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NORETHYNODREL BINDING TO HUMAN PLASMA PROTEINS

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SUMMARY

The results presented give evidence that norethynodrel binds to plasma proteins. The presence of a specific protein in the plasma has been analysed. The association constant of norethynodrel to the plasma was $1{\cdot}0 \times 10^6 M^{-1}$. The studies on the effect of temperature on the binding ability of plasma proteins with norethynodrel showed that the binding was not affected at 60°C for 30 min. The pH optimum of the plasma norethynodrel binding was 7–8. Among the steroids tested for the competitive displacement of norethynodrel, progesterone showed competition for the norethynodrel binding sites in the plasma. Analysis of the binding proteins showed that norethynodrel binding protein was eluted from the DEAE–cellulose column along with albumin and some other plasma proteins. Further analysis of norethynodrel binding protein on disc electrophoresis showed that norethynodrel binds to two proteins, a specific protein and to albumin.

INTRODUCTION

The specific binding proteins for corticoids, androgens, estrogens and progesterone have been demonstrated to be present in the human plasma [1–7]. The steroid protein interaction in the body apart from transport of the steroid, protects the steroid from rapid catabolism [8, 9]. Oral contraceptives provide an effective method for the control of fertility. However, not much is known about their transport in the blood, metabolism and their clearance from the body. The metabolic fate of norethynodrel in women showed that norethynodrel and its metabolites are retained in circulation over a prolonged period of time [10]. Recently it is reported that norethynodrel and its metabolites, chlormadinone acetate and norgestrel are cleared from the body with the clearance rate of 30, 152 and 458 l./day [10]. It is known that oestradiol and progesterone are cleared comparatively faster than contraceptive steroids. The binding of norethynodrel to plasma proteins, enterohepatic circulation and storage in the fat depot and subsequent slow release from there were three possibilities suggested by Laumas *et al.*[11] for the prolonged retention of norethynodrel and its metabolites in the plasma. Of these the binding of norethynodrel to plasma proteins has been investigated. The results of these investigations are presented in this paper.

MATERIALS AND METHODS

Source of plasma and plasma proteins. Blood from normal men, women and pregnant women was obtained from colleagues and also from pregnant women attending the Out Patient department of the All India Institute of Medical Sciences Hospital. Bovine serum albumin (BSA) fraction V was purchased from Nutritional Biochemical Corp. and also from Sigma Chemical Co.

Radioactive steroids and chemicals. [6,7-^{3}H]-norethynodrel (S.A. 112·5 mCi/mmol) was obtained from R. E. Ranney, G. D. Searle & Co., Chicago. [7-^{3}H]-progesterone (S.A. 4 Ci/mmol) was a generous gift of Dr. Marcel Gut. [6,7-^{3}H]-Testosterone (S.A. 10–15 Ci/mmol) was purchased from New England Nuclear Corp. Progresterone and cortisol were obtained from Sigma Chemical Co. Sephadex G-25 and 200 were procured from Pharmacia Fine Chemicals, Uppsala. DEAE–cellulose was from Sigma Chemical Co. Acrylamide, bisacrylamide and glycine were from Eastman Organic Chemicals. Tris (hydroxymethyl)-aminomethane was purchased from Nutritional Biochemical Corp. Dialysis sacs were purchased from Arthur Thomas Co.

Equilibrium dialysis. The plasma protein solution was dialysed in a dialysis bag at 4° or 37°C for 40 h against 25 ml of phosphate buffer pH 7·4 containing radioactive steroid. Duplicate aliquots were taken from inside and outside the bag for radioactivity determination at the end of equilibrium dialysis. Quenching corrections were made by adding an internal standard. The results presented are the average of four experiments. Calculation of bound steroids to plasma proteins was worked out according to Sandberg *et al.*[12].

Ion-exchange column chromatography. Ion-exchange DEAE–cellulose chromatography was carried in a step-wise fashion to test the binding of steroids to proteins. Plasma was added to the vials containing radioactive steroids and incubated at 37°C for 30 min. and the plasma protein–steroid complex was layered on the column. The proteins were eluted at 4°C in a step-wise fashion from the DEAE–cellulose column as described by Sober and Peterson[13]. Five ml fractions were collected, aliquots (0·5 ml) were taken from each fraction for counting and proteins were measured at 280 nm.

