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INFLUENCE OF ANTISERA ON THE ESTIMATIONS OF NORETHISTERONE AND LEVONORGESTREL

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SUMMARY

The specificity of six different antisera for norethisterone (NET) and ten different antisera for levonorgestrel (LNG) were studied. These antisera had varying degrees of cross-reactivities with the metabolites of the respective steroids. The antisera obtained from the same animal showed a trend towards higher cross-reactions with the length of the immunization period. Animal variation also seemed to be a major factor in deciding the specificity of antisera. Using these antisera radioimmunoassays (RIAs) for NET and LNG were standardized which fulfilled the necessary criteria for reliability. When NET and LNG concentrations were determined in pooled plasma, collected from women taking contraceptives containing the respective steroids, it was observed that the variation in the cross-reactivities of the metabolites with the antiserum used, had negligible effect on NET estimates, whereas they influenced the LNG estimates.

INTRODUCTION

The doses of contraceptive steroids have so far been fixed arbitrarily to get the desired effect with minimum side effects. The development of radioimmunoassay (RIA) for many of the contraceptive steroids has greatly enhanced our knowledge of the pharmacokinetics of these compounds, which would eventually help in modifying doses and delivery systems. The antisera used in RIA of synthetic steroids differ from one laboratory to another. The antisera, being biologic materials, have varying degrees of cross-reactivities, mainly with the metabolites of the original steroids. These metabolites are known to be present in the ether extracts of plasma collected from women following the steroid administration [1, 2]. In the literature the reliability of the steroid measurements has been assessed by studying the cross-reactivities of antisera with some of the metabolites and by studying recovery of the added steroid in plasma. However, neither of these methods assess the effect of the metabolites, that are also present in the plasma extracts on the estimation of the parent drug. Such information is necessary to compare the data reported from different research laboratories.

In this paper we describe our experience on the measurements of NET and LNG in pools of samples collected from women on NET and LNG steroid therapy, using different antisera.

EXPERIMENTAL

The source of the antisera for NET and LNG and the conjugates used for the production of these antisera are given in Tables 1 and 2. The tritiated steroids and the metabolites were received from Schering AG. Plasma pools A and B for NET were obtained by adding 100 pg and 1000 pg of the steroid to the male plasma, whereas pools C, D and E which contained different amounts of NET were obtained from three groups of women using various types of NET containing contraceptives. On similar lines, pools A′ and B′ were prepared by adding 100 pg and 1000 pg of LNG to male plasma and pools C′ and D′ were obtained from women taking contraceptives containing LNG.

Titres of the antisera were determined by incubating 100 μl of serially diluted antisera with fixed amounts of either tritiated NET or LNG (20,000 d.p.m.) overnight at 4–6°C. The reaction volumes for incubations were adjusted to 700 μl with 0.1 M phosphate buffered saline (PBS), pH 7.2. The separation of free and bound steroids were carried out by adding appropriate amounts of dextran charcoal suspension so as to keep the non-specific binding below 2%. Thus, 400 μl of a suspension of 0.6% charcoal (Norit A) and 0.06% dextran (MW 70,000) was needed for NET whereas 500 μl was needed for LNG. The radioactivity in the bound fraction was measured using 5 ml of toluene based scintillation medium (5.0 g PPO and 0.3 g POPOP in 1 l. of toluene), in a liquid scintillation spectrometer (Packard Tricarb Model 3255) with 50% efficiency for tritium.

The standard curves were obtained by incubating increasing amounts of unlabelled steroids (0–500 pg) with fixed amounts of tritiated steroids (20,000 d.p.m.) and appropriate dilutions of antisera. The bindings were expressed as percent of zero binding. Cross-reactivities of endogenous steroids such as cortisol, estradiol, progesterone, testosterone, dihidrotestosterone

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Table 1. Norethisterone antisera

Antisera Nos.	Norethisterone Conjugate	Sources
1	NET-3CMO-BSA	Royal Postgraduate Medical* School, London;
2	NET-11αhemisuccinate-BSA	Schering AG, Berlin;
3	NET-3CMO-BSA	University Hospital, Uppsala, Sweden
4, 5 & 6	NET-3CMO-BSA	I.R.R., Bombay Raised in rabbit No. 1031†

* Serial nos. of antisera may not correspond to the order of the sources mentioned.

