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ASSESSMENT OF THE SPECIFICITY OF NORETHISTERONE RADIOIMMUNOASSAYS

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SUMMARY

The specificity of four norethisterone antisera, obtained from four research laboratories, was examined. Antisera A, B and C were raised against norethisterone coupled to bovine serum albumin through position 3, antiserum D against 11α-hydroxynorethisterone coupled to bovine serum albumin through position 11. Using any of the 4 antisera, no significant deviation from parallelism could be found between graded doses of authentic norethisterone and increasing volumes of plasma from women taking norethisterone for contraceptive purposes. Cross-reaction studies with tetrahydro and dihydro derivatives of norethisterone indicated that antiserum D exhibited the lowest degree of cross-reaction. This antiserum, however, along with antisera A and B, gave a high plasma blank (interfering endogenous plasma compounds) when plasma from women not taking norethisterone was assayed. The blank was not measurable in the case of antiserum C. Furthermore, when plasma samples from subjects taking norethisterone were assayed, antisera A, B and D yielded significantly higher estimates than antiserum C.

Chromatography on celite decreased the plasma blank to non-measurable levels with all antisera. It also significantly decreased the norethisterone levels found with antisera A, B and D, but did not change those obtained with antiserum C. Calculation of the regression of the non-chromatographic (direct) on chromatographic measurements indicated that the magnitude of the overestimation was independent of the amount of norethisterone in plasma when antisera A, B and D were used. This suggests that the bulk of the material removed by chromatography consisted of endogenous compounds.

It is concluded that (1) the significance of cross-reaction studies as well as that of the parallelism test for the assessment of the over-all specificity of the assay is limited, (2) a single chromatography prior to the radioimmunoassay proper improves the assay specificity, but may not be sufficient to remove all interfering compounds, (3) a comparison of the direct and chromatographic assay procedures using several antisera is useful for the selection of the relatively most specific radioimmunoassay procedure. In the present study, this is the technique employing either antiserum C or antiserum D, the latter, however, only after chromatography.

INTRODUCTION

Norethisterone is a widely used contraceptive drug. The determination of its plasma levels is an important step in the clinical-pharmacological studies of this compound and of its different formulations.

The development of the radioimmunoassay of norethisterone was reported by several research groups[1–6]. In these studies, the over-all specificity of the assays was based on the specificity of the antisera and no purification of plasma prior to the radioimmunoassay proper was undertaken. Only in one paper[7] was the necessity of a chromatographic purification emphasized.

The aim of the present study was to assess the specificity of 4 different antisera by cross-reaction studies and to relate the antiserum specificity to the over-all specificity of the assay, as established by measurements of plasma levels in women regularly taking the microdose of norethisterone (300 μg per day).

EXPERIMENTAL

Clinical material. The blood plasma samples investigated in this study originated from a series of samples collected from 7 female volunteers (age 26–40 years) at the Department of Obstetrics and Gynecology, Karolinska Hospital, Stockholm. Following a

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Trivial names. The following trivial names were used in the present paper: Norethisterone: 17α-ethynyl-17β-hydroxy-4-estren-3-one, 3α,5α-tetrahydronorethisterone: 17α-ethynyl-5α-estrane-3α,17β-diol, 3α,5β-tetrahydronorethisterone: 17α-ethynyl-5β-estrane-3α,17β-diol, 3β,5α-tetrahydronorethisterone: 17α-ethynyl-5α-estrane-3β,17β-diol, 3β,5β-tetrahydronorethisterone: 17α-ethynyl-5β-estrane-3β,17β-diol, 5α-dihydronorethisterone: 17α-ethynyl-17β-hydroxy-5α-estran-3-one, 5β-dihydronorethisterone: 17α-ethynyl-17β-hydroxy-5β-estran-3-one, testosterone: 17β-hydroxy-4-androsten-3-one, dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one, ethynylestradiol: 17α-ethynyl-1,3,5(10-estratriene-3,17β-diol.

control cycle, the volunteers received norethisterone (300 μg) *per os* daily for 2 months. During the second month systemic blood was taken by venepuncture daily; it was collected in heparinized test tubes, centrifuged immediately and the plasma was frozen and stored at −20°C until the day of assay. The time interval between the pill ingestion and blood withdrawal varied between 3 and 22 h for individual subjects. This was due to the fact that the time of blood withdrawal was fixed between 8.00 and 11.00 h, whereas the time of norethisterone ingestion varied from subject to subject. Plasma samples collected on 5 different days were randomly chosen from each subject, i.e. a total of 35 samples were analyzed.

The plasma samples were obtained as a part of a clinical study which will be reported elsewhere (Landgren *et al.*, to be published).