Gel filtration: Sephadex G-25 (Coarse bead) or G-200 (fine) swollen gel was washed repeatedly and the fines were removed. Column 1 × 28 cm. of Sephadex G-25 or 2·5 × 52 cm. of Sephadex G-200 was prepared. The sephadex column was equilibrated with phosphate buffer 0·05 M, pH 7·4. Plasma incubated with radioactive steroid at 37°C for 30 min. was allowed to stand at 2 4°C for 20 min. and then layered on the top of the gel. After it has entered into the gel, the buffer was added without disturbing the top of the gel. Fractions of equal volume were collected. Aliquots were taken in vials, dried and counted in Packard liquid scintillation spectrometer Model 3314. Optical density was measured at 280 nm in Zeiss Spectrophotometer PMQ II.

Polyacrylamide gel electrophoresis. Disc electrophoresis was carried out according to Davis[14], using a 7·5% acrylamide gel. Serum equilibrated with radioactive norethynodrel was subjected to gel electrophoresis in Shandon Disc Electrophoresis Apparatus. At the end of the electrophoresis the gel was removed from the column cut into 2·5 mm lengths and dissolved in 30% hydrogen peroxide at 70°C for 20 h and then counted by adding diotol scintillation liquid (toluene 250 ml, dioxane 250 ml, methanol 150 ml PPO (2,5 diphenyloxazole)-benzene 3·25 g, dimethyl POPOP (1,4-bis-2-(4-methyl-5 phenyloxazolyl)-benzene 65 mg and naphthalene 52 g).

RESULTS

Effect of plasma protein concentration on binding of norethynodrel. The ability of total plasma and of plasma diluted to different concentrations to bind norethynodrel is shown in Table 1. It may be seen that undiluted plasma binds norethynodrel to the extent of 94–95%. When the same plasma was diluted to 5 times, the binding of norethynodrel to male, female and pregnancy plasma showed 88–90% binding. Plasma diluted to 20 times was able to bind about 65–72% of norethynodrel. Dilution of female plasma to 40 times showed 54% binding. At lower dilution of 1:20, the pregnancy plasma bound norethynodrel less than normal male and female plasma.

Table 1. Binding of norethynodrel to various plasma concentrations

Dilution	Per cent bound		
	Male	Female	Pregnancy
1·0	95	94	94
1:5	90	90	88
1:10	84	81	80
1:20	70	72	65
1:40	—	54	—

Plasma diluted to different concentrations was dialysed against 25 ml of phosphate buffer (pH 7·0, 0·1 M) containing radioactive norethynodrel 12,000 c.p.m.

Influence of temperature. Plasma heated at 60°C for 30 min on equilibrium dialysis with radioactive norethynodrel did not differ from control non-heated plasma. Both showed nearly 93% binding to plasma proteins.

Saturation of norethynodrel binding proteins. The kinetics of norethynodrel binding showed that at low concentrations the binding was apparently linear and steep and at high concentrations it attained a plateau indicating the presence of two classes of binding sites. The plateau values of the per cent bound/free (B/F) were subtracted from the first part of the curve. The values after subtraction was plotted gave a linear curve. The extrapolation of the first linear part of the curve gave an apparent association constant of the order of $1{\cdot}0 \times 10^{6} M^{-1}$ at 37°C and the number of binding sites was $0{\cdot}82 \times 10^{-6}$ mol/mg protein (Fig. 1).

Influence of pH on norethynodrel binding of plasma. Norethynodrel binding to plasma at pH values from 5–10 in different buffers is given in Fig. 2. Maximum binding was observed between pH 7–8. Beyond pH 8 a decrease in norethynodrel binding to plasma proteins was observed. The binding of norethynodrel to plasma proteins at 4°C was more than that of 37°C at all pH tested. The binding of norethynodrel was drastically affected at pH 8 and above.

