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SERUM PROTEIN BINDING CHARACTERISTICS OF CYPROTERONE ACETATE, GESTODENE, LEVONORGESTREL AND NORETHISTERONE IN RAT, RABBIT, DOG, MONKEY AND MAN

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Summary—Protein binding characteristics including percentage of total binding, total binding capacity (pmol/mg protein), degree of specific binding, competition with dihydrotestosterone (DHT) and estradiol (E_2) binding sites and dissociation constants (K_d) of low and high affinity binding sites were investigated for the progestins cyproterone acetate (CPA), gestodene (G), norethisterone (NET) and levonorgestrel (LN) in serum or plasma pools from man and four laboratory animal species (rat, rabbit, dog and monkey). Serum pools from animals were constructed from samples obtained either prior to or 1 day after pretreatment with ethinyl estradiol (EE_2) (5 μg/kg/day for 7 days). Human plasma pools differed by SHBG levels (normal/induced). All serum pools were characterized by protein content and distribution. Equilibrium dialysis or dextran-coated charcoal (DCC) methods were used to separate bound and free steroids labelled with tritium.

All progestins were highly (>80%) bound to proteins in all undiluted samples. Total binding capacity was highest in rat and lowest in moneky. Human plasma showed a capacity of 1.5–2.1 μg steroid/ml. In man, monkey and rabbit LN and G were specifically bound to the same degree as DHT, whereas NET binding was 50% lower. Specific binding of CPA to dog serum was 2–3 times higher than for other steroids. Two (high and low affinity) binding sites were found for LN, G and NET in man, monkey and rabbit and in dog for LN. K_d values for high affinity binding ranged from 3.5 (G in man) to 23 (NET in man) $\times 10^{-9}$ M. K_d values of low affinity binding varied from 0.5 (CPA in dog) to 4 (NET in man) $\times 10^{-6}$ M. E_2 and DHT competition experiments confirmed the concept of SHBG as a carrier protein of 19-nor-progestins and DHT and its occurrence in man, monkey and rabbit. A sex hormone binding protein (SBP) in the dog seems to be responsible for the relatively high specific binding of CPA. SHBG is inducible by means of EE_2 in man and monkey, but not in rabbit. EE_2 may induce SBP in the dog. Comparison of *in vitro* K_ds (high affinity binding) and *in vivo* metabolic clearance rates showed the same rankings for LN, G and NET in man, monkey and rabbit.

INTRODUCTION

Plasma protein binding of drugs is an essential part of overall pharmacokinetics. Interspecies differences in drug protein binding may contribute to interspecies variability of pharmacokinetic parameters which in turn are one of at least three variables in the search for an animal model most similar to man [1].

Extensive pharmacokinetic investigations in laboratory animals were carried out with four synthetic sex steroids used as progestins in oral contraceptives and showed that none of the species investigated (rat, rabbit, dog, cynomolgus monkey) can be regarded as pharmacokinetically similar to man for any of the substances tested, i.e. cyproterone acetate, gestodene, norethisterone, levonorgestrel [2]. However, data interpretation did not include possible changes in protein binding although there were some data published. A detailed review of publications [3–13] revealed some inconsistancies of data and conflicting results which might be due to differing experimental conditions and evaluating methods.

For the 19-nortestosterone derivatives levonorgestrel (LN; D-17α-ethinyl-13-ethyl-17β-hydroxy-4-gonen-3-one) and norethisterone (NET; 17α-ethinyl-17β-hydroxy-4-estren-3-one) a specific binding to SHBG (sex hormone binding globulin) is known and is anticipated for gestodene (G; 17α-ethinyl-13-ethyl-17β-hydroxy-4,15-gonadien-3-one). Dissociation constants (K_d) of LN in human plasma were reported be 1.1×10^{-7} M [3] or 7×10^{-7} M [13] for *d,l*-norgestrel. In rhesus monkey plasma K_d for LN varied between

