of AG (rat brain) has been reported[7] to produce hyperglycemia. The rather long T½ time noted in the present study (table 2) indicates that the turnover rate of AG in the body is slow, and suggests that the compound is not an energy metabolite.

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Binding of centchroman – a nonsteroidal antifertility agent to human plasma proteins[1]

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Summary. Centchroman, a non-steroidal antifertility agent showed a low affinity ($K_d = 13.19 \times 10^{-6}$ M) and nonsaturable binding to human plasma. Centchroman did not compete either with sex hormone binding globulin or corticosteroid binding globulin. Polyacrylamide gel electrophoresis and temperature dependent binding characteristics revealed that the protein responsible for centchroman binding to human plasma resembles albumin.

Contraceptive efficacy of centchroman [3,4-trans-2,2-dimethyl-3-phenyl-4 (p-pyrrolidinoethoxy-phenyl)-7-methoxy chroman hydrochloride], a non-steroidal antifertility agent, has been evaluated by Kamboj and his associates[2-3]. Centchroman binds to the estrogen receptor in rat uterus, which has been suggested as its molecular site of interaction[4]. However, the nature of centchroman binding to steroid binding proteins in plasma is yet unknown. The binding of contraceptive steroids to plasma proteins such as sex hormone binding globulin (SHBG), cortisol binding globulin (CBG) and albumin has been reported recently[5,6]. It is widely accepted that the unbound fraction of steroid in blood is biologically available to the target cells for its interaction with the receptor sites[7]. Therefore, in the present study, the binding of centchroman to steroid binding proteins in human plasma has been investigated using charcoal adsorption and polyacrylamide gel electrophoresis.

Methods. ^{14}C-centchroman (sp. act. 2.932 mCi/mmol) and unlabelled centchroman synthesized at this Institute were used. ^{3}H-Estradiol-17β, (sp. act.-110 Ci/mmol) and ^{3}H-Cortisol (sp. act.-114.5 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts, USA. Authentic steroid standards obtained by Prof. D.N. Kirk, Steroid Reference collection (Great Britain) were used. Purified human serum albumin (HSA) was procured from Sigma Chemical Co., USA.

Blood was drawn from healthy women and plasma was obtained by centrifugation at 800 × g for 15 min. To remove endogenous steroids, the plasma was treated with dextran-coated charcoal (DCC) suspension (0.5 g activated charcoal and 0.05 g of dextran/100 ml of Tris-HCl buffer, pH 7.4) for 15 min at 4 °C. Prior to the binding assay plasma was diluted (1:1) with 50 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 1 mM mercaptoethanol and 10% glycerol. 100 µl of diluted plasma (in duplicate) was incubated with ^{14}C-centchroman (2 µM to 60 µM) for 2 h at 4 °C in the absence or presence of a 1000-fold molar excess of unlabelled centchroman. Following incubation, protein-bound and free fractions were separated by addition of 500 µl of DCC suspension. After centrifugation at 800 × g for 10 min at 4 °C, the radioactivity was determined in the supernatant (bound fraction) as described earlier[7].

For competitive binding, incubations were carried out in the presence of a 1000-fold molar excess of unlabelled competitors using ^{14}C-centchroman, ^{3}H-estradiol and ^{3}H-cortisol as radioligands.

To study the effect of protein concentration on centchroman binding, the assay was carried out with diluted plasma in the ratio of 1:1, 1:5, 1:10, 1:20 and 1:50. The binding assay was carried out as described above. The effect of temperature on binding was studied by incubating centchroman with plasma at different temperatures of 4, 45 and 60 °C.

The characterization of centchroman bound complex was accomplished by polyacrylamide gel electrophoresis (PAGE). 100 µl of human serum albumin in buffer (50 mg/ml) or 100 µl of human plasma was incubated with ^{14}C-centchroman in the absence and presence of a 1000-fold molar excess of unlabelled centchroman at 4 °C and then subjected to PAGE at 4 °C as described earlier[9]. Following the run, gels were cut into 2 mm thick slices and radioactivity was assessed.

Results and discussion. Studies with ^{14}C-centchroman showed a non-saturable and low affinity binding in human plasma. The Scatchard analysis of binding data revealed a dissociation constant (K_d value) in the range of 13.19×10^{-6} M indicating low affinity and high capacity binding to human plasma (fig. 1). Incubations in the presence of unlabelled centchroman (1000-fold molar excess) failed to saturate the binding sites.

The results on binding studies in the various dilutions of human plasma revealed that ^{14}C-centchroman binding increased

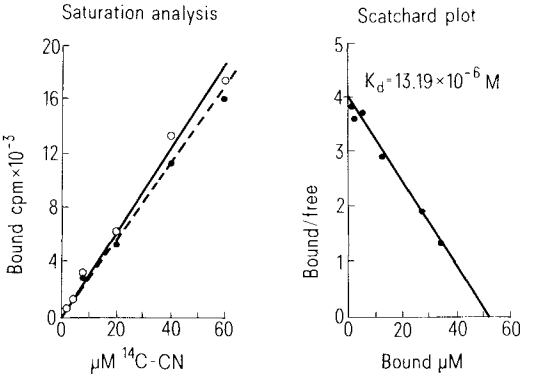

Figure 1. Saturation and Scatchard Plot analysis of centchroman (CN) binding in human plasma. 100 µl of diluted (1:1) plasma was incubated with increasing mass of ^{14}C-CN in the absence (○——○) or presence (●——●) of unlabelled CN at 4 °C for 2 h. Following incubation, bound and free CN were separated by DCC (see text).

in a linear fashion with increasing concentrations of plasma (fig. 2). These results suggest a nonsaturable nature of centchroman binding sites in plasma.

