Serum non-protein bound percentage and distribution of the progestin ST-1435: no effect of ST-1435 treatment on plasma SHBG and CBG binding capacities

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Abstract. The non-protein bound percentage of ST-1435 was measured by centrifugal ultrafiltration-dialysis in undiluted female serum at 37°C. It was found that as much as 13% of ST-1435 in serum is not bound to proteins. The results also show that the affinities of SHBG and CBG of ST-1435 are very low and that SHBG and CBG do not bind ST-1435 under physiological conditions in serum. We suggest that ST-1435 is bound mainly to serum albumin, and that the binding to that accounts for more than 87% of total serum ST-1435 concentrations. During the treatment period of 3 months, no change in SHBG or CBG binding levels was observed when ST-1435 was administered parenterally.

The lack of any interaction between ST-1435 and high affinity serum steroid binding proteins, and the very high percentage of non-protein bound ST-1435 in serum, probably explain its extremely high biological potency at the hypothalamic-pituitary level, when compared for example with d-norgestrel. For the same reason, practically all the ST-1435 in hepatic portal blood is probably taken-up and very rapidly metabolised by the liver. This may explain why oral administration of ST-1435 results in low and inadequate plasma concentrations for contraceptive purposes, while alternative parenteral routes of administration result in relatively much higher serum concentrations of the steroid. Finally, because ST-1435 does not bind to SHBG and CBG under physiological conditions, and does not change plasma SHBG and CBG binding capacities, ST-1435 treatment will not indirectly alter the amounts of endogeneous sex steroid hormones or their distribution in plasma. Therefore, side effects such as acne or hirsutism are unlikely to develop as a result of sustained administration of this potent progestin.

ST-1435 is a 19-norpregesterone derivative which has a potent anti-ovulatory action, mediated by the suppression of pituitary LH production (Lähteenmäki et al. 1981). Although inactive when taken orally (Coutinho et al. 1981), it has proven to be very effective as a progestin-only contraceptive when administered parenterally in sc capsules.

1 Trivial and systematic steroid nomenclature:
ST-1435 = 16-methylene-17α-acetox-19-nor-4-pregnene-3,20-dione;
d-norgestrel = levonorgestrel =
13β-ethyl-17α-ethynyl-17β-hydroxy-gon-4-en-3-one;
DHT = dihydrotestosterone =
17β-hydroxy-5α-androstan-3-one;
cortisol = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione;
ethynoestradiol = 17α-ethynyl-1,3,5(10)-oestratriene-3,17β-diol;
norethisterone = 17α-ethynyl-17β-hydroxy-oestr-4-en-3-one;
cyproterone acetate = 17-acetoxy-6-chloro-1α,2α-methylene-pregna-4,6-diene-3,20-dione.

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(Coutinho et al. 1976), and results in pregnancy rates of less than 0.5 pregnancies per 100 women years of use (Pearl index, 0.5), during treatment periods of more than 1 year (Coutinho et al. 1976).

We have recently developed a radioimmunoassay for the measurement of ST-1435 in blood samples (Lähteenväki et al. 1981), in order to monitor the pharmacokinetics and efficacy of the steroid during clinical trials as a contraceptive. However, in view of the opinion that only non-protein bound steroids in blood are able to enter target cells (Brien 1981; Anderson 1974), measurements of total steroid concentrations alone may not provide an adequate evaluation of biological potency. It has been our intention therefore to determine to what extent ST-1435 interacts with the major serum steroid binding proteins (sex hormone binding globulin (SHBG), corticosteroid binding globulin (CBG) and albumin), and to evaluate the distribution of ST-1435 between these proteins and the non-protein bound fraction in serum under conditions which mimic the physiological situation.