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9×10^{-8} M [6] and 3.2×10^{-9} M [7]. For NET K_d values of 4.5×10^{-7} M (man) or 3.6×10^{-7} M (r. monkey) were reported [3]. In dog plasma a specific binding site has not been demonstrated for NET but has for LN with a K_d of 2.2×10^{-6} M [3]. In rabbit plasma specific binding was observed for both NET and LN, and K_d values of 3.9×10^{-7} and 1.7×10^{-6} M were estimated, respectively [3]. No specific binding was found in rat plasma. Whereas for the 19-nor-progestins LN and NET, high and low affinity binding sites are known in animal species possessing SHBG or SHBG-like proteins [12, 14–18], no high affinity binding site on cyproterone acetate (CPA; 17α-acetoxy-6-chloro-1α,2α-methylene-4,5-pregnadiene-3, 20-dione) has been described [5, 8]. As was shown for another C_{21}-steroid, medroxy-progesterone acetate [4], and for progesterone [10] CPA does not displace testosterone or DHT from human SHBG [5].

SHBG is present in the plasma of humans, various monkey strains and rabbits [12]. While human and monkey proteins exhibit similar structures [10, 15–17] rabbit SHBG shows larger structural differences [14, 18]. In dogs the presence of a sex steroid binding protein (SBP) has been shown which reaches higher levels in female than in male animals [11]. Whereas Corvol and Bardin could not show a specific binding of testosterone to canine plasma proteins [12], Suzuki *et al.*[19] found a testosterone binding glycoprotein exhibiting a K_d of 5.6×10^{-8} M for testosterone and 1.4×10^{-8} M for dihydrotestosterone.

The aims of the present study were to reanalyze protein binding data published for LN, NET and CPA by means of a prospective study using identical samples from four animal species and man and to extend it to displacement studies and K_d determinations. In addition the new progestin gestodene (17α-ethinyl-13-ethyl-17β-hydroxy-4,15 gonadien-3-one), was included in order to fit it into the set of pharmacokinetic data published for NET, LN, CPA and G [2].

SHBG levels in serum are dependent on pretreatment with estrogens, which is ethinyl estradiol (EE_2) in combined oral contraceptives. Human plasma pools were therefore prepared from cycle day 2 and day 23 from women starting on Triquilar® (triphasic preparation with EE_2 and LN) and from animal species prior to and after s.c. pretreatment with 5 μg EE_2/kg/day for 7 days. In all animal species this EE_2 dose exhibits full estrogenic efficacy.

MATERIAL AND METHODS

Materials

LN (15,16 $^3H_2$, specific activity (SA) 28.4 Ci/mmol), NET (15,16 $^3H_2$, SA 24.9 Ci/mmol), G (9,11 $^3H_2$, SA 37.8 Ci/mmol) and CPA (17α acetoxy ^3H, SA 15.8 Ci/mmol) were available. [^{3}H]estradiol (E_2, SA 140 Ci/mmol) and [^{3}H]dihydrotestosterone (DHT, SA 180 Ci/mmol) were obtained from Amersham International. All tracers were more than 95% pure and all reagents of analytical grade.

Dextran-coated charcoal (DCC) suspensions were prepared by mixing 0.25% (w/v) charcoal and 0.025% (w/v) dextran in distilled water. 0.1 M phosphate saline buffer (PSB) of pH 7.4 containing 0.15 M sodium chloride was used for dilution of samples.

Serum and plasma samples. Prior to and 24 h after s.c. pretreatment with 5 μg EE_2/kg/day for 7 days serum was obtained from 5 female Wistar Han SPF rats, 5 female N.Z. White rabbits, 3 female beagle dogs and 3 female wild-captured rhesus monkeys. EE_2 was dissolved in castor oil/benzyl benzoate (6:4, v/v) at a concentration of 5 or 50 μg/ml. Samples were pooled for animal species to give 2 representative samples (± pretreatment). Human plasma samples were obtained from a pharmacokinetic study with the triphasic oral contraceptive Triquilar®. Two representative pools (day 2, day 23 of first treatment cycle) were constructed including identical sample volumes of 32 women. All sample pools were kept deep frozen until use (−20°C). Portions of samples obtained prior to EE_2 pretreatment or on day 2 of OC use (human) were stripped of low molecular substances by charcoal treatment (5% w/v, 25°C, 30 min, then centrifugation at 2000 *g*, 4°C, 20 min) and supernatants were kept deep frozen.