Competitive binding of other steroids. Table 2 shows the competitive binding of progesterone and cortisol to plasma norethynodrel binding proteins. It may be seen that progesterone competes for the binding sites whereas cortisol as much as 60 μg did not show any

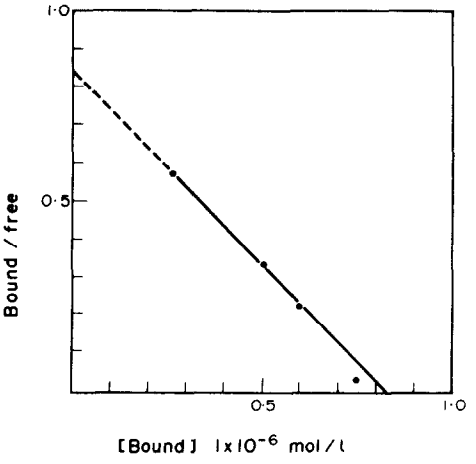

Fig. 1. Determination of number of binding sites and association constant of plasma proteins to norethynodrel. The binding of [6,7-^{3}H]-norethynodrel in the presence of increasing amount of norethynodrel to 2·5% plasma in 0·05 M phosphate buffer at 37°C was carried out. The results expressed were plotted according to Scatchard [15]. bound/free against the concentration of bound norethynodrel. The amount of protein in each dialysis bag was 1 mg/ml. On Scatchard plot the intercept on the X axis represent the number of binding site and the association constant was calculated by dividing the percent bound/free (B/F) norethynodrel by the concentration of bound norethynodrel (B).

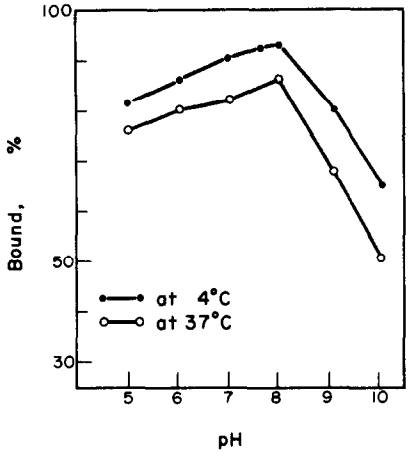

Fig. 2. Effect of pH on the binding of norethynodrel to plasma proteins.

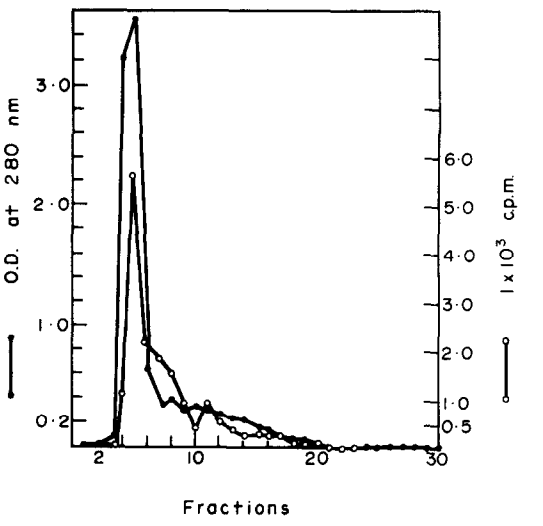

Fig. 3. [6.7-^{3}H]-norethynodrel binding to plasma protein by Sephadex G-25 Column Chromatography. Plasma (3 ml) was incubated with 1·0 μCi of [6.7-^{3}H]-norethynodrel at 37°C for 30 min. At the end of the incubation norethynodrel protein complex was layered on Sephadex G-25 column (1·0 × 28 cm) which was equilibrated with phosphate buffer 0·05 M, pH 7·4. Fractions of 3 ml were collected. Each fraction was monitored at 280 nm and 0·2 ml of aliquot was taken for determination of radioactivity.

competition. Progesterone in 20, 30 and 60 μg amounts was able to reduce norethynodrel binding from 54 to 37, 35 and 32% respectively, whereas, same amount of cortisol did not reduce the binding of norethynodrel.

Column chromatography on Sephadex G-25. Figure 3 shows elution pattern of plasma incubated with norethynodrel. The radioactivity showed an association with the protein peak. The protein and radioactivity were eluted in the void volume of the column. The superimposable protein and radioactivity peaks indicated norethynodrel binding to plasma proteins.