Reagents. [6,7-^{3}H]-Norethisterone (55 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). It was purified by chromatography on Sephadex LH-20 columns using the solvent system toluene–methanol (85:15, v/v).

Authentic non-radioactive norethisterone was donated by Syntex Laboratories Inc. (Palo Alto, CA, U.S.A.). The sample was of analytical purity. Samples of tetrahydro and dihydro derivatives of norethisterone were obtained through the courtesy of Schering AG (West-Berlin). Other steroids were purchased from Steraloids (Wilton, NH, U.S.A.) and purified as described previously[8].

Other reagents, materials and phosphate assay buffer (0.1 M; pH 7.4) were the same as published previously[8].

Antisera. The position of binding, final dilution and equilibrium constant of individual antisera are indicated in Table 1. The cross-reactions of various steroids with the antisera are shown in Table 2.

Radioimmunoassay. The principles of the radioimmunoassay method described previously[8] were adopted; the incubation of the standard or unknown solutions (0.3 ml) was performed at 60°C for 10 min., followed by 30°C for 30 min., and the bound and free radioactive material was separated by charcoal. The range of doses of norethisterone used for the preparation of the standard curve was 6.25 to 200 pg in all instances. All samples were measured in duplicate.

Direct procedure: Plasma (0.4 ml) was extracted with 10 volumes of ether by vortexing for 1 min. The ether solution was evaporated under nitrogen and the residue was dissolved in the assay buffer (0.6 ml). Aliquots of 0.1 ml were used for the radioimmunoassay.

Chromatographic procedure: Following the addition of internal standard (approx. 2000 d.p.m. in 0.1 ml buffer) to plasma (0.4 ml) and equilibration at 30°C

Table 1. Properties of the antisera investigated

Antiserum*	Position of binding to BSA†	Final dilution (in 0.3 ml)	Equilibrium constant × 10^9 (litres/mole)‡
A	3	1:264000	13
B	3	1:216000	14
C	3	1:163000	27
D	11	1: 46000	10

* The antisera were obtained by courtesy of Drs. K. Fotherby, London, K. Griffiths, Cardiff, A. Pala, Rome and R. F. Palmer, High Wycombe. The donors are listed in an alphabetic order which does not correspond to that in which the antisera are presented. † The conjugates were derived from the reaction of norethisterone 3-(O-carboxymethyl) oxime or 11α-hemisuccinate with bovine serum albumin. ‡ The equilibrium constants were estimated from Scatchard plots calculated from standard curves.

Table 2. Cross-reactions of various steroids with antisera

Compound	Cross-reaction* (%) with antisera			
	A	B	C	D
Norethisterone	100	100	100	100
3α,5α-Tetrahydronorethisterone	5.1	5.0	5.0	1.5
3α,5β-Tetrahydronorethisterone	8.1	0.3	1.6	0.1
3β,5α-Tetrahydronorethisterone	10.4	19.0	19.3	2.0
3β,5β-Tetrahydronorethisterone	7.0	2.6	12.9	0.4
5α-Dihydronorethisterone	18.5	35.8	31.2	24.9
5β-Dihydronorethisterone	27.5	8.1	23.2	5.5
Testosterone	0.5	0.3	0.2	0.1
Dihydrotestosterone	0.3	0.3	0.1	0.1
Ethynylestradiol	0.7	3.3	2.7	0.5

* The percentage of cross-reaction was estimated as a ratio of the mass of norethisterone (times 100) to the mass of the cross-reacting steroid when either mass brought about 50 per cent binding.

Table 3. Elution of norethisterone and its derivatives* from a celite–ethylene glycol (2:1, w/v) column

Solvent	Volume (ml)	Eluate
Isooctane	10	discarded (wash of the column)
Isooctane	1	discarded (sample applied)
Isooctane	7	5α-Dihydronorethisterone + 5β-dihydro-norethisterone; discarded
Isooctane–toluene (8:2, v/v)	3	discarded
Isooctane–ethyl acetate (85:15, v/v)	4	norethisterone + 3β,5α-tetrahydronorethisterone + 3β,5β-tetrahydronorethisterone

* The presence of norethisterone derivatives in the chromatographic fractions was established by radioimmunoassay. The individual norethisterone derivatives were chromatographed in nanogram quantities. Chromatographic fractions were then assayed using labelled norethisterone as tracer and antiserum C as immunoreagent.

for 15 min., the mixture was extracted with 10 vol. of ether. The ether solution was evaporated under nitrogen, the residue was dissolved in isooctane (1 ml) and transferred to a celite–ethylene glycol (2:1, w/v) column. The chromatography was performed as shown in Table 3. The fraction containing norethisterone was evaporated under nitrogen and the residue was dissolved in 0.6 ml assay buffer. A portion (0.1 ml) was used for the measurement of recovery and aliquots of 0.1 ml were used for the radioimmunoassay.