Methods

SHBG levels in human samples were analyzed radioimmunologically (Farmos Diagnostica, SF). Protein analysis was carried out by electrophoresis. Equilibrium dialysis was performed using a commercially available dialyzer (Dianorm, F.R.G.). Either normal cells (0.5 ml/chamber) or microcells (0.1 ml/chamber) were used. Chambers were separated by a membrane of 5000 D cutoff (Diachema, F.R.G.).

Equilibrium was reached after at least 4 h when cells were rotated at 16 rpm and ambient temperature. Drugs were dissolved in serum samples. All analyses were carried out for 6 h.

For binding capacity studies DCC-stripped undiluted samples and samples diluted 1:30 with PSB and containing 10 ng steroid/ml were dialyzed against PSB in microcells at room temperature. Duplicate countings were made from both chambers. In a separate experiment serum and plasma samples pretreated with DCC and spiked with 100 ng steroid/ml were dialyzed against PSB at various dilutions (1:4–1:128). Duplicate measurements were done.

For binding site analysis DCC-stripped and 1:30 diluted samples obtained after EE_2 pretreatment of animals or on day 23 of OC ingestion were used. Samples were spiked with a constant amount of radiolabelled steroid (8000 dpm/ml) and varying amounts of unlabelled steroid (25–1400 nM/l). Microcells were used for dialysis against PSB.

For E_2 and DHT binding site experiments DCC-stripped samples and samples diluted 1:1 with PSB

Table 1. Total protein content and relative distribution of albumin and globulins in serum (plasma) pools from different animal species and man. All results are means ± SD of 5 determinations

Species	Man (plasma)	Monkey (serum)	Dog (serum)	Rabbit (serum)	Rat (serum)
Number of female individuals	36	3	3	5	5
Total protein g/100 ml	6.9 ± 0.2	7.6 ± 0.4	6.0 ± 0.7	6.3 ± 0.4	5.9 ± 0.5
Albumin%	67.8 ± 3.2	63.1 ± 1.8	57.5 ± 3.0	72.3 ± 6.0	61.1 ± 1.9
α-total globulin%	9.5 ± 1.9	9.2 ± 0.9	16.2 ± 2.0	9.0 ± 2.1	22.2 ± 4.3
β-total globulin%	7.0 ± 0.9	13.5 ± 1.6	19.0 ± 0.9	8.6 ± 2.2	12.2 ± 1.7
γ-total globulin%	15.8 ± 1.8	14.2 ± 2.2	7.4 ± 0.9	10.1 ± 2.9	4.5 ± 1.8

Pools were constructed from samples obtained prior to pretreatment with ethinyl estradiol (animal species) or from samples containing normal SHBG levels (man).

(0.1 ml) were used. [^{3}H]E_2 or [^{3}H]DHT were added to give a final concentration of 4 nM. Except in the case of standard samples, unlabelled steroids (E_2, DHT, CPA, G, NET, LN) were added to give a 100-fold higher concentration. After 2 h at 4°C bound and free steroids were separated by mixing with 0.5 ml of the DCC suspension. Suspensions were kept at 4°C for 15 min and then centrifuged at 2000 *g* (4°C, 20 min). Supernatants were counted for radioactivity.

The determinations of the percentage of specific binding were carried out as described for competition experiments with the modification that no unlabelled steroids were added. Besides radiolabelled E_2 and DHT the binding of [^{3}H]LN, [^{3}H]NET, [^{3}H]G and [^{3}H]CPA was also investigated.

Evaluation. The percentage of binding was calculated according to Yoa-Pu and Curry[20]. The overall binding capacity (method: dialysis) was calculated by least square regression analysis of binding at dilutions of 1:4–1:128. The dilution of sample able to bind 50% of steroid was used to calculate the binding capacity expressed as pmol steroid bound per mg of protein.

The percentage of specific binding was calculated by dividing the cpm of the supernatant after DCC treatment by the total radioactivity.