† The antisera 4, 5 & 6 were obtained by bleeding after 75, 104 and 188 days following the first injection. The animal was immunized by giving 3 injections weekly, one fortnightly and then at monthly intervals. The days of bleeding correspond to the 10th day after the last injection.

etc. as well as of the metabolites of NET and LNG with these antisera were determined according to the method of Abraham[3].

One millilitre of each plasma pool containing added tritiated steroids (2000 d.p.m.) were extracted with 10 volumes of diethylether purified by passing through basic alumina column, as suggested by Vermeulen (personal communication). The dried ether extracts were suspended in 2 ml of PBS. Aliquots of 0.5 ml were counted for recovery and 0.5 ml volumes in duplicates were processed for the assay.

The plasma concentrations of the steroids were calculated using logit-log transformation of the data with a programmable pocket calculator (Hewlett–Packard, Model 67). The values were corrected for procedural losses.

RESULTS AND DISCUSSION

Specificity of NET antisera

Norethisterone antisera did not cross-react with any of the endogenous steroids studied. Table 3 shows the percent cross-reactivities of the metabolites were different for different antisera. The over-all cross-reactivities of the NET antisera with the metabolites showed an increase with the length of the immunization period. However, only one metabolite 17αethinyl-5αestran-3β,17β diol showed a drop in cross-reactivity with the antiserum obtained at 105 days duration of immunization. The cross-reactivity of the antiserum obtained at 188 days of immunization was higher than those obtained at 75 and 105 days.

Our observations regarding the cross-reactivities of the endogenous steroids with the NET antiserum received from Dr. Johansson were similar to those reported from his laboratory [4]. However, no data was available to us regarding its cross-reactivities with the NET metabolites. The data from Schering laboratory regarding their NET antiserum specificity were comparable to those observed by us with the same antiserum [5]. The cross-reactivities observed by Warren and Fotherby[6] using their antiserum, with endogenous steroids were similar to those observed by us, whereas those of two synthetic compounds studied were lower in our laboratory. However, their system differs from ours in the buffer used (Tris, pH 8.5 instead of PBS, pH 7.2) and the reaction volume (0.2 ml instead of 0.7 ml).

Table 2. Levonorgestrel antisera

Antisera Nos.	LNG Conjugates	Sources
1	LNG-3CMO-BSA	Royal Postgraduate Medical* School, London;
2	LNG-3CMO-BSA	Schering AG, Berlin
3, 4, 5 & 6	LNG-3CMO-BSA	I.R.R., Bombay Raised in rabbit No. 931†
7, 8, 9 & 10	LNG-3CMO-BSA	I.R.R., Bombay Raised in rabbit No. 939‡

* Serial nos. of antisera may not correspond to the order of the sources mentioned.

† The antisera 3, 4, 5 & 6 were obtained by bleeding the animal after 32, 175, 265 & 350 days respectively following the first injection.

‡ The antisera 7, 8, 9 and 10 were obtained by bleeding the animal after 175, 235, 265 and 350 days respectively following the first injection.

Both the animals were immunized by giving four injections at weekly and then at monthly intervals. The days of bleeding corresponded to the 10th day following the last injection.

Table 3. Characteristics of norethisterone antisera

Antisera Nos.*	1	2	3	4	5	6
Day of bleeding after first injection				75	104	188
Antisera dilution (in thousands)	70	105	175	140	210	210
Slope of standard curve	−1.94	−2.02	−2.19	−2.37	−2.23	−2.45
Sensitivity†	15	5	7	3	4	3
Value (in pg) at 50% intercept	145	44	63	34	45	30
Percent cross-reaction with						
17αethinyl-5αestran-3β, 17β diol	34	4.1	7.1	26.7	20.0	28.3
17αethinyl-5βestran-3β, 17β diol	5.7	1.7	9.6	3.6	3.3	6.1
17αethinyl-5αestran-3α 17β diol	14.8	7.2	4.6	11.9	12.1	17.0
17αethinyl-5βestran-17β OH-3 one	8.1	16.5	23.7	9.7	9.4	11.7
17αethinyl-5αestran-17β OH-3 one	29.6	23.9	17.3	3.5	33.5	50.0
Norethisterone acetate	<1.45	<0.44	<0.63	<0.34	<0.45	<0.30

* The numbers refer to the antisera as listed in Table 1.
† Amount of norethisterone in pg in assay tube that can be distinguished from Zero at 95% confidence limit.

