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RADIOIMMUNOASSAY SYSTEM FOR NORETHISTERONE USING ^{3}H- AND ^{125}I-LABELLED RADIOLIGANDS

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SUMMARY

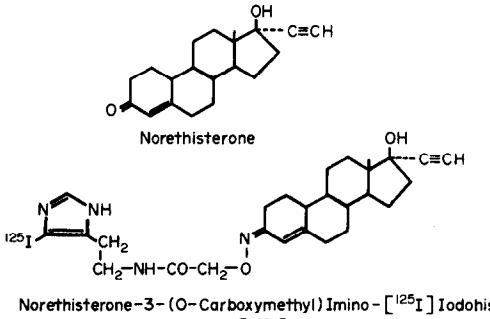
The use of both ^{3}H- and ^{125}I-labelled radioligands was compared in the radioimmunoassay of norethisterone. At various time intervals, plasma hormone concentrations were measured in male volunteers after the oral administration of 10 mg norethisterone acetate (SH 420). Values obtained using both radioligands were found to be in good agreement and the effect of alumina thin-layer chromatography upon these titres was shown to be negligible.

These results suggest that the use of a ^{125}I-radioligand may be generally applicable for the radioimmunoassay of synthetic steroids.

INTRODUCTION

The radioimmunoassay of naturally occurring steroids in blood plasma is facilitated by the availability of commercially prepared (normally ^{3}H-labelled) radioligands.

Assays for synthetic steroids are in a less fortunate position and since laboratory synthesis of high specific activity ^{3}H-labelled synthetic steroids is extremely difficult, alternative radioligands such as those containing radioiodine have been investigated. The formation of steroid-tyrosyl-methyl esters and their subsequent radioiodination has already been described [1, 2] and recently Nars and Hunter[3] reported a procedure involving the preliminary radioiodination of histamine followed by its coupling to oestradiol (17β)-6-(O-carboxymethyl)oxime. The latter method was used in a preliminary investigation of a number of assay systems involving five different steroids, and their antisera [4].



Norethisterone-3-(O-Carboxymethyl)Imino-[^{125}I]Iodohistamine
(Norethisterone-3-OCMO-[^{125}I]Iodohistamine)

Fig. 1.

As part of a larger study on the effects of administration of synthetic steroids upon naturally occurring hormone concentrations in plasma, it became necessary to establish a radioimmunoassay for norethisterone (19-nor-17α-ethynyl-17β-hydroxyandrost-4-en-3-one) (Fig. 1) but the establishment of such an assay using both ^{3}H-and ^{125}I-labelled radioligands (Fig. 1) was also intended to serve as a "model" for future assays of synthetic steroids for which no ^{3}H-radioligand existed. Norethisterone is a synthetic progestagen (administered as the 17β-acetate) which can be used to treat breast cancer [5] is also effective in the treatment of endometriosis [6, 7] and as a contraceptive agent [8–11].

MATERIALS AND METHODS

All common reagents used were as described by Hillier, Brownsey and Cameron[12].

The phosphate buffered saline (PBS) solution was 0·05 M sodium phosphate (pH 7·5) in 0·15 M sodium chloride, containing 0·01% merthiolate and 0·5% human serum albumin. The Dextran-charcoal reagent was prepared as a 0·5% suspension of Norit-A charcoal in a 0·05% solution of Dextran T-70 in PBS. All stock solutions of non-radioactive steroids were stored at a concentration of 5 μg/ml in methanol and diluted as required in methanol. [^{3}H]-Norethisterone (specific activity 20 Ci/mmol) was purchased from NEN GmbH, Frankfurt am Main, Germany and was stored as a stock solution at a concentration of 0·5 μCi/ml in ethanol at 4°C. A more dilute solution (0·1 μCi/ml in ethanol) was also prepared for use in the monitoring of recoveries.

Aquasol scintillator was used as supplied by NEN GmbH at a ratio of 500 μl aqueous solution/5 ml scintillator.