Assay design. In order to exclude the influence of the day-to-day variation on the comparison of antisera, aliquots (0.1 ml) of the same samples were always assayed by the direct and chromatographic procedures on the same day using all 4 radioimmunoassay systems (i.e. all 4 antisera).

Calculations. All calculations of radioimmunoassay results were performed as described previously[8, 9] using a logit-log transformation of the data. The results of the direct procedure were not corrected for extraction losses which were approx. 5%; the recovery of the internal standard added was 95.4 ± 1.7% (standard deviation); $n = 20$. In the chromatographic procedure the extraction and chromatography losses were monitored by the internal standard measurements (see above), and a correction for the mass of the internal standard added[9] was applied.

In the calculation of the results of norethisterone measurements in plasma a lognormal distribution of individual measurements[10] was assumed.

RESULTS

Reliability criteria

The recognized criteria of reliability[8, 9] were checked for the 4 radioimmunoassay systems. In none of the systems was any measurable solvent blank found. The same was true for plasma blank (assay of a plasma pool of normally menstruating women; cf. Table 4) when the chromatographic procedure was adopted. On the other hand, a plasma blank was found in the systems A, B and D when the direct radioimmunoassay was employed. It can be concluded on the basis of an analysis of variance that there were significant differences between the plasma blanks yielded by the 3 antisera (Table 4).

In all "direct" systems (i.e. in the radioimmunoassays employing no chromatographic purification) statistically valid parallelism was obtained between 3 increasing doses of the standard and 3 increasing volumes of plasma. The F-values for parallelism and linearity were in the systems A, B, C and D 0.70 and 0.38, 0.95 and 0.11, 0.15 and 1.25, 0.10 and 0.10, respectively (tabulated $F_{0.95(1,15)} = 4.54$). The within-assay and the between-assay coefficients of variation were 6.5 to 7.2% ($n = 37$) and 7.9 to 9.3 ($n = 25$ on 3 occasions), respectively, for the 4 radioimmunoassay systems.

The cross-reaction of 6 norethisterone reduction products and of ethynylestradiol, testosterone and

Table 4. Means ± standard deviations ($n = 5$) of plasma blank measurements (in pg/ml) using the 4 antisera in the direct radioimmunoassay of norethisterone in a female plasma pool*; analysis of variance of the measurements.

Antiserum†			
A	B	C	D
205 ± 6.99	153 ± 13.5	not measurable‡	252 ± 8.26

Analysis of variance§		
Mean square means ($df = 2$)	Mean square within ($df = 12$)	F-value
13470	99.30	136

* For the assay of the plasma blank a plasma pool was used which had been collected from 16 normally menstruating women and contained 1.2 ng/ml of progesterone and 98 pg/ml of estradiol. The women have not been using any contraceptive drugs. † For the properties of the individual antisera cf. Tables 1 and 2. ‡ The responses were found between zero and the first dose of the linearized standard curve (cf.[8]). Since the first dose was 6.25 pg and—similarly to the assay of norethisterone containing plasma—the radioimmunoassay results had to be multiplied by a factor of 15 in order to obtain the concentration of plasma blank per ml, the limit between the non-measurable and measurable values was 93.75 pg/ml. § When appropriate contrasts were calculated on the basis of the analysis of variance, significant ($P < 0.001$) differences between means A and B, A and D, B and D were found.

Table 5. Geometric means and 95% confidence limits of norethisterone levels (pg/ml) in blood plasma of female subjects*; comparison of 4 antisera using the direct radioimmunoassay procedure or that involving chromatography

Antiserum†	A		B		C		D	
Procedure	Direct	Chromatography	Direct	Chromatography	Direct	Chromatography	Direct	Chromatography
	504 (427–596)	356 (281–450)	410 (336–500)	353 (283–440)	317 (249–404)	314 (242–408)	532 (458–619)	295 (230–379)

* Norethisterone levels were measured in 35 plasma samples collected from 7 subjects (5 samples per subject); the samples were chosen at random from the group of samples collected in each subject throughout one menstrual cycle; the samples of one subject were always assayed using both the direct and chromatographic procedure and all 4 antisera on one day. † For the properties and cross-reactions of individual antisera cf. Tables 1 and 2, respectively.

dihydrotestosterone with the 4 antisera investigated were as demonstrated in Table 2. The lowest degree of cross-reaction was found with antiserum D.