The administration of another contraceptive 19-norprogestin, d-norgestrel, results in a decrease in the serum binding capacity of SHBG (van Kammen et al. 1975). This is probably due to a decrease in SHBG synthesis mediated by the inherent androgenic nature of this, and other, synthetic progestins (Bullock & Bardin 1977). This reduction in serum SHBG binding capacity undoubtedly causes increases in serum non-protein bound testosterone and 5α-dihydrotestosterone (DHT) concentrations (Anderson 1974), which may in part account for androgenic side-effects (acne and hirsutism) occasionally observed after the administration of d-norgestrel. We therefore also considered it important to establish whether or not the continuous administration of ST-1435 to normal women, over a period of several months, has any influence on plasma binding capacities of SHBG and CBG.

**Materials and Methods**

**Materials**

Unlabelled ST-1435 and [3H]ST-1435 (Merck AG, Darmstadt, FDR) were kindly supplied by the Population Council, New York. Tritiated ST-1435 was purified twice immediately prior to use on thin-layer chromatography (TLC) plates (13179 Silica Gel, Eastman Kodak, Rochester, USA), using chloroform: acetone (12:1) as the solvent system, and was estimated to be > 98% pure. The specific activity (4.8 Ci/mmol) of the purified [3H]ST-1435 was determined by measuring the concentration of [3H]ST-1435 by a radioimmunoassay in which [125I]ST-1435 was used as the labelled ligand (Lähteenväki et al. 1981). [1,2-3H]cortisol (53.3 Ci/mmol) and [1,2-3H]5α-dihydrotestosterone (50.6 Ci/mmol) were purchased from New England Nuclear (NEN) Chemicals GmbH (Dreieich, FDR) and purified by Lipidex-5000™ (Packard Becker, B.V., Groningen, The Netherlands) column chromatography, using petroleum ether (b.p. 66–69°C) and chloroform as the elution solvents. Levo-norgestrel (d-norgestrel, Schering AG, Berlin, FDR), cortisol and 5α-dihydrotestosterone (Steraloids, Inc., Wilton, N.H., USA) were used without further purification. [14C]glucose (13.9 mCi/mmol) was obtained from NEN and was stored and used as supplied.

**Blood samples**

A normal follicular phase serum was used to study the relative affinities of SHBG and CBG for ST-1435 and d-norgestrel. Blood samples (2 follicular and 1 luteal phase) from normal women were taken for the determination of the percentage of non-protein bound ST-1435, and distribution of ST-1435 between various steroid binding proteins in serum. Plasma SHBG and CBG binding capacities were measured from blood samples taken from 6 healthy women treated with either ST-1435 sc capsules (n = 5) or an ST-1435 intracervical device (n = 1). Samples were collected prior to the initiation of treatment, and once a month for 3 months thereafter. All serum and plasma samples were stored at −20°C until assayed.

**Determination of the percentage of non-protein bound ST-1435 in serum**

The equipment required for the centrifugal ultrafiltration-dialysis method used to determine the percentage of non-protein bound ST-1435 in undiluted serum has been described in detail (Hammond et al. 1980). Serum samples (650 µl) were incubated (1 h at 37°C with 52.6 pmol of the purified [3H]ST-1435, together with approximately 15 000 DPM [14C]glucose. The exact concentrations of radiolabelled compounds are not important since only isotope ratios are used in subsequent calculations. Triplicate aliquots (200 µl) of these incubations were then placed in centrifugal ultrafiltration vials and centrifuged at 3000 x g for 1 h, at 37°C. The percentage of non-protein bound ST-1435 was determined by the ratio of [3H]ST-1435: [14C]glucose in the ultrafiltrate, divided by the corresponding ratio in the serum retained by the dialysis membrane.

In order to assess any possible degradation of [3H]ST-1435 as a result of 37°C incubations prior to, and during centrifugation, 350 µl of serum retained by the membranes after centrifugal ultrafiltration-dialysis was extracted with 2 ml of ether, and the extract was run alongside non-labelled ST-1435 on a 13179 Silica Gel
TLC plate, as described above. The radioactivity moved as one spot with an Rf value (0.8) identical to that of unlabelled ST-1435, and the purity of the [3H]ST-1435 extracted was estimated to be more than 98%. Thus, no detectable destruction of [3H]ST-1435 occurred during the incubations. This was important to ascertain, because impurities constitute a major source of error in the determination of non-protein bound steroids.