As can be seen from Table 3 all the antisera gave ideal standard curves as assessed by slope, sensitivity and 50% intercept.

Norethisterone estimates

Using these antisera, NET values obtained with the different plasma pools are given in Table 4. The table shows that the values estimated in all the plasma pools with different antisera were comparable. This suggests that the varying degrees of cross-reactions of antisera with the metabolites do not have any appreciable effect on the estimated values of NET. This is in agreement with the observation of Morris and Cameron[7], who showed that though the cross-reactivities of metabolites in the assay systems using ^{125}I and ^{3}H labelled NET differed, the NET values estimated in plasma pools using both the labels do not differ significantly. This also confirms our findings.

However, recently Bedolla-Tover *et al.*[8] have shown that the values found in plasma pools from women taking NET containing contraceptives differed widely according to the antiserum used. It appears from their data that the values were proportional to the high blanks which they obtained in their system, even after using an extract equivalent to 0.075 ml plasma. In our system even using extract equivalent to 0.25 ml plasma, we have not seen any detectable blank. This might explain why we obtained comparable values with the various antisera.

Specificity of LNG antisera

Endogenous steroids did not show any cross-reactions with the LNG antisera. However, the antisera tested had varied degrees of cross-reactivities with the metabolites of LNG, as shown in Table 5. A trend towards higher cross-reaction with the duration of immunization was also apparent. A similar observation was made by Walker *et al.*[9]. Several reports in the literature however, indicate an increase in specificity with the duration of immunization [10–12], but Jean and Wickings[13] failed to confirm this finding. Our data shows that animal variation is also a major factor in deciding the specificity of an antiserum.

Table 4. Norethisterone values (pg/ml) estimated in plasma pools using various antisera

Antisera Nos.†		1	2	3	4	5	6
Plasma Blank		*	*	*	*	*	*
Plasma pool A	Mean	127	113	99	115	113	123
	S.D.	15.2	10.4	7.2	7.1	13.9	9.9
	C.V.	12.0	9.2	6.1	6.2	12.3	8.1
Plasma pool B	Mean	1062	1047	1102	1165	1193	1076
	S.D.	138.7	78.0	71.5	90.8	135.9	86.3
	C.V.	13.1	7.5	6.5	7.8	11.4	8.02
Plasma pool C	Mean	219	200	192	210	224	208
	S.D.	20.2	20.5	16.3	16.1	21.4	23.4
	C.V.	9.2	10.2	8.0	7.6	9.5	11.3
Plasma pool D	Mean	2166	1996	1969	1960	1966	1923
	S.D.	240.8	198.0	181.2	177	155.2	124.5
	C.V.	11.1	9.9	9.2	9.1	7.9	6.5
Plasma pool E	Mean	4755	4703	4543	4465	4630	4448
	S.D.	197	168	285	301	269	264
	C.V.	4.1	3.6	6.2	6.7	5.8	5.9

* The values were below the sensitivity limit in 0.25 ml plasma extract.
† The numbers refer to the antisera as listed in Table 1.
Mean values represents eight observations for each pools.

Table 5. Characteristics of levonorgestrel antisera

Antisera Nos.*	1	2	3	4	5	6	7	8	9	10
Day of bleeding after first injection	—	—	32	175	265	350	175	235	265	350
Antisera dilution (in thousands)	28	175	70	175	175	70	70	70	140	70
Slope of standard curve	−2.02	−2.07	−2.14	−2.01	−2.02	−2.27	−2.15	−2.33	−2.26	−2.21
Sensitivity†	4	5	8	4	4	5	3	7	4	4.5
Value (in pg) at 50% intercept	39	47	67	33	30	40	25	40	32	35
Percent cross-reaction with										
17αethinyl-18 methyl-5β estran-3α, 17β diol	2.6	1.3	<0.67	<0.33	<0.30	<0.40	2.8	2.5	5.0	3.5
17αethinyl-18 methyl-5α estran-3α, 17β diol	18.3	33.3	7.7	14.6	18.1	22.0	34.1	33.6	37.5	44.3
17αethinyl-18 methyl-5α estran-3β, 17β diol	56.9	100	42.6	60.6	69.0	66.0	82.8	84.0	79.0	100

* The numbers refer to the antisera as listed in Table 2.
† Amount of LNG in pg in assay tube that can be distinguished from Zero at 95% confidence limit.