Column chromatography on DEAE–cellulose. The association of norethynodrel to plasma proteins is shown in Fig. 4. It may be seen that from the chromatographic column four major protein peaks were eluted. The major proteins eluted at pH 7 from the column was γ-globulin. The second protein eluted at pH 5·9 was β-globulin. The third protein peak eluted at pH 5·8 was mainly albumin and some other proteins. The fourth peak was α-globulin. It was eluted at pH 5·2. The protein-norethynodrel association was observed mainly in the third protein peak. Experiments carried out had shown that testosterone associated specific protein-sex steroid binding protein (SBP) was eluted in the β-globulin region and the progesterone specific protein was eluted in albumin-corticosteroid binding globulin (CBG) region of the column. It was found that the free radioactivity was eluted after the first protein peak (γ-globulin). Therefore, before starting the second buffer the column was eluted with sufficient amount of starting buffer.

Sephadex G-200 column chromatography. Figure 5 shows the chromatographic separation of plasma proteins on Sephadex G-200 column. A small radioactivity peak was associated with the first protein peak. The major radioactivity was associated with the third protein peak where albumin was eluted. However, it was found only on the descending shoulder of the third protein peak.

Electrophoretic pattern of norethynodrel binding protein. Serum incubated with radioactive norethynodrel on electrophoresis resolved into number of proteins. The gel was then cut into nearly 2·5 mm slices and counted. Figure 6 shows norethynodrel binding at 5th and at 14–15th fractions. The relative mobility of the norethynodrel binding protein with respect to bromophenol blue was 0·31 and albumin had the relative mobility of 0·9. The binding protein at 14–15th fraction appeared to be albumin which moved in this region of the gel.

Table 2. Effects of steroids on norethynodrel binding

Steroid μg	Per cent bound		
	Norethynodrel	Progesterone	Cortisol
0	54	54	54
20	36	38	54
30	35	35	53
60	33	32	55

Plasma diluted 1:40 (nearly 1 mg protein/per dialysis tubing) was equilibrium dialysed against 25 ml of phosphate buffer (pH 7·0, 0·01 M) containing radioactive norethynodrel 12,000 c.p.m. and the cold steroid.

DISCUSSION

The binding of steroids varies depending on the levels of the circulating estrogens. The binding of norethynodrel to pregnancy plasma was less than that

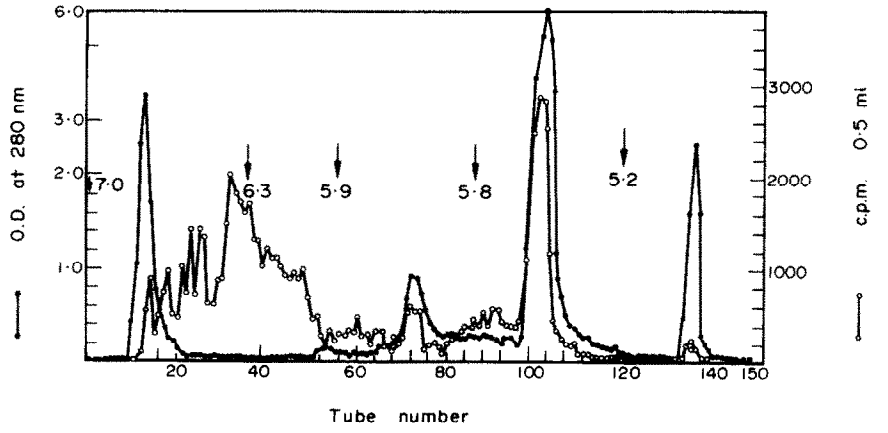

Fig. 4. Binding of [6,7-^{3}H]-norethynodrel to plasma proteins by DEAE-cellulose column chromatography.
Dialysed plasma was incubated with [6,7-^{3}H]-norethynodrel as described in "Materials and Methods" and then layered on DEAE-cellulose column (2·5 × 52 cm.) equilibrated with the starting buffer. Elution was carried out step-wise with sodium phosphate buffers: 0·005 M, pH 7·0; 0·0175 M, pH 6·3; 0·04 M, pH 5·9; 0·1 M, pH 5·8 and 0·4 M, pH 5·2. Absorbance of the effluent was monitored at 280 nm (light path 1 cm) for protein. The four peaks correspond to γ-globulin, β-globulin, albumin and α-globulin in that sequence of elution. Aliquots (0·5 ml) were taken for radioactivity counting.