Distribution of ST-1435 between protein and non-protein bound fractions

The centrifugal ultrafiltration-dialysis assay was performed on each of the samples after heat-treating the serum (1 h at 60°C) to destroy heat-labile, high-affinity binding proteins (SHBG and CBG). In this way, it is possible to determine whether these binding proteins regulate the amount of non-protein bound ST-1435 in blood, and to also assess the relative binding of ST-1435 to heat-stable steroid binding proteins (i.e. albumin and α1-acid glycoprotein) in serum (Hammond et al. 1982).

Assays of SHBG and CBG binding capacity

In brief, 20 µl of plasma was incubated (30 min at room temperature with intermittent mixing) with 2 ml dextran-coated charcoal (DCC) solution (1.25 g Norit A and 0.125 g Dextran-T-70 in 500 ml 0.1 M phosphate buffered saline with 0.1% gelatin (PBS) pH 7.4) to remove endogenous steroids. The mixture was then centrifuged (2000 × g, 10 min) to sediment the DCC. The resulting supernatant (1:100 dilution) was then taken for the SHBG assay, and further diluted (1:5) in PBS for the CBG assay. Duplicate aliquots (100 µl) of the respective serum dilutions were then added to dried tubes containing 3H-labelled steroids (1 pmol [3H]5α-dihydrotestosterone + 200 pmol cortisol for the SHBG assay, and 1 pmol [3H]cortisol for the CBG assay) which represent total binding activity, and also to tubes containing a 200-fold excess of the respective non-radiolabelled steroids in addition to the radiolabelled steroids to determine non-specific binding (NSB) activity. After vortex mixing, the tubes were incubated at room temperature for 1 h, and then at 0°C for 15 min before addition of 900 µl of DCC solution at 0°C. After 10 min at 0°C the tubes were centrifuged at 3000 × g for 3 min. Aliquots (650 µl) of the supernatant were then counted in a liquid scintillation spectrometer in vials containing 5 ml RiaLuma (LKB/Wallac, Turku, Finland). The concentration of specifically bound 3H-labelled steroid was calculated by subtracting NSB from total binding activity, and extrapolated to zero serum dilution to give binding capacity in pmol/ml. A correction factor of +12% was made for CBG measurements to account for a 12% loss in total binding which occurs during the separation of CBG bound from non-bound cortisol. All samples were measured in the same assays for SHBG and CBG, and the precision (CV%) of the measurements were < 10%.

Assessment of ST-1435 and d-norgestrel binding to SHBG and CBG

Competition studies were conducted to determine the relative affinities of SHBG and CBG for ST-1435 and d-norgestrel, by using 5α-dihydrotestosterone (DHT) and cortisol as the respective reference ligands for these two binding proteins. A normal female plasma was diluted with 0.1 M PBS (1:100 for SHBG and 1:500 for CBG), and duplicated aliquots (100 µl) were incubated (1 h at 25°C) with 1 pmol of [3H]DHT (for SHBG) or [3H]cortisol (for CBG) and increasing concentrations of ST-1435, d-norgestrel or the corresponding unlabelled reference steroids. Thereafter, the method used was similar to that described for the measurement of SHBG and CBG binding capacities.