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Antigens and antisera

Norethisterone, the corresponding 11α-hemisuccinate and all derivative standards were very kindly supplied by Schering AG, Berlin. Norethisterone-11α-BSA was prepared as described by Erlanger, Borek, Beiser and Lieberman[13].

Antisera were raised in New Zealand White rabbits by immunisation with the appropriate antigens (1 mg/2 ml Freund's complete adjuvant:saline, 7:3, v/v emulsion) injected into multiple s.c. sites at monthly intervals. Test-bleeds were taken from a marginal ear vein ten days after the second and all subsequent injections until a suitable antiserum titre was reached. After separation, sera were stored at −15°C and a suitable dilution was prepared in PBS solution for use in each assay as required.

^{125}I-Labelled radioligands

Norethisterone-3-(O-carboxymethyl)oximino-[^{125}I] iodohistamine(norethisterone-3-OCMO-[^{125}I] iodo histamine), and norethisterone-11α-hemisuccinyl-[^{125}I]iodohistamine(norethisterone-11α-HS-[^{125}I]iodohistamine) were prepared according to the method of Nars and Hunter[3]. Histamine was first iodinated, then coupled to the steroid hapten by the mixed anhydride procedure [13] and following extraction of the ^{125}I-histamine conjugates with toluene, aliquots (100 μl) of the extracts were chromatographed as required on methanol-washed alumina-precoated plastic sheets. Norethisterone-3-OCMO-[^{125}I]iodohistamine was run in the solvent system benzene:ethanol:acetic acid (75:24:1, by vol.) and norethisterone-11α-HS-[^{125}I]iodohistamine in ethyl acetate:acetic acid (70:2·5, v/v) and all chromatograms were scanned on a Berthold radiochromatogram scanner. Spots corresponding to the desired products were eluted [12] with methanol and stock solutions prepared by dilution in assay buffer to a concentration of approximately 8 × 10^5 c.p.m./ml.

Plasma samples

Norethisterone acetate (SH420) was taken orally in a single dose (10 mg) by healthy male volunteers and blood samples (10 ml) were taken by venepuncture at convenient time intervals thereafter. Samples were placed in tubes containing sequestrene and following centrifugation at room temperature for 5 min the plasma separated and was stored at −15°C until analysis.

Extraction of steroids from plasma

[^{3}H]-Norethisterone in PBS solution (100 μl, containing approximately 4500 d.p.m.) was pipetted into each tube (1·5 × 13 cm., fitted with stopper) containing a suitable volume of plasma for extraction (where less than 500 μl was used for extraction the volume was made up to this amount with distilled water) and into two extra tubes containing 500 μl distilled water (water blank). Diethyl ether (2 ml) was added to each tube and the contents buzzed for approximately 20 min. The stoppers were removed and tubes, covered with a sheet of aluminium foil, placed in the deep freeze for 30 min. The ether extracts could then be decanted from the frozen aqueous layer into the appropriate standard rimless glass bacteriological tubes (75 × 12 mm assay tubes) and evaporated to dryness in a stream of nitrogen at 37°C. To each dried residue a volume of PBS solution was added (1 ml). Tubes were then buzzed on a vortex mixer and allowed to incubate at 37°C for a period of 15 min. Suitable aliquots were taken from each tube for recovery (500 μl) which was normally > 90% and assay determinations (100 μl). In some instances, plasma extracts were purified on thin layer chromatography prior to being assayed.

Chromatography of plasma extracts

Dried ether extracts were dissolved in methanol (20 μl) and applied to a methanol-washed alumina-precoated plastic sheet (20 × 20 cm.) which was developed in an appropriate solvent system. Testosterone was used as a reference standard detectable by localisation in short-wave (254 nm) ultra-violet light. The requisite area was cut out and the radioactive material eluted with methanol [12]. The eluate was taken to dryness under a stream of nitrogen and redissolved in PBS solution (1000 μl) for assay and recovery determinations.

