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PROTEIN BINDING OF THE CONTRACEPTIVE STEROIDS GESTODENE, 3-KETO-DESOGESTREL AND ETHINYLESTRADIOL IN HUMAN SERUM

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Summary—The protein binding of ethinylestradiol (EE_2), gestodene (GEST) and 3-keto-desogestrel (KDG) has been determined by ultrafiltration in the serum of women who had either taken a gestodene ($n = 37$) or desogestrel ($n = 28$) containing oral contraceptive for a time period of at least 3 months. GEST and KDG were analyzed in individual serum pools whereas EE_2 was repeatedly measured in two serum pools, each one representing one treatment group. The respective free fractions of the three steroids were $0.6 \pm 0.1\%$ (GEST), $2.5 \pm 0.2\%$ (KDG), $1.7 \pm 0.6\%$ (EE_2, in the gestodene-group) and $1.5 \pm 0.2\%$ (EE_2, in the desogestrel-group). EE_2 was exclusively bound to albumin, whereas GEST and KDG were also bound to sex-hormone-binding globulin (SHBG). The distribution of the two progestins over the serum binding proteins was determined after heat-treatment of serum samples. For GEST, the contribution of albumin and SHBG was 24.1 ± 9.1 and $75.3 \pm 9.1\%$, respectively and for KDG it was 65.9 ± 11.9 and $31.6 \pm 12.0\%$, respectively. SHBG and corticosteroid-binding globulin (CBG) concentrations were measured in the serum samples obtained from both treatment groups. In the gestodene-group 180 ± 61 nmol/l (SHBG) and 89 ± 13 mg/l (CBG) were measured, the corresponding values in the desogestrel-group were 226 ± 64 nmol/l (SHBG) and 93 ± 14 mg/l (CBG).

SHBG concentrations were correlated with the total concentration of GEST and its free fraction and a positive ($r = 0.395$) and negative ($r = -0.491$) correlation respectively was found. Only a weak negative correlation ($r = -0.291$) was found for SHBG and the free fraction of KDG in the serum. These data demonstrate that the three contraceptive steroids EE_2, GEST and KDG were all bound extensively to serum proteins, however, with pronounced differences concerning their distribution over the various binding proteins.

INTRODUCTION

Gestodene and desogestrel are two synthetic progestins which are used in combination with ethinylestradiol as oral contraceptives.

The administration of synthetic steroids can cause pronounced alterations in some of the proteins which are present in serum, particularly the concentrations of sex-hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG). Ethinylestradiol (EE_2) will cause a dose-related increase in both SHBG and CBG concentration in serum. The extent of this increase seems to depend not only on the absolute dose of EE_2, but also on the nature of the coadministered progestin and the dose ratio of EE_2 and progestin [1–3].

In serum, SHBG and albumin are the major binding proteins for contraceptive steroids, whereas the contribution of CBG does not seem to be significant [4, 5]. The distribution of a particular steroid between the different binding proteins may have consequences for the drug's free fraction, its distribution in the body, uptake into target cells and its metabolic conversion.

In the present study an ultrafiltration technique was employed to determine the fraction unbound of the two progestins gestodene (GEST) and 3-keto-desogestrel (KDG), the latter being the pharmacologically active metabolite of desogestrel, as well as the fraction unbound of EE_2 in the serum of women who had been taking monophasic oral contraceptives containing either GEST or desogestrel for at least 3 months. In addition, by determination of the unbound fraction of each of these steroids in native as well as in heat-treated serum, the distribution of the protein-bound drug between albumin and SHBG was calculated.

EXPERIMENTAL

Subjects and samples

The design of the human study is described in detail elsewhere [6]. Altogether 69 healthy women

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were evaluated in the study and all of them were already under established treatment with either of two oral contraceptives. One group ($n = 39$) had been taking a preparation containing 75 μg gestodene and 30 μg EE_2 for 11 ± 5 months, the other group ($n = 30$) had been taking a preparation containing 150 μg desogestrel and 30 μg EE_2 for 38 ± 24 months. For the determination of serum protein binding, however, only 37 women in the first group and 28 in the latter group could be included because from two women of each group serum had been completely used for other analyses [6].