Results

Percentage of non-protein bound ST-1435 and its distribution in serum

The percentages of non-protein bound ST-1435 measured at 37°C in undiluted serum samples from 3 normal women were all very similar (i.e.
10.6%, 12.9% and 14.3%), and the mean ± sd percentages of non-protein bound ST-1435 in these sera before (12.6 ± 1.9%) and after heat-treatment at 60°C for 1 h (12.3 ± 1.5%) were practically identical. Thus under physiological conditions, it appears that heat-labile serum steroid binding proteins (SHBG and CBG) play no role in regulating non-protein bound concentrations of ST-1435 in blood. This is also supported by the competition studies which compare the relative binding affinities of SHBG and CBG for ST-1435 and d-norgestrel in normal female serum (Fig. 1). As expected 1 pmol of unlabelled DHT and cortisol displace approximately 50% of the [3H]DHT and [3H]cortisol in the SHBG and CBG competition binding assays, respectively. The SHBG competition curves clearly demonstrate that increasing concentrations of unlabelled ST-1435 (up to 100 000-fold excess) do not displace any of the [3H]DHT bound with high affinity to SHBG, while the SHBG binding affinity for d-norgestrel is about 10% of the relative affinity of SHBG for DHT.

According to the CBG competition curves for ST-1435 and d-norgestrel, relative to that of cortisol, it seems that the interactions between these progestins and CBG is very weak: ST-1435 and
d

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<th>SHBG pmol/ml</th>
<th>CBG pmol/ml</th>
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* Concentration of ST-1435 in capsules was 1.33 mg/mm.
** Sample taken prior to initiation of treatment.
d-norgestrel only displaced 49% and 58% of the [3H]cortisol bound to CBG, when 100 000-fold excess of the unlabelled steroids was used. These data therefore indicate that ST-1435 is bound almost exclusively by heat-stable binding components in serum, the binding to which accounts for more than 87% of total serum ST-1435 concentrations.

Effect of ST-1435 treatment on plasma SHBG and CBG binding capacities

The binding capacities of SHBG and CBG were determined in plasma taken from 6 women before and at 3 monthly intervals during treatment with ST-1435. All the treatment cycles were anovulatory as assessed by serial determinations of plasma progesterone concentrations (data not shown), which indicates that plasma levels of ST-1435 were high enough for contraceptive purposes. When administered as sc implant the plasma concentrations of ST-1435 are known to fluctuate (Lähteenmäki et al. 1982), and because of this the mean plasma concentrations of ST-1435 were calculated from several blood samples drawn throughout each treatment months (Table 1). In contrast, the plasma SHBG and CBG binding capacities in individual subjects show practically no variation with respect to time of sampling. Therefore, plasma SHBG and CBG binding capacities were measured in blood samples taken just prior to the treatment and at monthly intervals thereafter (Table 1). These results demonstrate that treatment with various doses of ST-1435, which result in different plasma levels of ST-1435, has no effect on the plasma SHBG or CBG binding capacities in any of the subjects over the 3-month treatment period investigated.

Discussion

In this investigation we have determined the proportion of non-protein bound ST-1435 in serum, and assessed the interaction between this potent progestin and serum steroid binding proteins. It was found that as much as 13% of ST-1435 in serum is not bound to proteins. This is much greater than the serum non-protein bound percentages of many natural steroid hormones (Dunn et al. 1981) and most of the commonly used contraceptive steroids (Hammond et al. 1982). In the latter study, it was reported that norethisterone and d-norgestrel bind well to both SHBG and albumin, while cyproterone acetate and ethinylestradiol are bound only by albumin. The present data indicate that ST-1435 does not bind to SHBG and CBG under physiological conditions, and this is substantiated by the fact that the percentage of non-protein bound ST-1435 does not change if high affinity binding proteins in serum are destroyed by heat-treatment. Thus, like cyproterone acetate and ethinylestradiol, the main binding of ST-1435 in serum appears to be a heat-stable component, and it is most likely that this is almost entirely attributable to serum albumin, because α1-acid glycoprotein, which is also heat-stable, has only limited binding capacity and is present in much lower concentrations when compared to albumin (Westphal 1971).