Binding site analysis and K_d estimation were performed according to Weder *et al.*[21]. Competition for E_2 and DHT binding sites was expressed as % displacement of radioligand with the tracer alone as 100%.

RESULTS

Protein binding

Protein contents and distributions in the plasma (serum) pools used (before EE_2 pretreatment and on day 2 of cycle, respectively) are given in Table 1. SHBG determinations revealed a level of 60.0 nmol/l and 210 nmol/l in human plasma pools constructed from samples on day 2 and day 23, respectively. Serum pools from animal species were also investigated but failed to give an answer in the assay, which is specific for human SHBG.

Protein binding experiments revealed high binding of all four progestins (10 ng/ml) in undiluted samples and no influence of EE_2 pretreatment on binding data (Table 2). Binding was above 92% for all progestins in samples from man, dog and rabbit. In monkey and rat samples the binding ranged from 83–93% with CPA showing the lowest values.

Binding of 10 ng/ml of progestins in 1:30 diluted samples again was not influenced by EE_2 pretreatment of dog, rabbit and rat. However, in the human plasma pool with elevated SHBG-level NET, LN and G were significantly ($P < 0.01$, t-test) bound to a higher degree than in the sample pool with a low SHBG-level (Table 2). The increase in binding was 6–10%. A similar effect could not be shown for CPA. Slight increases in binding of 5–7% for 19-norprogestins were also found in the monkey sample pool ($+EE_2$).

In contrast to man and monkey a slight increase of

Table 2. Serum (plasma) protein binding of progestins in undiluted (A) and 1:30 diluted (B) samples from man and animal species

Species		Man		Monkey		Dog		Rabbit		Rat	
Sample		A	B	A	B	A	B	A	B	A	B
Levonorgestrel	−	97	54	92	54	98	47	97	66	93	64
(LN)	+	97	63*	91	59	98	51	98	66	94	65
Norethisterone	−	92	51	87	44	95	41	97	60	90	54
(NET)	+	95	57*	89	51	95	44	95	63	90	55
Gestodene	−	96	56	86	56	96	53	98	63	83	61
(G)	+	96	66*	89	63	96	53	97	66	88	61
Cyproterone acetate	−	94	61	86	57	96	58	94	48	83	48
(CPA)	+	95	56	83	58	95	65	94	53	83	53

−/+ indicates pools constructed from samples obtained prior to (−) or after (+) pretreatment of individuals with ethinyl estradiol. *Significant difference ($P < 0.01$, t-test) between −/+ pool at the same dilution.

Table 3. Total binding capacity of four progestins in serum samples from animal species and a human plasma pool

Species	Human	Monkey	Dog	Rabbit	Rat
Compound					
LN	76	58	76	116	291
NET	69	55	55	100	170
G	97	64	80	128	175
CPA	109	80	92	59	117

All samples were freed from endogenous hormones by charcoal pretreatment and then spiked with 100 ng/ml of respective steroid. Values are given as pmol/mg of protein (means of duplicate analysis). Pools were constructed from samples obtained prior to pretreatment of individuals with ethinyl estradiol (no SHBG induction).

4–7% in CPA binding was found in samples from dog, rabbit and rat after EE_2 treatment. Total binding of all three C_{19}-steroids was similar when compared between species but different from CPA.

Comparing the overall (specific and non-specific) binding capacity for the progestins in all species, similar rankings were found for man, monkey and dog samples with highest figures for CPA, lowest for NET and with G and LN in between (Table 3). In rabbit serum the highest binding capacity was found for G and LN followed by NET and CPA (50% of LN). In rat serum a high capacity was observed throughout; for LN the capacity was about 300 pmol/mg protein, which was the highest of all. The capacities for NET and G were almost identical (approx. 170 pmol/mg), whereas the capacity to bind CPA was lowest (Table 3).

Binding sites

In Table 4 dissociation constants (K_d) estimated from Scatchard plot analyses are given for high affinity and low affinity binding sites.

Both binding sites were found for the 19-nor-progestins LN, NET and G in man, monkey and rabbit samples. Whereas the low affinity site showed K_ds in the range $1–4 \times 10^{-6}$ M, respective data for the high affinity binding site were between 3.5 and 23×10^{-9} M.