The interaction of centchroman with two major steroid binding plasma proteins, namely sex hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG), was studied. We allowed centchroman to compete with ^{3}H-estradiol-17β and ^{3}H-cortisol as markers used for characterization of SHBG and CBG respectively (table 1). It is evident that centchroman did not compete either with ^{3}H-estradiol-17β or with ^{3}H-cortisol binding sites; conversely, estradiol and cortisol failed to compete for ^{14}C-centchroman binding sites. These results indicated that centchroman does not interact with either SHBG or CBG in human plasma. Further confirmation of this observation was obtained from the temperature-dependent binding characteristics of centchroman. In the present experiment (fig. 3), the temperature of incubation had no effect on centchroman binding to plasma proteins as the binding remained unaltered at 4, 45 and 60 °C suggesting that the protein responsible for centchroman binding in human plasma is heat-stable. In contrast, SHBG and CBG are irreversibly denatured, as these proteins lose their binding capabilities at temperatures between 60 and 65 °C[10].

The characterization of ^{14}C-centchroman bound human plasma complex with PAGE resolved a single peak of radioactivity (relative mobility, Rf 0.68; fig. 4). ^{14}C-centchroman incubated with purified HSA was also subjected to PAGE under similar conditions, and showed a single peak (Rf 0.68) identical to that of plasma-bound ^{14}-C-centchroman complex, suggesting that centchroman is bound to albumin in human plasma.

We concluded from our studies that centchroman, a non-steroidal antifertility agent, binds to a protein in human plasma whose characteristics are very similar to that of albumin. This is shown in the present study by the non-saturable nature of the binding, the lack of competition with binding sites for SHBG or CBG and the fact that the binding properties are unaffected by heating human plasma up to 60 °C. In a recent study another non-steroidal antiestrogenic compound, tamoxifen, showed no significant effect in displacing steroids having an affinity for SHBG in plasma[11], whereas the compound is known to interact with estrogen receptor in the target cell[12]. The lack of centchroman binding to SHBG or CBG may be advantageous for this non-steroidal contraceptive agent, as it is unlikely to cause any displacement of steroids from specific steroid-binding plasma proteins.

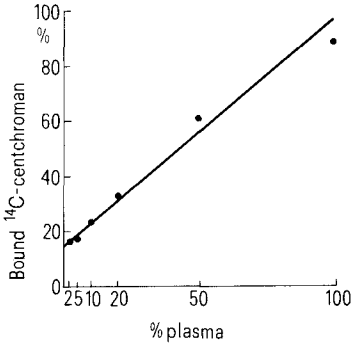

Figure 2. Effect of plasma dilution of ^{14}C-CN binding. 100 µl of plasma diluted in the ratio of 1:1, 1:5, 1:10, 1:20 and 1:50 with buffer was incubated with 2 µM ^{14}C-CN at 4 °C. Following incubation bound and free CN were separated by DCC. Details in text.

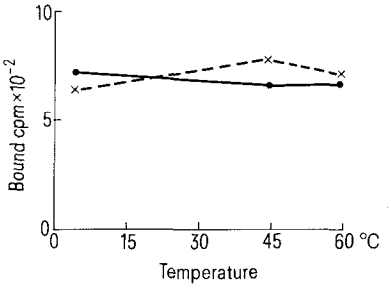

Figure 3. Temperature-dependent binding characteristics of ^{14}C-CN. 100 µl of plasma (diluted 1:4) was subjected to treatment for 30 min at 4, 45 and 60 °C. Tubes were allowed to cool at 4 °C and then incubated with ^{14}C-CN in the absence (●——●) or presence (×-----×) of unlabelled CN, as usual.

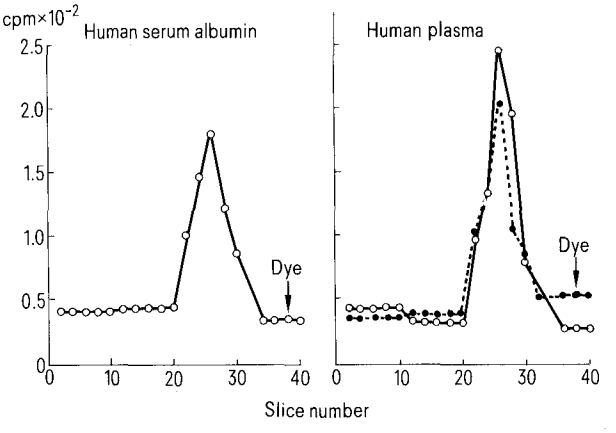

Figure 4. PAGE profile of centchroman binding to HSA and human plasma. ^{14}C-CN was incubated with HSA or human plasma for 2 h at 4 °C in the absence (○——○) or presence (●------●) of unlabelled CN. Following incubation, ^{14}C-CN bound HSA or plasma complex was briefly treated with DCC to remove free ^{14}C-CN. The bound complex was then subjected to PAGE. Bromophenol blue was used as a marker to calculate the relative mobility.

Radioligand specificity of centchroman binding to human plasma

Competitor	Competing efficiency ^{14}C-centchroman	^{3}H-Estradiol-17β	^{3}H-Cortisol
nil (no competitor)	0	0	0
Centchroman	0	0	0
Cortisol	0	25.32	100
Estradiol-17β	0	100	2.0735

100 µl of Human plasma (diluted 1:4) was incubated with 2 µM ^{14}C-CN, 5 nM ^{3}H-estradiol-17β and 5 nM ^{3}H-cortisol, in the absence or presence of 1000-fold molar excess of unlabelled competitor, for 2 h at 4 °C. Binding was assessed as described in text.

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