificity, accuracy, precision and sensitivity. The specificity of the antiserum, in that almost no cross-reaction occurred with norethisterone, is surprising; it might have been expected that the antigen would have undergone some hydrolysis *in vivo* with loss of the oenanthate group and hence the generation of

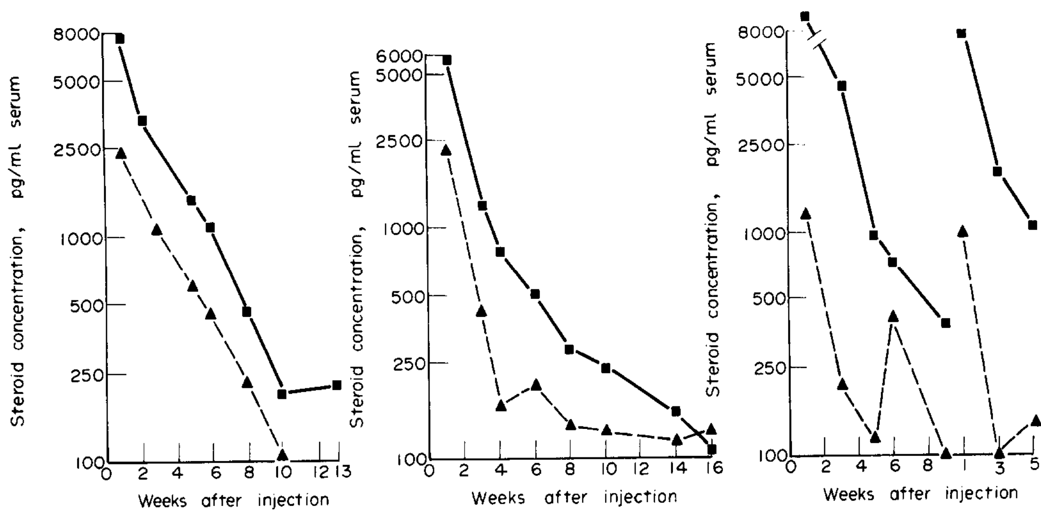

Fig. 1. Serum concentrations of norethisterone oenanthate (NET-OEN, broken line) and norethisterone (NET, continuous line) in three women given intramuscular injections of 200 mg NET-OEN. Values for NET are from Howard, Warren and Fotherby [2].

antibodies interacting with norethisterone. Since this did not seem to occur presumably the binding of norethisterone oenanthate to bovine serum albumin hindered hydrolysis of the ester.

Since the efficiency of the charcoal separation method is apparently dependent upon the concentrations of protein and lipid in the incubation medium [7] it was necessary to maintain similar protein and lipid concentrations in the standard curve assay tubes as in the sample tubes by addition to the former of supernatant obtained by methanol precipitation of blood bank plasma.

In the development and investigation of new contraception drugs, a knowledge of the blood levels of the compound after its administration is often helpful in the interpretation of the biological effects of the drug. Norethisterone oenanthate has been little investigated compared to the more widely used injectable preparation DepoProvera, a microcrystalline suspension of medroxy-progesterone acetate [1]. The suppressive effect of norethisterone oenanthate on the pituitary-ovarian axis appears to be less intense than that of DepoProvera. Ovulation returns in most women within 8–13 weeks after injection of norethisterone oenanthate by which time not only are plasma levels of norethisterone low [2, 3], but, as shown in this paper, so are levels of the administered steroid whereas after injection of DepoProvera, ovulation may not occur for some considerable time [1]; peak levels of medroxyprogesterone acetate are much higher than those of norethisterone and detectable levels of medroxyprogesterone acetate are found in plasma for up to 300 days after injection [7].

Although at all times after injection the plasma levels of norethisterone were higher than those of NET-OEN, it was surprising to find such high concentrations of norethisterone oenanthate (one half to one quarter of those of norethisterone) in plasma; even when low concentrations of norethisterone oenanthate were present in plasma, hydrolysis to norethisterone was not complete. *In vitro* norethisterone oenanthate is rapidly hydrolysed by a number of tissues [5]. It had been anticipated that with the slow release of norethisterone oenanthate from the site of injection the activity of the blood esterases would have been sufficient to have rapidly hydrolysed the ester to free norethisterone. That this did not occur might suggest that norethisterone oenanthate released into the circulation becomes bound to plasma proteins and that this binding prevents complete hydrolysis. This suggestion would be in agreement with the finding that the antigen used for immunisation appeared not to undergo hydrolysis as evidenced by the lack of interaction of norethisterone with the antiserum. The rate of decline of the concentration of norethisterone oenanthate in the circulation was similar to that of norethisterone.

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