Assay procedure

Standards were prepared, in duplicate, from working standard solutions in methanol (5 ng/ml), the appropriate volume being pipetted into each assay tube and taken to dryness in a stream of nitrogen. An aliquot (100 μl PBS solution) was added to each tube and to six extra tubes, two to which no standard would be added, "zero" tubes; two to which buffer would be added instead of antibody and charcoal suspension "total" tubes; two to which buffer would be added instead of antibody, "blank" tubes. All tubes were buzzed and incubated at 37° for 15 min. A suitable dilution of antibody (100 μl; PBS solution) was then added to all tubes with the exception of the "total" and "blank" tubes. Finally, the radioactive solution (50 μl ^{3}H- or ^{125}I-labelled radioligand) in PBS solution was added to all tubes which were then buzzed once more and left at 4°C overnight.

The remainder of the assay was then performed at 4°C. A volume (100 μl) of PBS solution was added to all tubes, followed by 500 μl of the Dextran-charcoal suspension (the suspension was stirred on ice while aliquots were transferred to the assay tubes) to all except the "total" tubes. All tubes were buzzed and then centrifuged (15 min incubation at 4°C before centrifugation for ^{125}I-radioligand) at 4°C for 15 min. An aliquot (500 μl) of the supernatant was taken from each tube for counting as described earlier. The "blank" was used in the assay to monitor the efficiency of the charcoal in separating the bound and free steroid fractions and was invariably negligible.

The "water" blank however was occasionally troublesome and as it proved to be an unsatisfactory procedure to subtract this "blank" the assay in such instances was always repeated.

Calculation

Standard curves were plotted as percentage of "zero" standard against mass of steroid.

If M is the mass (pg) of steroid read from the standard curve $R_{(s)}$ the c.p.m. in 100 μl of the [^{3}H]-norethisterone recovery solution, $R_{(R)}$ the c.p.m. in the aliquot taken for recovery determination, $V_{(R)}$ the volume taken to measure recovery, $V_{(a)}$ the volume taken for the assay and $V_{(p)}$ the volume (ml) of plasma extracted, then the concentration of steroid is given by the expression:-

$$\frac{M \times R_{(s)} \times V_{(R)}}{R_{(R)} \times V_{(a)}} \times \frac{1}{V_{(p)}} \times \frac{1}{1000} \text{ ng/ml}$$

RESULTS AND DISCUSSION

Anti-norethisterone-11α-BSA/^{3}H-norethisterone assay system

The titration curve (Fig. 2) indicated that the antiserum might be used at a dilution of approximately 1:4000 at which point a 50% displacement of the ^{3}H-norethisterone radioligand (150 pg) per tube was observed. Simultaneous construction of a second titration curve in which 500 pg non-radioactive norethisterone had been added to each dilution tested showed that substantial displacement of label occurred. This indicated that the assay system should give a reasonably sensitive standard curve over a 0–500 pg working range. Figure 3 shows the type of standard curve obtained and Fig. 4 the corresponding Scatchard plot [14] of the data. Calculation of the mean binding affinity of the antiserum gave a value of 2·62 × 10^9 l/mol.

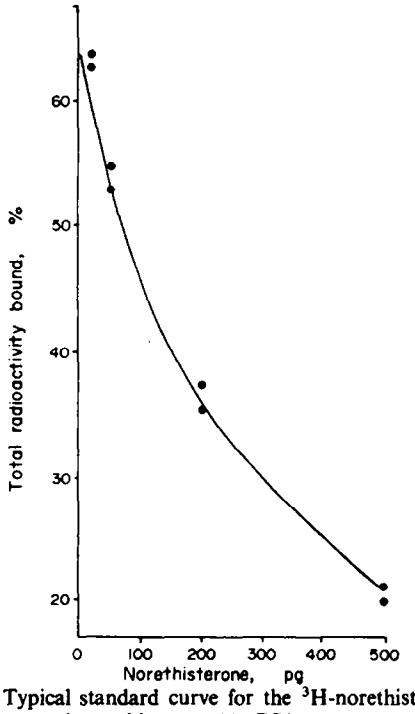

Fig. 3. Typical standard curve for the ^{3}H-norethisterone/anti-norethisterone-11α-BSA system.