Assay of norethisterone

Thirty-five plasma specimens (5 samples per subject) were randomly chosen from a series of samples collected from 7 subjects using norethisterone as a contraceptive at a microdose level of 300 μg per day. These samples were assayed using the 4 antisera by both the direct and chromatographic procedure.

The results of these assays are demonstrated in Table 5. A statistical evaluation by means of the computation of an anlysis of variance and of appropriate contrasts [11] indicated (Table 6) that antiserum C yielded the significantly lowest result when the direct assay was used. There was no significant difference between the direct and chromatographic procedures as far as this antiserum was concerned. In this respect antiserum C differed from the three other antisera. The chromatographic procedure yielded statistically non-distinguishable results with antisera C and D. Furthermore, the results obtained with these 2 antisera were significantly lower than those found using antisera A and B.

When a regression of the direct on chromatographic measurements was calculated (Table 7), correlation coefficients close to unity and slopes not distinguishable from unity were found in the case of all 4 antisera. Except for antiserum C, all antisera exhibited a significant positive y-intercept of the regression line. Hence the use of antisera A, B or D resulted in a systematic overestimation of norethisterone values when the direct procedure was employed. The independence of the magnitude of overestimation on the amount of norethisterone (as demonstrated by the slopes not distinguishable from unity) points to compounds of endogenous character (plasma blank) as the cause of overestimation. As a matter of fact, the magnitude of the y-intercepts was positively correlated to the magnitude of plasma blanks found by the direct procedure in plasma not containing norethisterone (Table 4).

DISCUSSION

The specificity of the 4 antisera investigated was tested by means of cross-reaction studies with tetrahydro and dihydro derivatives of norethisterone (cf. Table 2) and with ethynylestradiol. These compounds

Table 6. Analysis of variance of norethisterone plasma levels assayed in 35 plasma samples (from 7 subjects) using 4 various antisera in the direct and chromatographic radioimmunoassay procedure, respectively; significance of differences between antisera and procedures, as established by the calculation of appropriate contrasts

Between samples (df = 34)	Between procedures (df = 7)	Residual (df = 238)	F-value procedures	Significance
0.5703	0.3217	0.0061	52.7	$P < 0.001$

Contrast*	F-value	Significance
Cd vs. Ad	116.9	$P < 0.001$
Cd vs. Bd	35.7	$P < 0.001$
Cd vs. Dd	145.5	$P < 0.001$
Cc vs. Cd	0.1	not significant
Ac vs. Ad	66.0	$P < 0.001$
Bc vs. Bd	12.1	$P < 0.001$
Dc vs. Dd	188.6	$P < 0.001$
Cc vs. Dc	2.2	not significant
Cc, Dc vs. Ac, Bc	24.9	$P < 0.001$

* The capital letters denote the 4 antisera, c stands for the chromatographic procedure and d for the direct one.

Table 7. Regression of the direct on chromatographic procedures*, as measured in 35 plasma samples collected from 5 subjects

Antiserum	A	B	C	D
Slope	0.9075	1.0222	0.9352	1.0013
95% Confidence limits of the slope	0.8019 ... 1.0131	0.9469 ... 1.0975	0.8703 ... 1.0002	0.8865 ... 1.1161
y-Intercept (pg/ml)	163	43.7	18.9	210
95% Confidence limits of the *y*-intercept (pg/ml)	109 ... 218	6.57 ... 80.9	−12.3 ... 50.19	158 ... 262
Correlation coefficient	0.950	0.979	0.981	0.951

* The direct and chromatographic procedures yielded the dependent (Y) and independent (X) measurements, respectively.

were shown to be present in plasma or urine in the unconjugated[12] or conjugated[12, 13, 14] form. Other potential metabolites of norethisterone (4-estrene-3,17-dione[15], 17α-ethynyl-17β-hydroxy-4β,5β-epoxyestran-3-one[16, 17], 17α-ethynyl-17β-hydroxy-5α-estrane-3,6-dione[16]), which were found in *in vitro* studies, were not tested in the present investigation.

In the present study, marked cross-reactions of the tetrahydro and dihydro derivatives of norethisterone were found with antisera A, B and C, whereas anti-serum D (raised against 11-hydroxynorethisterone conjugate) exhibited a low degree of cross-reaction. However, when the over-all specificity of the 4 radioimmunoassay systems was tested by assaying plasma samples, the significance of the information obtained from the cross-reaction studies became questionable.