The highest affinity was found for G in the human sample ($K_d = 3.5 \times 10^{-9}$ M). The affinity found for LN was somewhat higher than for G in monkey and almost identical in the rabbit sample. Compared to LN and G the affinity found for NET was lowest in all three species ($K_d = 14–23 \times 10^{-9}$ M). In the dog

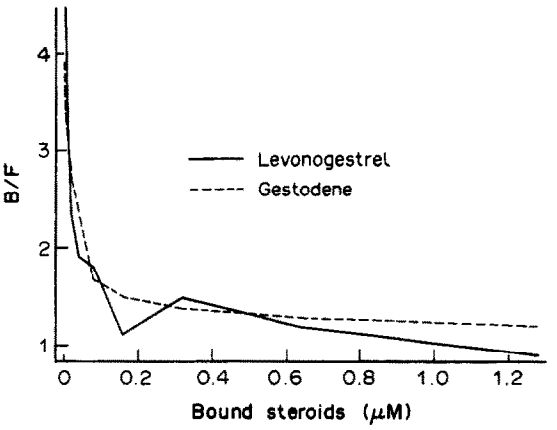

Fig. 1. Scatchard plots of LN binding (hook-shaped) and G (curvilinear) in monkey serum.

sample, a high affinity binding site was found only for LN ($K_d = 10 \times 10^{-9}$ M), but not for NET and G.

In rat, only low affinity binding sites were obtained ranging from $K_d = 1.2 \times 10^{-6}$ M (LN) to 3.7×10^{-6} M (NET). No high affinity binding site for CPA was present in any animal species or in the human sample. K_d-values for the low affinity binding sites were in the range $0.5–2.6 \times 10^{-6}$ M with highest affinity in dog and lowest in the rabbit sample. A hook-shaped curve often resulted in Scatchard plot analysis. This phenomenon was observed in all species and for all steroids except for NET and G in monkey serum and for G in rabbit serum. Examples of the two different forms of the curve (hooked/curvilinear) are given in Fig. 1.

Competition with E_2 and DHT binding sites

The results on competition experiments for the E_2 binding site are given in Table 5. In all samples the binding of $[^3H]E_2$ was displaced by about 60–70% when E_2 was added in 100-fold excess. The addition of DHT did not influence $[^3H]E_2$ binding in dog and was below 10% in monkey, rabbit and rat samples. A roughly 20% reduction in $[^3H]E_2$ binding by DHT was observed in the human pool sample. The addition of LN, NET and G did not show marked competition with $[^3H]E_2$ binding in all samples. Again almost no effect on $[^3H]E_2$ binding was observed by

Table 4. Dissociation constants (K_d) of high (A: $\times 10^{-9}$ M) and low (B: $\times 10^{-6}$ M) affinity binding sites in serum samples obtained from EE_2-pretreated animals or a human plasma pool containing elevated SHBG level

Compound	LN		NET		G		CPA	
Species	A	B	A	B	A	B	A	B
Human	5.1	3.4	23.0	4.0	3.5	3.3	—[b]	1.2
Monkey	4.6	1.0	14.2	1.6	5.8	2.9	—[b]	2.2
Dog	10.1	3.8[a]	—[b]	2.1	—[b]	1.5[a]	—[b]	0.5[a]
Rabbit	9.8	3.2	14.8	1.5	10.0	2.1	—[b]	2.6
Rat	—[b]	1.2[a]	—[b]	3.8	—[b]	1.7[a]	—[b]	1.8

Estimation of data was carried out using the Scatchard plot method.
[a] Figures have to be taken as rough estimates due to inconsistent binding data and only 2 or 3 points for calculation.
[b] No respective binding site.