Animal 931 from which antisera 3–6 were obtained responded to the immunization faster than the animal 939 from which antisera 7–10 were obtained. The titre of antisera obtained on 32nd day following the first injection, from the first animal was 1:70,000 whereas that from the second animal was 1:7,000. The antisera obtained from animal 931 were more specific than those obtained from animal 939. This suggests that animals which respond faster to immunization may give antiserum of better specificity. However this needs to be verified since the impression is based on one single observation.

Our observations on the cross-reactivities of LNG metabolites with the antiserum supplied by Schering AG were comparable to those observed in the Schering laboratory [5]. The data about the cross-reactivities of the antiserum received from Dr. Fotherby is available to us from his laboratory only with ^{125}I linked LNG [14]. These cross-reactivities were higher than those observed by us with tritiated LNG which has been reported with NET [6, 7].

Levonorgestrel estimates

Table 6 gives the LNG estimates in four different plasma pools and the statistical analysis of their variations are presented in Table 7. From both the tables it is apparent that RIAs using all the antisera fulfill the reliability criteria, especially plasma blanks, precision and accuracy as assessed by the recovery of the steroid added to male plasma (pools A′ and B′). However, LNG estimates in pools C′ and D′ obtained from women on LNG containing contraceptives varied significantly with the different antisera used. Probable reasons for this may be the variation in cross-reactivities of the antisera with the metabolites of LNG. The metabolites of LNG are known to be present up to 20% in the ether extracts of plasma obtained from women receiving the steroid [1].

The antisera 3–6 and 7–10 were raised in two different rabbits. They showed varying degrees of cross-reactivities with the LNG metabolites. The cross-reactivities of antiserum 3 were less than those of antisera

Table 6. Levonorgestrel values (pg/ml) estimated in plasma pools using various antisera

Antisera Nos.†		1	2	3	4	5	6	7	8	9	10
Plasma Blank		*	*	*	*	*	*	*	*	*	*
Plasma pool A′	Mean	112	104	111	99	107	102	116	100	98	96
	S.D.	14.6	10.8	13.2	14.1	6.8	9.2	13.2	13.6	8.3	8.0
	C.V.	13.0	10.4	11.9	14.2	6.4	9.6	11.4	13.6	8.5	8.3
Plasma pool B′	Mean	1112	1179	1156	1081	1115	1062	1134	1160	1126	1171
	S.D.	42.8	91.2	57.9	88.1	95.4	55.2	52.4	47.4	125	112
	C.V.	3.8	7.7	5.0	8.2	8.6	5.2	4.6	4.1	11.2	9.6
Plasma pool C′	Mean	219	286	257	316	296	312	314	316	321	293
	S.D.	17.9	19.9	18.2	24.9	30.9	17.2	25.3	21.2	18.1	39.4
	C.V.	8.2	7.0	7.1	7.9	10.4	5.5	8.1	6.7	5.6	13.4
Plasma pool D′	Mean	1184	1467	1372	1555	1459	1524	1698	1623	1741	1702
	S.D.	90.3	127	119	122	121	112	98.0	86	107	112
	C.V.	7.6	8.7	9.5	7.9	8.3	7.3	5.8	5.3	6.2	6.6

* The values were below the sensitivity limit in 0.25 ml plasma extract.
† The numbers refer to the antisera as listed in Table 2.
Mean values represent eight observations for each pools.

Table 7. Statistical estimate (using ANOVA test) for the differences in LNG values estimated in plasma pools with different antisera

Plasma Pool	Antisera*	F-ratio	P-value
A′	1–10	1.35	>0.05‡
B′	1–10	0.89	>0.05‡
C′	1–10	14.33	<0.01†
	3–6	10.50	<0.01†
	4–6	1.40	>0.05‡
	7–10	1.68	>0.05‡
	4–10	1.35	>0.05‡
D′	1–10	20.73	<0.01†
	3–6	4.28	<0.05†
	4–6	1.39	>0.05‡
	7–10	1.87	>0.05‡
	4–10	7.46	<0.01†

* The numbers refer to the antisera as listed in Table 2.
† The difference is significant.
‡ The difference is not significant.