Blood samples were drawn from all subjects immediately before and at regular time intervals after intake of the respective oral contraceptive on one day between day 10 and 21 of the cycle. Blood was kept at 4°C until coagulation, the serum was separated and stored at −18°C until analysis.

Serum samples which were obtained from individual subjects up to 4 h post administration, were combined in such a way that the pool represented the average concentration of each of the drugs (GEST, KDG) over the sampling period of 4 h. For the determination of the fraction unbound of EE_2, aliquots of these individual pools were combined to form one serum pool for each treatment group and each of the two pools was analyzed 6-fold.

Radiolabeled compounds

[9,11-^{3}H]gestodene (2.05 TBq/mmol) and [15,16-^{3}H] 3-keto-desogestrel (1.67 TBq/mmol) were available (Isotope chemistry, Schering AG, F.R.G.) and purified immediately prior to use by HPLC on a reversed-phase system (column: Spherisorb ODS II, 3 μm, 250 × 4.6 mm; mobile phase: acetonitrile/water, 60/40 v/v at a flow rate of 1 ml/min). [6,7-^{3}H] ethinylestradiol (2.19 TBq/mmol) was purchased from NEN (Dreieich, F.R.G.) and its radiochemical purity was also checked by HPLC. The purity of the labeled steroids was ⩾99% in all three cases.

Ultrafiltration of serum samples

The MPS-1 ultrafiltration unit of Amicon (Danvers, U.S.A.) was used. Nonspecific binding of radiolabel to the ultrafiltration unit was tested by ultrafiltration of buffer-solutions, which only contained the pure radiolabel. About 23% (EE_2), 16% (GEST) and 27% (KDG) of the tracer activity was adsorbed to the filtration device.

250 μl of serum were spiked with 5×10^4 cpm of the labeled steroid and the sample was then equilibrated for 1 h at 37°C. Subsequently 150 μl of the sample were transferred into a MPS-1 unit and centrifuged in a pre-warmed (37°C) fixed angle rotor for 15 min at 1500 *g* (Heraeus-Christ, type 5915 centrifuge). Aliquots (50 μl) of the serum sample and the ultrafiltrate were each mixed with 10 ml of scintillation cocktail (Atomlight, New England Nuclear) and the total radioactivity was counted in a liquid scintillation counter (Betaszint, Berthold, F.R.G.). The unbound fraction of the respective steroid was calculated as quotient of radiolabel in the filtrate and the serum, respectively and was not corrected for nonspecific binding.

Distribution of steroids in serum

The free fraction of the steroids was determined in native and heat-treated serum. Heat treatment of serum samples and the calculation of the distribution of the steroids between albumin and the heat-labile proteins (SHBG, CBG) were performed according to a published procedure [5].

Determination of total gestodene in serum

Total concentrations of GEST in serum were determined with a specific radioimmunoassay and [9,11-^{3}H] gestodene was used as tracer. The antiserum (Schering) was obtained by immunization of rabbits with the immunogen GEST 3-*O*-(carboxymethyl)-oxime-BSA, the dilution was 1:200,000 in the assay. Serum samples were extracted with diethylether and the extracts were submitted to radioimmunological analysis analogous with a published procedure [7].

Determination of total protein, albumin and globulin fraction and of SHBG, CBG in serum

Total protein content of the serum samples was determined by Biuret-reaction and the relative proportions of the albumin- and the globulin fraction were obtained by quantitative electrophoresis on cellulose acetate gels and subsequent staining with amidoblack. SHBG and CBG concentrations in pooled serum samples were measured radioimmunologically with two commercially available assay-kits (SHBG: Diagnostic Products Corp., Los Angeles, U.S.A.; CBG: IRE Medgenix, Fleurus, Belgium).

RESULTS

Data on the mean percentages of non-protein bound GEST, KDG and EE_2 in serum of women who had either taken a gestodene or desogestrel containing oral contraceptive for an extended period of time are presented in Table 1 together with the proportion of these steroids bound to albumin and SHBG.