Table 5. Competition between DHT and progestins for E_2 binding and between E_2 and progestins for DHT binding in serum of four animal species and human plasma

Sample		Human (plasma)	Monkey (serum)	Dog (serum)	Rabbit (serum)	Rat (serum)
Radioligand				[^{3}H]Estradiol		
Competitor						
	E_2	70 ± 2	64 ± 3	65 ± 5	60 ± 6	57 ± 8
	DHT	18 ± 7	6 ± 3	0 ± 5	8 ± 2	6 ± 4
	LN	6 ± 4	9 ± 3	3 ± 9	9 ± 5	−2 ± 6
	NET	5 ± 4	2 ± 9	2 ± 6	4 ± 6	−9 ± 2
	G	3 ± 2	8 ± 3	−1 ± 8	3 ± 6	0 ± 11
	CPA	11 ± 7	3 ± 3	3 ± 7	7 ± 8	10 ± 4
Radioligand				[^{3}H]Dihydrotestosterone		
Competitor						
	DHT	80 ± 4	76 ± 4	34 ± 12	59 ± 7	−47 ± 48
	E_2	66 ± 4	56 ± 9	25 ± 9	10 ± 6	−28 ± 15
	LN	56 ± 5	68 ± 5	41 ± 6	23 ± 6	−45 ± 18
	NET	41 ± 4	48 ± 4	32 ± 5	33 ± 7	−1 ± 4
	G	68 ± 5	75 ± 6	36 ± 6	46 ± 3	−1 ± 6
	CPA	13 ± 11	18 ± 6	7 ± 4,	6 ± 1	−10 ± 6

All samples were stripped using charcoal and diluted 1:1 with phosphate saline buffer. Serum pools constructed from samples obtained after EE_2 pretreatment of animals and a human plasma pool containing high SHBG level were used. [^{3}H]E_2 and [^{3}H]DHT were 4 nM and unlabelled competitors 400 nM. Analyses were run in triplicate and results (% decrease in radioligand binding) given as mean ± SD. Negative numbers stand for an increase in E_2 or DHT binding after addition of competitors.

the addition of CPA. At most, a 10% decrease was detected in human and rat samples.

The data on the [^{3}H]DHT binding site are included in Table 5.

A 100-fold excess of DHT decreased the [^{3}H]DHT binding by about 80% in human and monkey and by 60 and 35% in rabbit and dog samples, respectively. In rat serum there was an increase in [^{3}H]DHT binding of about 50%. The addition of E_2 displaced [^{3}H]DHT from its binding by more than 50% in human and monkey samples, but did not markedly interfere with binding in dog and rabbit serum (Table 5). In rat serum [^{3}H]DHT binding was increased by about 30%. As far as CPA is concerned there was no pronounced effect on the [^{3}H]DHT binding in any species. In addition, NET and G exhibited no effect on radioligand binding in rat serum, but LN increased binding of [^{3}H]DHT to the same extent as DHT (45%). 19-nor-progestins exhibited similar effects on [^{3}H]DHT displacement in human, monkey, dog and rabbit samples. The highest competition was found for G in samples from human and monkey, followed by LN and—well behind—NET.

In dog serum LN, G and NET displaced [^{3}H]DHT to a similar degree of 30–40%, whereas in rabbit LN showed the lowest effect and G the highest (Table 5).

Degree of specific binding

Using the charcoal method to separate more and less tightly bound steroids the figures given in Table 6 resulted.

Estradiol was bound to about 40% in all samples, whereas large differences were found for DHT. Almost no strong binding was seen in rat and low specific binding in dog serum. Some 40% binding was obtained in rabbit and human and 60% in monkey samples. Rather similar figures were obtained for LN and G with 40% binding in humans, 60% in monkey and 20–30% in rat, dog and rabbit serum. The degree of specific NET binding turned out to account for approximately only half of that in all species.

Table 6. Percentage of specific binding of E_2, DHT and progestins to proteins of animal serum and pooled human plasma

Sample [^{3}H]Radioligand	Human (plasma)	Monkey (serum)	Dog (serum)	Rabbit (serum)	Rat (serum)
E_2	38 ± 1	42 ± 2	40 ± 3	41 ± 3	37 ± 2
DHT	44 ± 4	60 ± 8	18 ± 2	36 ± 10	5 ± 0
LN	39 ± 7	62 ± 9	28 ± 4	30 ± 4	28 ± 4
NET	18 ± 3	30 ± 6	16 ± 5	16 ± 3	14 ± 2
G	42 ± 7	65 ± 12	22 ± 2	22 ± 5	19 ± 4
CPA	38 ± 12	34 ± 6	51 ± 2	34 ± 2	36 ± 1

Samples as outlined in legend of Table 5 were used. Radiolabelled steroids were added to give a concentration of 4 nM and bound and free steroids were separated by charcoal treatment. Results of triplicate analysis (means ± SD) are given as % bound steroid.