Cross-reaction [15] data are given in Table 1 together with that for the anti-norethisterone-11α-BSA/norethisterone-3-OCMO-[^{125}I]iodohistamine assay system (see below). It was noted that the cross-reaction of norethisterone-17β-acetate (one form in which the drug is administered) was extremely high

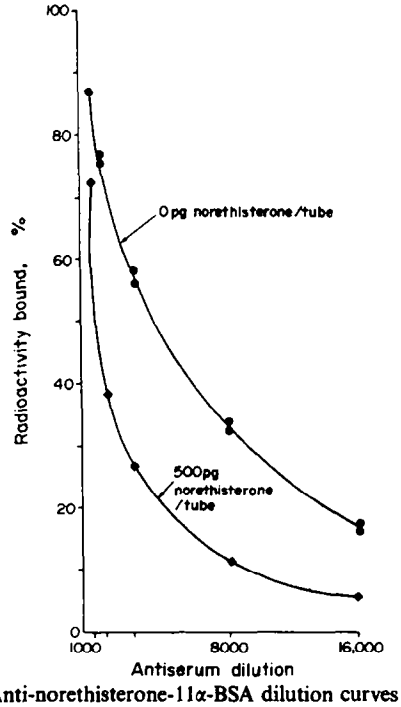

Fig. 2. Anti-norethisterone-11α-BSA dilution curves: effect of addition of norethisterone (Radioligand: 150 pg [^{3}H]-norethisterone).

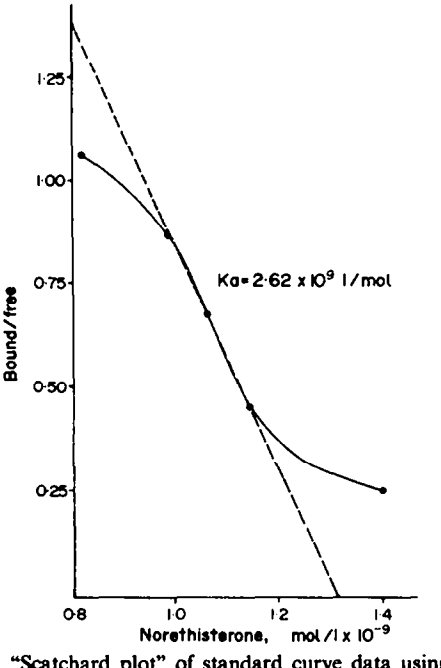

Fig. 4. "Scatchard plot" of standard curve data using an anti-norethisterone-11α-BSA serum with ^{3}H-norethisterone ligand displaced by increasing masses of norethisterone.

Table 1. Cross-reactions of various steroids with the anti-NE-11α-BSA serum using a NE-3-^{125}I or a ^{3}H-NE radioligand

Steroid	% Cross-reaction NE-3-^{125}I	^{3}H-NE
Norethisterone (NE)	100	100
NE acetate	104	126
5α-dihydro-NE	63	18
5β-dihydro-NE	23	2·6
5α-dihydro-NE-3α-ol	3·7	<0·8
5α-dihydro-NE-3β-ol	4·4	<0·8
5β-dihydro-NE-3α-ol	1·6	<0·8
5β-dihydro-NE-3β-ol	3·9	<0·8
NE oenanthate	0·3	1·7
D-Norgestrel	16·0	55·0
17α-ethynyl oestradiol	0·6	<0·5
19-nor-testosterone	0·3	<0·5
Testosterone	<0·2	<0·5

(126%) and that *D*-norgestrel cross-reacted to the extent of 55%. The ring-A reduced metabolites of norethisterone, 5α- and 5β-dihydro-norethisterone exhibited cross-reactions of 18% and 2·6% respectively. Norethisterone is also administered as the oenanthate ester and the low cross reaction of this material (1·7%) is in direct contrast to that observed for the acetate. However, the cross-reaction curve for the oenanthate ester was not parallel to the other curves investigated suggesting that a different population of antibodies was involved in the binding process and that the degree of cross-reaction was, to some extent, concentration dependent.