The unbound fraction of GEST was only 0.6 ± 0.1% and the contribution of SHBG and albumin to serum protein binding was 75.3 ± 9.1 and 24.1 ± 9.1%, respectively. For KDG an unbound fraction of 2.5 ± 0.2% was found and the contribution of SHBG and albumin to the total protein binding in serum amounted to 31.6 ± 12.0 and 65.9 ± 11.9%, respectively. The free fraction of EE_2 was measured 6-fold in both pools of the treatment groups and was found to be 1.7 ± 0.6 and 1.5 ± 0.2%, respectively. Protein binding was exclusively due to albumin and the percentage bound was 98.3 ± 0.6 and 98.5 ± 0.2%, respectively. The contribution of SHBG, if there was any, was below the limit of detection of the method used.

Table 1. Free and protein-bound fractions of three synthetic steroid hormones (EE_2, GEST, KDG) in the serum of women who had taken either a gestoden- or a desogestrel containing oral contraceptive over a period of 11 ± 5 and 38 ± 24 months, respectively. Concentration of serum proteins in the same samples

Compound	Fraction unbound (%)	Protein bound fraction (%) Albumin	SHBG	Total protein (g/l)	Albumin fraction (%)	Globulin fraction (%)	SHBG nmol/l	CBG (mg/l)
EE_2^a	1.7 ± 0.6	98.3 ± 0.6	—	—	—	—	—	—
EE_2^b	1.5 ± 0.2	98.5 ± 0.2	—	—	—	—	—	—
GEST	0.6 ± 0.1	24.1 ± 9.1	75.3 ± 9.1	0.71 ± 0.04	62 ± 3	38 ± 1	180 ± 61	89 ± 13
KDG	2.5 ± 0.2	65.9 ± 11.9	31.6 ± 12.0	0.71 ± 0.04	62 ± 3	39 ± 1	226 ± 64	93 ± 14

Determined in the group of women taking the gestodene containing[a] and the group taking the desogestrel containing[b] oral contraceptive. Each serum pool was analyzed 6-fold.

SHBG and CBG concentrations were measured in the serum samples obtained from both treatment groups and amounted to 180 ± 61 nmol/l and 89 ± 13 mg/l in one group (gestodene) and to 226 ± 64 nmol/l and 93 ± 14 mg/l in the other group (desogestrel) (Table 1). SHBG concentrations in the serum samples were correlated with total concentrations and the free fractions of GEST (Fig. 1) and the correlation coefficients were found to be $r = 0.395$ and $r = -0.491$, respectively. When free KDG fractions were correlated with the respective SHBG concentrations, a correlation coefficient of $r = -0.291$ was calculated (Fig. 2).

DISCUSSION

In the present study, serum samples of 65 women who had taken either a gestodene or desogestrel containing oral contraceptive were collected and the protein binding of the two progestins GEST and KDG as well as of the estrogenic steroid EE_2 was determined by an ultrafiltration technique. In order to closely simulate a physiological environment, ultrafiltration was performed at 37°C because it has been demonstrated [8, 9] that temperature is a critical factor with respect to the free fraction of steroids in serum samples. Another precaution which had been taken was the use of serum instead of plasma, as it has been shown for a number of drugs as well as for endogenous steroids [10, 11] that the free fatty acids which are present in plasma due to heparin-induced lipase activity, can effectively complete for the same binding sites and eventually cause an increased free fraction of the steroid. Finally nonspecific binding of the radiolabeled steroids to the ultrafiltration unit was investigated. Although pure tracer activity was partly adsorbed to the filtration device, this was not considered as a serious restriction, because in actual serum samples a large excess of the unlabeled drug, endogenous steroids and matrix constituents will compete for the same binding sites on the surface of the device and therefore any noticeable effect on the determination of the drug's free fraction will be rather unlikely. Obviously, there were no differences in the mean percentages of unbound EE_2 in the serum of women belonging to either of the two treatment groups. Furthermore it is evident that the fraction unbound did not change after heat treatment of the samples, thus confirming the observation already reported by others [5, 12] that EE_2 is more or less exclusively bound to albumin. An increase of the SHBG concentration in the serum, caused by EE_2 induced hepatic synthesis of SHBG in oral contraceptive users does therefore neither change the free fraction of EE_2 nor its distribution between albumin and SHBG. This was also confirmed by others [5] who found no difference between the free fraction of EE_2 in serum of non-pregnant women and women in the third trimester of pregnancy.