Thus, when plasma from normally menstruating women was assayed without chromatographic purification, a significant plasma blank (i.e. interfering endogenous compounds) was detected using anti-serum D. Significant blanks were also obtained with antisera A and B. On the other hand, a non-measurable blank was found in the case of antiserum C. The same discrepancy between the results of cross-reaction studies and plasma measurements was seen when plasma samples from subjects treated with norethisterone were assayed. In the direct assay, the use of antiserum D yielded an obvious overestimate. The significantly lowest and probably most accurate results were obtained using antiserum C.

The chromatographic purification decreased the plasma blank to non-measurable levels in the case of antisera A, B and D (in the case of antiserum C, non-measurable blank values were obtained even without chromatography). The chromatographic procedure also diminished substantially the norethisterone levels when these 3 antisera were used. A regression analysis of the direct on chromatographic measurements demonstrated that—within the range of values found—the amount of interfering compounds was on the average constant and independent of the amount of norethisterone. This indicates that the interfering compounds were very probably of endogenous origin (plasma blank).

Only a small portion of the interfering material might be due to norethisterone metabolites. This portion would consist of compounds which had not been removed by celite chromatography and which were detected in the form of elevated norethisterone levels when antisera A and B were used. It seems unlikely, however, that the derivatives of norethisterone investigated in the present study could play an important role in causing an overestimate. Thus, tetrahydro derivatives of norethisterone are eluted—probably all 4 of them—from the column together with norethisterone (Table 3), and they significantly cross-react with anti-serum A and B. However, if these compounds were significantly represented in the interfering material, also antiserum C would yield elevated results after chromatography, since the cross-reactions of these compounds with antiserum C were of the same order of magnitude as with antisera A and B, respectively. This conclusion is in agreement with recent gas chromatographic–mass spectrometric measurements of norethisterone metabolites[12]. According to these studies, the amount of unconjugated tetrahydro derivatives of norethisterone in plasma 3 h after taking the drug represents approximately 5% of the free norethisterone concentration. Hence with cross-reactions of some 5–10%, the real interference would be within the limits of the precision of the method.

The dihydro derivatives do not seem to be responsible for the over-estimates either, as can be inferred from the following observations: the dihydro derivatives can be effectively separated from norethisterone by chromatography on celite (cf. Table 3). There was no significant difference between the direct and chromatographic measurements of plasma levels when antiserum C was used. Again, these observations are not contradicted by the fact that the amount of 5β-dihydronorethisterone was found to be about 10% of the norethisterone concentration in plasma [12], and that the magnitude of cross-reaction of this metabolite with antiserum C was about 20%.

Several conclusions may be drawn from the results of the present study. The plasma appears to contain compounds which caused significant overestimates in the direct measurements when 3 out of the 4 antisera investigated were used. The celite chromatography,

although it significantly reduced the false high values, was not completely successful in separating all interfering compounds from norethisterone, as can be inferred from the fact that both antiserum A and antiserum B yielded higher results after chromatography than antiserum C and D, respectively. The nature of the interfering compounds is not known. They may in part be of endogenous origin, and in part they may be norethisterone metabolites.

There is a certain possibility that there exists some additional material which is not separated from norethisterone by the chromatographic procedure employed and which still causes some overestimation when antisera C and D are used. Such a possibility can be investigated by comparison of the results with those obtained by another analytic method, such as gas liquid chromatography–mass spectrometry. Such studies are now in progress.

The data of the present study also seem to indicate that the character of norethisterone antigen, as given by the position of the attachment to bovine serum albumin, is of relatively minor importance for the over-all specificity of the radioimmunoassay, and that cross-reaction tests involving a few known or suspected metabolites provide inconclusive evidence of the assay specificity.

Furthermore, the test of parallelism is equally insufficient as a sensitive criterion of specificity and accuracy. The limitations of this test were earlier recognized[9] and now again confirmed. In spite of the obvious lack of specificity in some of the assay systems, no significant deviation from parallelism could be found between graded doses of authentic norethisterone and increasing volumes of plasma.

The present approach, involving the comparison of several antisera using the direct and chromatographic procedures, seems to be useful in identifying the relatively most specific radioimmunoassay procedure. In the present study, this is the procedure employing either antiserum C or antiserum D, the latter only after chromatography. For reasons of practicability, the direct procedure using antiserum C was chosen for further studies on norethisterone levels in the blood of women taking this drug (Landgren *et al.*, to be published).

The present study cannot answer the question why antisera A, B and C, prepared in 3 different laboratories by the use of the same type of antigen, differed with regard to their specificity. However, it is a generally known fact that the specificity of antisera obtained from individual animals within one laboratory and using the same antigen shows appreciable differences. The results reported in the present study suggest that this is not necessarily a disadvantage.

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