Preparation of ^{125}I-labelled radioligands

The purified aliquots of each radioligand were frequently used up within a relatively short space of time but the shelf life of this material exceeded 4 months. The degree of binding of radioligand to antiserum even at low serum dilution was always greater than 80% and normally 100%. It was calculated that the specific activity of the ligands was of the order of 3 Ci/mmol and that the mass of radioligand used per assay tube was approximately 1 pg (~ 20,000 c.p.m.).

Anti-norethisterone-11α-BSA/norethisterone-11α-HS-[^{125}I]iodohistamine assay system

Dilution curves using this system exhibited very high titres (≫ 1:20,000), and addition of 500 pg of non-radioactive norethisterone completely failed to produce significant displacement of label. This meant that no standard curve could be produced since norethisterone could not compete adequately for the binding sites on the antibody. Such phenomena have been previously described for other *homologous* assay systems [16]. The explanation may lie in the fact that the structure of the radioligand and the original hapten are so similar that the binding affinity of the ligand for the antibody grossly exceeds that of the free steroid.

Anti-norethisterone-11α-BSA/norethisterone-3-OCMO-[^{125}I]iodohistamine assay system

Although the structure of this radioligand (Fig. 1) is, in immunoassay terms, quite different from that of the free steroid it still bound to the anti-norethisterone-11α-BSA serum. Figure 5 shows the titration curves obtained with and without the addition of 500 pg norethisterone per tube. The addition of the non-radioactive steroid caused substantial displacement of label predicting the construction of useful standard curves over the 0–500 pg range. Figure 6 shows such a curve over the 0–200 pg range at a dilution of 1:8000.

As mentioned earlier, the cross-reactions of a number of steroids in this assay system are compared with those of the anti-norethisterone-11α-BSA/^{3}H-norethisterone system in Table 1. With the notable exceptions of norethisterone acetate and *D*-norgestrel, the use of the norethisterone-3-OCMO-[^{125}I]-iodohistamine radioligand appeared to result in signifi-

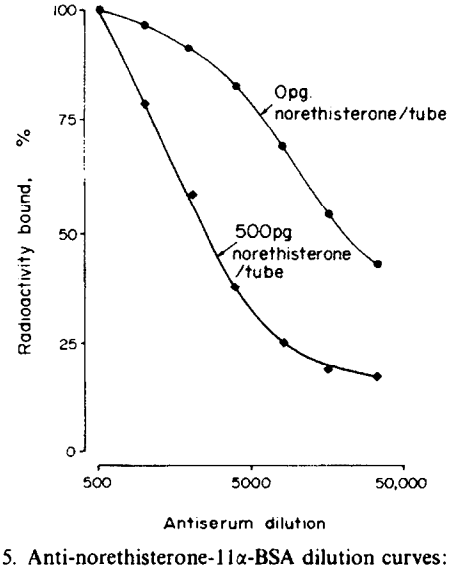

Fig. 5. Anti-norethisterone-11α-BSA dilution curves: effect of addition of norethisterone (Radioligand: norethisterone-3-OCMO-[^{125}I]-iodohistamine.