to normal male and female plasma. Corticosteroid binding globulin or SBP might not have been involved in norethynodrel binding because binding of norethynodrel was lower during pregnancy than normal status. The binding of corticoids to CBG and sex steroids to SBP is known to be higher in pregnancy plasma than non-pregnancy plasma [1, 2, 16, 17]. The apparent association constant $1{\cdot}00 \times 10^6 M^{-1}$, observed for norethynodrel with plasma protein was considerably less than CBG and SBP for the specific steroids which were more than $3{-}8 \times 10^8 M^{-1}$ and thus 200 times higher than the norethynodrel association constant to plasma proteins [1, 2, 4, 6, 9, 16]. The dissociation constant observed for norethynodrel with plasma proteins was closer to the dissociation constant of α_1-acid glycoprotein (AAG) to progesterone $9 \times 10^5 M^{-1}$ and was much higher than the albumin association constant $10^4 M^{-1}$ [10, 16]. The association constant of norethynodrel to plasma proteins, the thermostability of the binding protein at 60°C and the optimum pH for norethynodrel at 7–8 were similar to AAG binding to progesterone [6, 18] and different from SBP, CBG and albumin to the specific steroids [19–22]. Thus it may be possible that CBG and SBP may not be the binding proteins for norethynodrel. Norethynodrel binding protein in plasma may be specific one which has similarities to AAG.

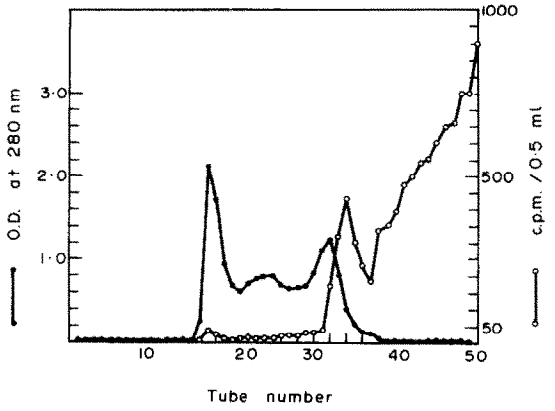

Fig. 5. Elution behaviour of norethynodrel binding protein on Sephadex G-200 column.
Plasma (3 ml) incubated with [6,7-^{3}H]-norethynodrel (0·5 μCi) was applied on Sephadex G-200 column. Elution was performed with phosphate buffer pH 7·4. Flow rate of the column was 24 ml/h. Five ml fractions were collected from the column. After measuring the optical density at 280 nm, aliquots (0·5 ml) were taken of radioactive counting.

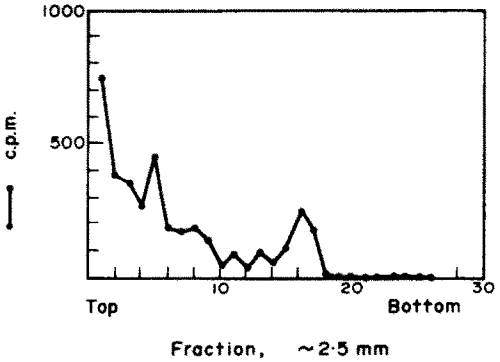

Fig. 6. Electrophoretic pattern of norethynodrel binding to plasma proteins.
Plasma (0·2 ml) was incubated with 0·5 μCi of [6,7-^{3}H]-norethynodrel and electrophoresed on 7·5% acrylamide gel at 4°C at 1 mA per gel. Gel pieces (2·5 mm) were cut, dissolved in 30% hydrogen peroxide and counted.

The steric configuration and the three-dimensional structure of the protein and the steroid determine the protein–steroid interactions. Certain groups of the steroid nucleus have been attributed for the action of steroid and the specific steroid–protein interactions [23–27]. Several investigators [25–27] have suggested that steroids having 17β-hydroxyl group would bind with SBP. Further the binding of steroids to SBP is facilitated if the axial 19-methyl group is absent in the steroid molecule [26]. However, the evidence does not suggest the possibility of SBP-norethynodrel interaction. In the steroid–protein interactions the 17α-ethynyl group of norethynodrel may be a steric hindrance for the interaction of the 17β-hydroxyl group with SBP.

In conclusion the norethynodrel binding plasma protein on the basis of association constant, competitive binding, pH, gel filtration, DEAE–cellulose ion-exchange chromatography and electrophoretic pattern appear to be a distinct protein other than albumin, CBG and SBP. Norethynodrel binding protein has similarities to AAG but confirmation of the nature of the protein would require its isolation and further characterization.

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