A completely different pattern was obtained for CPA with specific binding of about 35% in all species except the dog serum where specific binding of 50% was observed.

DISCUSSION

In the present series of studies four progestins were characterized for their protein binding properties in four laboratory animal species and man. Besides total binding in undiluted and diluted serum samples, the total binding capacity of plasma or serum, the number of and affinity for binding sites, competition with the binding of E_2 and DHT and the degree of specific binding were investigated. The selection of compounds tested and the animal species used were predetermined in a series of studies on comparative pharmacokinetics [2]. The present study can, therefore, be regarded as an extension of these pharmacokinetic investigations.

All four progestins were highly (>80%) bound to serum (plasma) proteins in all animal species and man. The binding capacity was highest in rat and lowest in monkey. In human plasma the capacity was between 70–110 pmol/mg protein which is equivalent to 1.5–2.1 μg steroid/ml plasma. Low dosed combined oral contraceptives containing one of the progestins investigated led to progestin plasma levels of 1.5–20 ng/ml at most. Therefore effective plasma progestin concentrations in human are around the 1% level of total steroid binding capacity. In addition, the serum binding capacity will not be exhausted in acute or chronic tolerance studies in any animal species [22].

Although total steroid binding and total binding capacity is high in all animal species and man, differences exist in binding characteristics. The method used to evaluate the degree of specific binding (DCC treatment) has not been sufficiently validated in terms of the dynamics of binding and of the power to differentiate between various binding affinities. Therefore results given in Table 6 have to be discussed carefully. It may be concluded that in man, monkey and rabbit samples the degree of specific binding of LN, G and DHT is identical but 2-fold higher than that of NET. Interestingly, the degree of specific binding of CPA in dog serum turned out to be 2–3 times higher than for other progestins or DHT, although no high affinity binding site could be demonstrated for CPA in any species.

However, the "low" affinity binding site showed a K_d of 5×10^{-7} M in dog serum and so was close to K_d values of high affinity binding sites. In line with the present study on DHT, in the dog no stable testosterone-globulin complex was found [12, 19] but a sex steroid binding protein (SBP), which seems to bind CPA with high affinity. As compared to 19-norprogestins the metabolic clearance rate and elimination half-life of CPA in the dog are drastically reduced and prolonged, respectively.

In line with the results on the degree of specific binding for LN, G and NET two binding sites were found in samples from man, monkey and rabbit, which is in agreement with published data [10, 15–17]. Human SHBG (hSHBG) and monkey SHBG (nSHBG) are structurally closely related [17] but nSHBG levels are about 4-fold higher than hSHBG levels which might be an explanation for the highest absolute specific binding of LN, G and DHT in monkey serum as compared to human plasma (Table 6).

As is demonstrated by the K_d-values and [^{3}H]DT displacement studies hSHBG and nSHBG indeed seem to possess very similar binding characteristics.

For the specific binding protein in rabbits (rSHBG) large differences in physico-chemical and structural properties were reported [10, 18] as compared to hSHBG or nSHBG. The specific binding capacity and binding affinity found for LN and G, but not for NET, were lower than in human samples. Consequently, both sex steroids showed a lower degree of [^{3}H]DHT displacement in rabbit serum.

In dog serum a high affinity binding site was only found for LN ($K_d = 1 \times 10^{-8}$ M), which—together with CPA binding and [^{3}H]DHT displacement studies—point to a sex steroid binding protein exhibiting completely different binding characteristics as compared to h-, n-, or rSHBG.