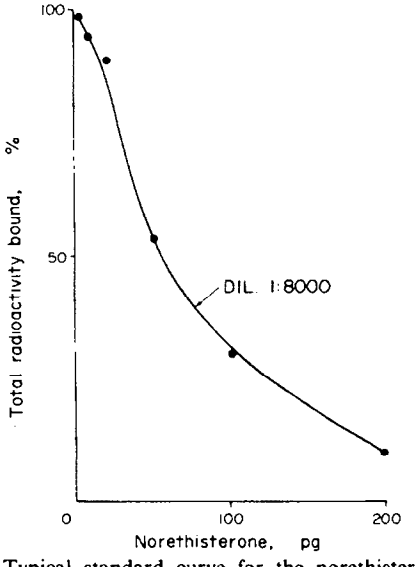

Fig. 6. Typical standard curve for the norethisterone-3-OCMO-[^{125}I]-iodohistamine/anti-norethisterone-11α-BSA system.

Table 2. R_f values of steroids on alumina-pre-coated plastic sheets in the solvent system, benzene:cyclohexane:ethanol (70:27:3 by vol.)

Steroid	Rf
NE	0·57
5α-dihydro-NE	0·70
NEA	0·86
5α-dihydro-NE-diols	0·40
5β-dihydro-NE-diols	0·40
D-NG	0·70
testosterone	0·51
5α-dihydrotestosterone	0·61

cantly increased cross-reaction for the steroids tested. In particular, the 5α- and 5β-dihydro-norethisterone derivatives showed increases from 18% to 63% and from 2·6% to 23% respectively.

Effect of chromatography on plasma titres for norethisterone

As shown above, some steroids structurally related to norethisterone cross-reacted quite markedly in the assay systems used and Table 2 gives thin-layer chromatogram R_f values in the solvent system chosen to separate them, of a number of the compounds considered most likely to interfere. With the possible exception of 5α-dihydrotestosterone present in female plasma in relatively low concentrations [17, 18] none of the steroids ran very close to norethisterone.

A series of plasma samples from one of the volunteers was then assayed using the anti-norethisterone-11α-BSA/^{3}H-norethisterone system with and without the inclusion of the chromatography step and the results are shown in Fig. 7. It can be seen that the two sets of values closely agree and resemble data obtained by Gerhards, Hecker, Hitze, Nieuweboer and Bellmann[19] who performed a similar experiment using isotope dilution techniques to determine the norethisterone. It can be concluded, therefore, that

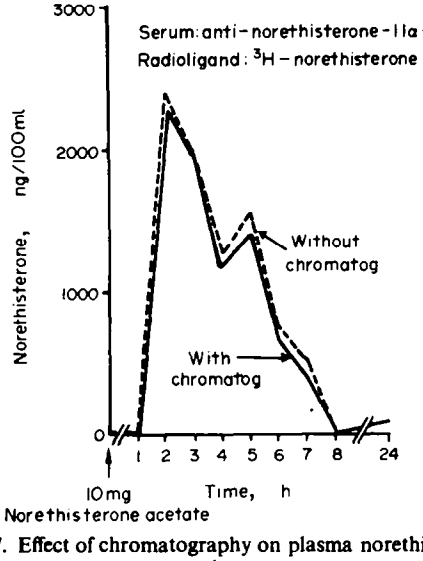

Fig. 7. Effect of chromatography on plasma norethisterone values.

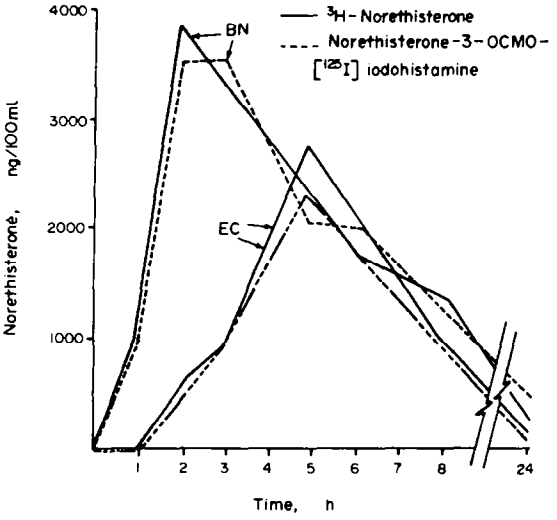

Fig. 8. Concentrations of norethisterone in plasma of volunteers given 10 mg norethisterone orally. Serum: anti-norethisterone-11α-BSA (^{3}H-norethisterone, dil. 1:4000), (norethisterone-3-OCMO-[^{125}I]-iodohistamine, dil. 1:10,000).

the chromatography step is unnecessary to ensure a specific analysis with this assay system.