A different situation as regards the distribution between the binding proteins in serum was observed for the two progestins. More than 99% of GEST were protein-bound, mainly to SHBG and less extensively to albumin. GEST binds to SHBG with a relative high affinity (40% relative to 5α-dihydrotestosterone) [13] and one would expect not only an influence on the fraction unbound but also on the total drug concentration in serum. However, only a weak negative (SHBG vs drug unbound $r = -0.491$), respectively positive correlation (SHBG vs total drug concentration $r = 0.395$) was obtained (Fig. 1). This is probably due to the fact that in the present study the women had already taken the GEST/EE_2-containing oral contraceptive for several months and therefore they all exhibited high serum levels of SHBG, covering only a narrow range. Only recently, in another study, female volunteers were treated with the same oral contraceptive over a period of 12 months and the serum concentrations of GEST and SHBG were monitored throughout the whole treatment period [14]. A wider range of GEST and SHBG concentrations could be evaluated in this study and in fact a close correlation ($r = 0.749$) of the two parameters was reported by the authors.

In contrast to GEST, KDG was mainly bound to albumin and to a lesser extent to SHBG. Also the observed free fraction was somewhat higher for KDG as compared to GEST and the free fraction of KDG showed only a poor correlation (Fig. 2) with the SHBG-levels in the serum ($r = -0.291$). A similarly weak correlation of KDG total concentrations in the serum ($r = 0.339$), even over a relatively wide range of SHBG concentrations has been reported recently [14]. All of these results are quite in agreement with the relatively low binding affinity of KDG