As is known from the literature [12] and confirmed by the present study there is no protein in rat plasma specifically binding sex steroids. Low affinity binding was found for the progestins investigated and its influence on E_2 binding was meaningless. It could be shown, however, that rat serum has a much higher binding capacity than serum from other species. Displacement studies on [^{3}H]DHT binding clearly revealed increased binding of [^{3}H]DHT by an excess of LN, DHT and E_2. Therefore, it may be concluded that in rat serum the non-specific binding to proteins has a different character than in other animal species or in man.

Effects of pretreatment of animals with EE_2 or induction of SHBG in man can be judged from differences in total binding in diluted (1:30) serum samples. There was a clear increase in binding of LN, G and NET by induction of hSHBG and a tendency towards increase in monkey serum obtained after EE_2 pretreatment which is in agreement with earlier observations [23, 24]. No similar effect was observed for the rabbit indicating a missing EE_2-inducibility towards rSHBG. The tendency towards increased CPA binding in dog serum might indicate a possible induction of SBP/CBG by EE_2.

High affinity binding site characteristics observed in the present study are partly in disagreement with published ones. Whereas the results of competition for DHT binding sites in monkey, dog and rabbit serum are almost identical to those published previously [3, 8], a high displacement of [^{3}H]E_2 by CPA and LN in monkey serum [8] could not be confirmed.

Low competition of LN and CPA for E_2 binding sites were also found by others [5, 25, 26]. K_d-values found in the present study were close to progestogen receptor affinities with K_ds of 10^{-9}–10^{-10} M [27]. K_d-values found for NET in serum of man, monkey and rabbit were at least one order of magnitude lower than those found before [3]. The same is true for respective LN data when compared to results from the same authors [3]. However, K_d values reported for LN in human [28] and monkey [7] serum were almost identical to those of the present study.

As outlined before, data on plasma protein binding in various animal species and in man are important for the interpretation of pharmacokinetic parameters and their interspecies differences. High and specific binding of drugs to serum proteins may limit the diffusion into hepatic cells and thereby protect against rapid biotransformation. Because all progestins investigated are almost totally metabolized before excretion a correlation between the metabolic clearance rate (MCR) and binding affinity to serum proteins might exist for various compounds in a single animal species or in man.

Indeed, for 19-nor-progestins, which are structurally closely related and are metabolized by similar pathways, the same rankings of K_d-values and MCR were observed in species with high affinity (SHBG) binding. CPA, however, does not fit into this ranking (Table 7). Because CPA undergoes other metabolic degradations than 19-nor-progestins and does not bind to SHBG this finding is not surprising. Attempts to correlate MCR with K_d-values of low affinity protein binding or percentages of specific protein binding of various species failed, although both, MCR and K_d, were lowest in dogs, then came man (Table 7).

Table 7. Comparison of *in vivo* metabolic clearance rate (MCR; ml/min/kg) and *in vitro* dissociation constant of high affinity binding site (K_d; $\times 10^{-9}$ M) of four progestins in various animal species and man

Species	Ranking of compounds			
Human				
MCR	G	< CPA	< LN	< NET
	0.8	1.8	2.2	6.2
K_d	G	< —	LN	< NET
	3.5	—	5.1	23
Monkey				
MCR	LN	= G	< CPA	< NET
	1.6	1.4	4.7	9.3
K_d	LN	= G	< —	NET
	4.6	5.8	—	14.2
Rabbit				
MCR	CPA	< G	= LN	< NET
	2.9	27	27	35
K_d	—	G	= LN	< NET
	—	9.9	9.8	14.8
Dog				
MCR	CPA	< G	< LN	< NET
	0.6	5.2	7.5	9.0
K_d	CPA	< G	< NET	< LN
	0.5	1.5	2.1	3.8

Values for MCR were taken from [2].
[a]In dog K_d of low affinity binding site ($\times 10^{-6}$ M) was used for comparison.

The same ranking in MCR and K_d-values for the three 19-nor-progestins in human, monkey and rabbit may indicate a direct influence of plasma protein binding on the pharmacokinetics of these structurally closely related and highly protein bound substances and thereby contribute to the discussion about animal models in preclinical safety evaluation and animal pharmacology.

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