(Since it has been reported that solvent residues can interfere with radioimmunoassay determinations [20, 21], "blank" eluate from chromatograms were added to a series of standards and the resulting curve compared with a normal one. No significant difference could be discerned between the two and the problem was not considered further.)

Comparison of ^{3}H- and ^{125}I-labelled radioligand assays

Two series of plasma samples were analysed simultaneously for norethisterone using the anti-norethisterone-11α-BSA serum with both the ^{3}H-norethisterone and norethisterone-3-OCMO-[^{125}I]iodohistamine radioligands. Figure 8 shows the results obtained and it can be seen that little difference can be detected between the two sets of data in either case. Consequently, since a chromatographic step is unnecessary for a specific analysis using the ^{3}H-norethisterone radioligand the same must be true of the system involving the use of the norethisterone-3-OCMO-[^{125}I]iodohistamine.

Accuracy, precision and sensitivity

Accuracy was assessed by determination of recovery of 500 pg norethisterone added to eight samples of water (500 μl). Recoveries ranged from 93–113%.

Assay precision was determined by the method of Snedecor[22] in which the difference between values for duplicate assays is used to estimate the standard deviation(s) by means of the formula $\sqrt{\Sigma d^2/2N}$ (where d is the difference between duplicates and N is the number of samples). The estimated values of s for three concentration ranges using both the ^{3}H-norethisterone and norethisterone-3-OCMO-[^{125}I]iodohistamine radioligands are given in Table 3. In the two lower ranges the standard deviations were less

Table 3. Estimates of standard deviations for assay systems using anti-norethisterone-11α-BSA serum with [^{3}H]-norethisterone and NE-3-^{125}I radioligand

Concentration range (ng/100 ml)	estimated standard deviation (S)*	
	[^{3}H]-norethisterone	NE-3-^{125}I
0–1000	32 (N = 8)	57 (N = 5)
1000–1500	92 (N = 7)	68 (N = 4)
1500–∞†	115 (N = 15)	188 (N = 9)

* $s = \pm\sqrt{\Sigma d^2/2N}$, where d is difference between duplicates and N is number of samples.

† for [^{3}H]-norethisterone 10 samples had values in excess of 2000 ng/100 ml. For NE-3-^{125}I 8 samples had values in excess of 2000 ng/100 ml.

than 10% of the mid-range value. This was also the case for the upper range when ^{3}H-norethisterone was the radioligand. The figure for the upper range with the norethisterone-3-OCMO-[^{125}I]iodohistamine radioligand was ±188 which initially appears to be high. However, 8 out of 9 of the sets of data used to calculate this value exceeded 2000 ng/100 ml and consequently it was of the same order as the rest in relation to the data from which it was derived.

The specificity of the analyses was established as described earlier by the observation that chromatographic purification of the extracts on thin-layers of alumina did not affect the observed values of the steroid in plasma. Sensitivity of assay was not a problem in the experiments undertaken since the plasma concentrations were such that quite small volumes contained sufficient masses of steroid to avoid any difficulty.

CONCLUSION

Radioimmunoassay systems using ^{3}H-norethisterone and norethisterone-3-OCMO-[^{125}I]iodohistamine radioligands with an anti-norethisterone-11α-BSA serum give comparable results of adequate specificity, precision, accuracy and sensitivity when used to analyse crude ether extracts of plasma samples from subjects receiving the drug norethisterone acetate (SH420). Similar heterologous systems using ^{125}I-labelled ligands may prove useful for the determination of other synthetic steroids etc. for which there is no suitable commercially available ^{3}H-labelled radioligand in existence.

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