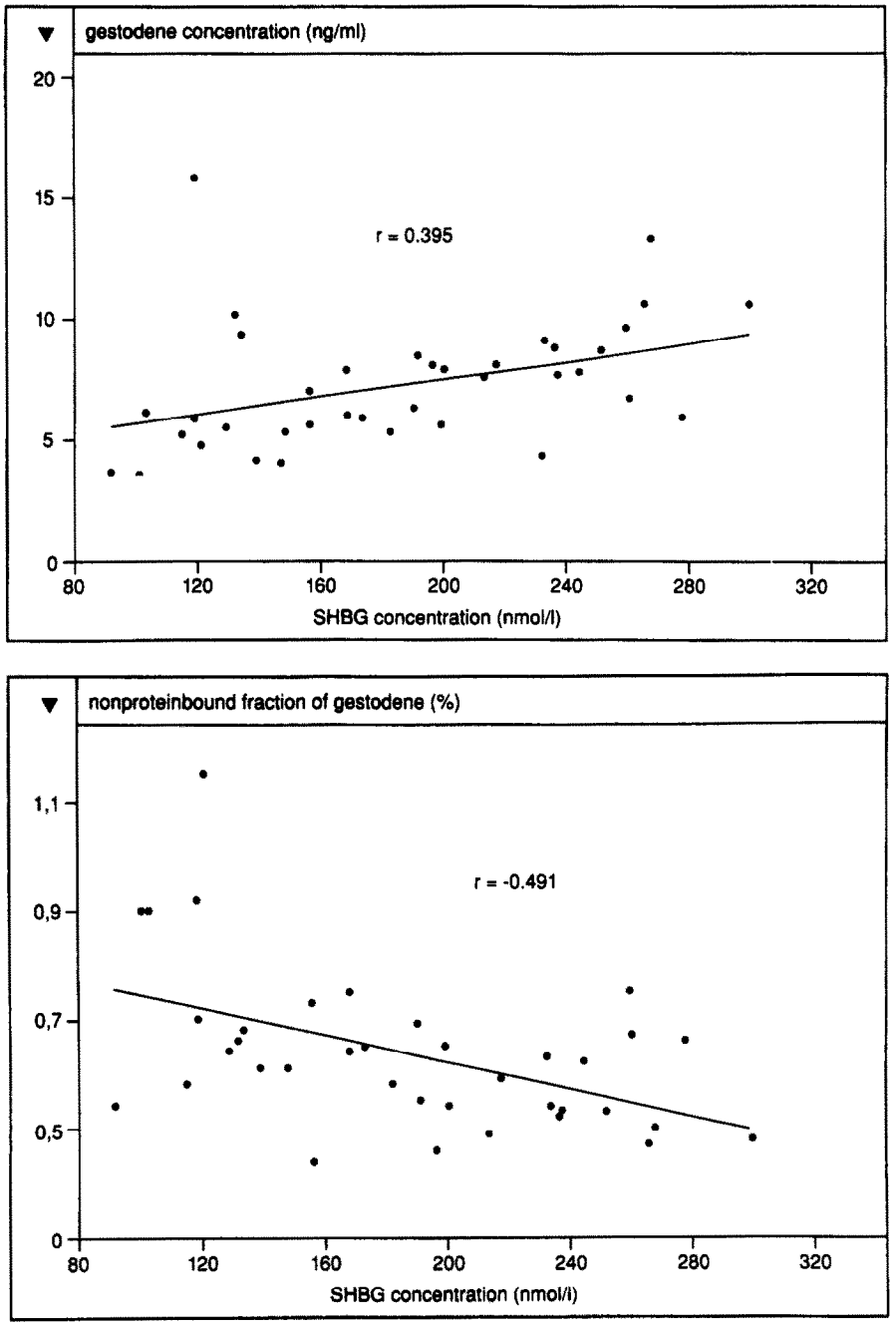

Fig. 1. Correlation of SHBG-concentrations and either total gestodene concentrations (top) or free fractions of gestodene (bottom) in the serum of 37 women who had taken a gestodene-containing oral contraceptive over a period of 11 ± 5 months.

to SHBG (3–5% relative to 5α-dihydrotestosterone [13, 16].

However, some of our data are not consistent with the results published by Hammond *et al.*[16]. In their study 10 women received a desogestrel containing oral contraceptive over a treatment period of 3 months. In agreement with the present study, the authors found the same free fraction of KDG in the serum samples obtained during treatment (2.4%), however, they reported a different distribution of the protein bound moiety, with a higher proportion (61.9%) of KDG bound to SHBG and a smaller proportion (35.7%) bound to albumin. Except for the free fraction, the distribution of KDG over the different binding proteins in our study resembled closely the distribution Hammond *et al.* reported for their collective of women, before drug treatment was commenced.

So far we have no explanation for this discrepancy since in both studies, not only the same oral con-

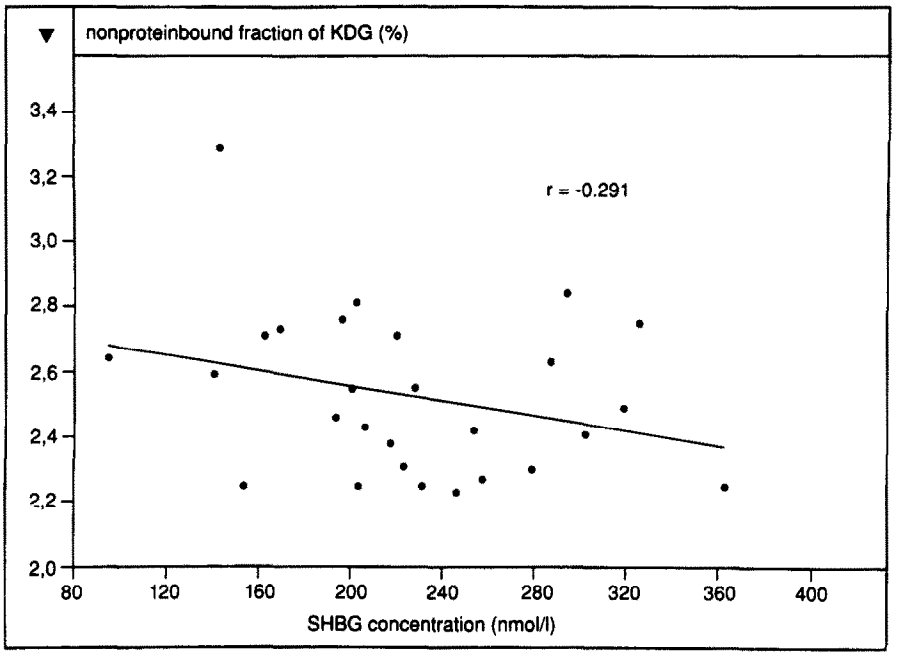

Fig. 2. Correlation of SHBG-concentrations and free fractions of 3-keto-desogestrel in the serum of 28 women who had taken a desogestrel containing oral contraceptive over a period of 38 ± 24 months.