When the lowest plasma concentrations of ST-1435 and d-norgestrel which inhibit ovulation are compared as indicators of their biological activities at the level of the hypothalamo-pituitary-ovarian axis, it is possible to estimate their relative potencies in vivo. Plasma concentrations above 20 pg ST-1435/ml (55 nmol/l) are sufficient to cause a constant inhibition of ovulation (Lähteenmäki et al. 1982), whereas 150 pg d-norgestrel/ml (480 nmol/l) does not inhibit ovulation in all subjects (Nilsson et al. 1980). Thus, in terms of total plasma concentrations, ST-1435 appears to be at least seven times more potent than d-norgestrel in this respect. However, the present data indicate that the non-protein bound percentage of ST-1435 is more than 5 times greater than that of d-norgestrel (Hammond et al. 1982). This may therefore account for the greater biological potency of ST-1435 as compared to d-norgestrel. It therefore seems that relationship exists between the non-protein bound percentages of these two progestins and their relative in vivo biological potencies. This complements the observations of Raynaud et al. (1973) who investigated the interactions between several natural and synthetic oestrogens and serum binding proteins, with respect to their biological activities in the immature rat uterus. They concluded that plasma binding proteins modulate the activity of oestrogens, and that only the non-protein bound steroids are active on the uterus.

Despite the elegant design of the latter study, the debate continues as to which proportion of total plasma steroids actually enters target cells, particularly as recent evidence indicates that albumin bound steroids, in addition to non-protein bound steroids, are taken-up by the rat brain under in vivo
conditions (Pardridge 1981). Whether this also occurs in other target tissues, such as the uterus, which may differ markedly from the brain in terms of their capillary transit time, lipid composition and permeability of cell membranes, and intracellular steroid receptor titers is not known. However, it should be noted that the proportion of ST-1435 bound to albumin in female serum (87%) is also approximately twice as great as the corresponding proportions of albumin bound d-norgestrel (50%) (Hammond et al. 1982). Furthermore, when comparing the percentages of non-protein bound ST-1435 (12.3%) and d-norgestrel (4.6%) (Hammond et al. 1982) in heat-treated serum from normal women, it appears that the relative affinity of albumin for ST-1435 is also considerably less than for d-norgestrel. Thus, even if the albumin bound fraction is available for uptake by a specific target cell, the data also indicate that practically all of the ST-1435 in plasma will enter cells as opposed to less than half of the total plasma concentrations of d-norgestrel. In this respect, the liver has also been shown to readily take-up albumin bound as well as non-protein bound steroids (Pardridge 1981), and therefore practically all the ST-1435 in the hepatic portal blood may be very rapidly taken up and metabolised by the liver. This may explain why oral administration of ST-1435 results in very low plasma ST-1435 concentrations, while alternative parenteral routes of administration result in a sustained supply, of much higher concentrations of the steroid, which are adequate for contraceptive purposes.

It is well known that androgens and synthetic progestin derivatives of 19-nortestosterone (e.g. d-norgestrel) decrease the binding capacity of SHBG, and that this amplifies endogenous androgen activity via a concomitant increase in the non-protein bound fraction of plasma testosterone (Anderson 1974; van Kammen et al. 1975). In some cases this alteration in the normal distribution of androgens in blood is so severe that it is manifest by the onset of acne and hirsutism (Rosenfield 1971; Lawrence et al. 1981). In contrast, the use of synthetic oestrogens, such as ethinylestradiol results in an apparent induction of SHBG synthesis which is reflected by an increase in serum SHBG binding capacity (Weiner & Johansson 1976). These changes in SHBG binding capacity in serum induced by oestrogenic and androgenic steroids, occur over a similar time period of about 2–3 weeks (Victor & Johansson 1977). During the treatment period of 3 months, no change in plasma SHBG or CBG binding capacities was observed when ST-1435 was administered parenterally. This, together with the evidence that ST-1435 does not bind to high-affinity serum steroid binding protein, suggests that its administration as a contraceptive steroid will probably not alter the proportions of non-protein bound endogenous steroid hormones or their distribution in plasma.

Acknowledgments

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Dunn J F, Nisula B C & Rodbard D (1981): Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and cortico¬


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