4–6 which in turn were less than those of antisera 7–10 (Table 5).

It can be seen from Table 6 that the values found in pools C′ and D′ using antiserum 3 were less than those found with antisera 4–6. Also the values observed in pool D′ using antisera 4–6 were less than those observed with antisera 7–10. Table 7 reveals that the values estimated in pools C′ and D′ using the antiserum 3 were significantly different from the values obtained with antisera 4–6. This is evident from the fact that the values obtained with antisera 3–6 showed significant variations but those obtained with antisera 4–6 did not. Similarly, it can also be inferred from the table that the values estimated in pool D′ with antisera 4–6 were significantly different from those estimated with the antisera 7–10. But the values estimated in pool C′ using antisera 4–10 did not show any significant variation. This may be because the pool C′ which has low concentration of LNG might also have low concentration of its metabolites. The variation in cross-reactivities of antisera 4–10 might not have influenced the estimated values of LNG in the presence of a low quantity of metabolites. These observations lead us to the conclusion that LNG values estimated in plasma are influenced by the cross-reactivities of the metabolites of the steroid with the antiserum used.

It can be seen from Table 6 that the values estimated with antiserum 3 of the pools C′ and D′ were higher than those obtained with antiserum 1. But from Table 5 it is also evident that the cross-reactivities of the LNG metabolites were lower with antiserum 3 as compared to antiserum 1. However, we have studied the cross-reactivities of only three of the metabolites. It is possible that there might be other compounds derived from the original steroid, that are present in the plasma extracts in proportions capable of causing these discrepancies.

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REFERENCES

1. Sisenwine S. F., Kimmel H. B., Liu A. L. and Ruelins H. W.: The presence of *dl*, *d*- and *l*-norgestrel and their metabolites in the plasma of women. *Contraception* **12** (1975) 339–353.
2. Gerhards E., Hecker W., Hitse H., Nieuweboar B. and Bellmann O.: Zum stoffwechsel von norethisteron and *dl*-sowie *d*-Norgestrel beim menschen. *Acta endocr., Copenh.* **68** (1971) 219–248.
3. Abraham G. E.: A solid phase radioimmunoassay for estriol-17-beta. *J. clin. Endocr. Metab.* **29** (1969) 866–870.
4. Nygren K. G., Lindberg P., Martinsson K., Bosu W. T. K. and Johansson E. D. B.: Radioimmunoassay of norethindrone: Peripheral plasma levels after oral administration to humans and rhesus monkeys. *Contraception* **9** (1974) 265–278.
5. Personal communication from Schering AG.
6. Warren R. J., Fotherby K.: Iodine labelled ligands in the radioimmunoassay of synthetic progestin. *J. steroid Biochem.* **6** (1975) 1151–1155.
7. Morris S. E. and Cameron E. H. D.: Radioimmunoassay system for norethisterone using ^{3}H- and ^{125}I-labelled radioligands. *J. steroid Biochem.* **6** (1975) 1145–1150.
8. Bedolla-Tovar N., Rahman S. A., Cekan S. Z. and Diczfalusy E.: Assessment of the specificity of norethisterone radioimmunoassays. *J. steroid Biochem.* **9** (1978) 561–568.
9. Walker C. S., Clark S. J. and Wotiz H. H.: Factors involved in the production of specific antibodies to estriol and estradiol. *Steroids* **21** (1973) 259–283.
10. Haning R., McCracken J., St. Cyr. M., Underwood R., Williams G. and Abraham G.: The evolution of titer and specificity of aldosterone binding antibodies in hyperimmunized sheep.
11. Abraham G. E.: Radioimmunoassay of steroids in biological fluids. *J. steroid Biochem.* **6** (1975) 261–270.
12. Schmied U., Siegenthaler W., Nussberger J., Beckerhoff R. and Vetter W.: Characteristics of aldosterone antisera related to the duration of immunization. *Horm. Res.* **7** (1976) 28–33.
13. Jean Wickings E., Tafurt C. A., Hoogen H. and Nieschlag E.: The development of the titre and specificity of antibodies to testosterone. *J. steroid Biochem.* **8** (1977) 147–150.
14. Warren R. J. and Fotherby K.: Radioimmunoassay of synthetic progestogens, norethisterone and norgestrel. *J. Endocr.* **62** (1974) 605–618.