traceptive was administered but also nearly identical analytical methods were used. The only marked difference between both studies was the duration of treatment, which was 3 months and more than 12 months, respectively and this may probably account for the divergent results. Kuhl *et al.*[15] reported increasing SHBG concentrations in the serum up to 3 months of treatment, thereafter SHBG-levels remained more or less unaltered up to the 12th month of treatment, when corresponding days of the respective cycles were compared. In the same study total KDG concentrations in the serum, expressed as area under the plasma drug concentration–time curve (AUC (0–4 h)), increased up to 3 months of treatment, however, subsequently they decreased significantly towards the 12th treatment cycle, reaching values which were close to those found at the end of the first treatment cycle. The reason for this unexpected behaviour remained unclear. When the SHBG-bound fractions of KDG as determined by Hammond *et al.*[16] up to 3 months and by us up to more than 12 months of treatment are together put into context with these findings, it becomes apparent that the total drug concentration and the fraction bound to SHBG show similar changes over the time of observation. These results, taken together, indicate a redistribution of KDG between SHBG and albumin during long-term treatment without affecting the free fraction of KDG. The possible causes of such a redistribution, however, remain to be identified.

The extent to which a drug is bound to serum proteins and its affinity for these binding sites, has an impact on both the drug's pharmacodynamic and pharmacokinetic characteristics. The pharmacological activity can be related to the free concentration rather than to the total concentration of the drug in serum, because only the former will at steady-state reflect the drug's availability at the target cells. However, it is generally assumed that due to a relatively low binding affinity, the albumin-bound fraction of the drug may also contribute to the free fraction [17]. The affinities of several endogenous and synthetic steroids for SHBG are usually about 3 orders of magnitude higher than those for albumin and hence the pharmacological activity of the hormone is suppressed as long as it is bound to the protein. Since protein binding is characterized by a dynamic equilibrium, the protein bound steroid represents a pool which can replenish free steroid cleared from plasma by distribution or metabolic inactivation [18]. Similarly, specific and high-affinity binding of a drug to serum proteins affects its uptake in the liver and other organs and thus influences the drug's metabolic clearance rate (MCR).

Pardridge[17, 19] has shown for endogenous steroids that organ uptake depends on the relation between organ transit time and half-life of dissociation of the hormone from its specific binding site. Subsequently Petra *et al.*[20] reported direct evidence for the effect of SHBG on the MCR of testosterone in rhesus monkeys. Infusion of human SHBG to female rhesus monkeys decreased testosterone clearance, infusion of human SHBG antibodies, known to cross-react with rhesus SHBG, increased clearance.

Bearing these data in mind and taking into account that GEST is characterized by a restrictive hepatic extraction, obviously systemic clearance will depend on the free concentration of GEST which in turn is closely related to the SHBG concentration in serum. Since SHBG levels increase during multiple dosing

due to induction of hepatic synthesis by concomitant EE_2 administration, the following changes can be expected in the pharmacokinetics of GEST, if calculated from total drug concentrations in the serum:

—total systemic clearance decreases
—total drug concentration in the serum increases
—apparent volume of distribution decreases
—the terminal half-life may be altered or may remain unaffected.

In fact our own studies [21] and those carried out by others [14] have confirmed increasing GEST concentrations up to the 3rd cycle of treatment, until steady-state concentrations of SHBG were reached. Furthermore, single i.v. administration of GEST to humans revealed a considerably smaller apparent volume of distribution than has been reported for KDG [22], thus again demonstrating the different interactions of both progestins with SHBG.

The influence of changes in SHBG concentrations on the terminal half-life ($t_{1/2}$) of GEST cannot easily be predicted, because $t_{1/2}$ is a function of volume of distribution and clearance, which both themselves are subject to alterations.

For steroids which are less extensively bound to SHBG, like KDG, or which are exclusively bound to albumin, like EE_2, the above mentioned implications on pharmacokinetics are either less stringent (KDG) or don't apply at all (EE_2). In conclusion, the present study has clearly demonstrated that the three synthetic steroids EE_2, GEST and KDG were all bound extensively to serum proteins, however, there were pronounced differences concerning their distribution over the relevant binding proteins. These differences account for different pharmacokinetic properties of each individual steroid and consequently any changes in the protein binding will have different consequences for each of the three hormones.

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