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BINDING OF ESTROGEN-3-SULFATES TO STALLION PLASMA AND EQUINE SERUM ALBUMIN

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Summary—The binding of estrone-3-sulfate (E_1-3-S) and estradiol-3-sulfate (E_2-3-S) to adult stallion plasma was determined and compared with the binding to equine serum albumin (ESA). On the ESA molecule, two binding sites for E_1-3-S with an association constant of $1.3 \times 10^5\ M^{-1}$ and several sites of weaker affinity were found; the data for E_2-3-S showed the existence of four binding sites of moderate affinity ($1 \times 10^5\ M^{-1}$) and several sites of weaker affinity. The removal of albumin from the stallion plasma resulted in the absence of binding of E_1-3-S or E_2-3-S, whereas the removal of glycoproteins resulted in binding parameters similar to those obtained with whole plasma. These results indicate that ESA is the only estrogen sulfate binder in horse plasma. Under physiological conditions, 95% of E_1-3-S was bound to ESA.

INTRODUCTION

In various species, the availability of steroids from the circulation may be influenced by the presence of high affinity binding proteins such as corticosteroid-binding globulin (CBG) and sex steroid-binding protein (SBP) as well as low or moderate affinity binding proteins such as orosomucoid and albumin [1, 2]. Previously, it was generally accepted that only the free (unbound) fraction of the steroid was available to target tissues and that the CBG- and SBP-bound fractions served as a pool of readily available hormone. However, some workers have observed that steroids bound to CBG or SBP can interact with or enter target cells [3–5]. We have previously reported the absence of binding of testosterone or dihydrotestosterone by SBP in the stallion and in pregnant and non-pregnant mares [6, 7]. Renoir *et al.* [8] have shown the absence of SBP in the horse by immunoelectrophoresis performed with a monospecific antiserum against human SBP. Despite the absence of a specific binding to proteins, the action of steroid hormones takes place normally in the horse, thus raising the question as to the true function and necessity of this glycoprotein. In contrast, we have demonstrated the presence of CBG in the plasma of the mare [9].

Less is known about the role of albumin-bound unconjugated steroid, although it has been suggested that it may supplement the pool of free hormone in tissues because of the rapid dissociation of unconjugated steroids from albumin [3, 10] and that the biologically active fraction of hormone in plasma consists of the free plus albumin-bound fractions. Rosenthal *et al.* [11, 12] have demonstrated that estrogen-3-sulfates do not bind to SBP but are bound by human serum albumin (HSA).

In the stallion, estrogen-3-sulfates are synthesised in the testes and are present in massive amounts in peripheral blood and urine [13–16]. Peripheral blood levels of estrone-3-sulfate (E_1-3-S) (40–100 ng/ml) are about 20-fold higher than those of estradiol-3-sulfate (E_2-3-S) (2–5 ng/ml). The present work was designed to investigate the binding of E_1-3-S and E_2-3-S to stallion plasma and equine serum albumin (ESA).

MATERIALS AND METHODS

[6,7-^{3}H]E_1-3-S (60 Ci/mmol) was purchased from Dupont de Nemours. [6,7-^{3}H]E_2 with a specific activity of 10 Ci/mmol was purchased from CEN (Saclay, France) and [4-^{14}C]E_1 (56 mCi/mmol) from Amersham (France) E_1-3-S and E_2-3-S were obtained from Sigma (France). The purity of steroids was tested by TLC [cyclohexane–ethyle acetate (1:1)] and by

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HPLC as described previously [17]. ESA, fatty acid free-ESA and bromocresol green reagents were obtained from Sigma (France). Prepacked disposable columns with Sephadex G-25 (PD-10), Concanavalin A (Con-A) Sepharose, Sephacryl S-200 high resolution, DEAE-A25 gels and blue Dextran 2000 were obtained from Pharmacia. Affi-gel blue and chemicals employed for SDS–PAGE electophoresis were purchased from Bio-Rad. Dialysis bags (Tetrapor 4, cutting off at 12–14,000) were obtained from Spectrum Medical Industries. All other chemicals and solvents were of analytical grade and were obtained from Sigma (France) and Merck.

Preparation of [6,7-^{3}H]E_2-3-S

This steroid was prepared according to a modified procedure of Hobkirk *et al.* [18]. Endogenous steroids were removed from the cytosolic fraction of stallion testis by 1 h charcoal treatment (50 mg/ml). After centrifugation (10,000 *g* for 30 min), the supernatant was filtered (micropore filter 0.22 μm) and purified on a PD-10 column. The elution was performed with 50 mM Tris–HCl buffer, pH 7.4 (Buffer I). The fractions were then tested for sulfotransferase activity in the presence of 80 mM 3′-phosphoadenosine 5′-phosphosulfate (PAPS), 10 mM monothioglycerol, 25 mM $MgCl_2$ and 2 μM [4-^{14}C]E_1 in a total volume of 500 μl of Buffer I during 1 h at 30°C. The sulfotransferase activity was estimated by counting the radioactivity remaining in the aqueous phase after extraction of unconjugated steroids by 4 × 6 vol diethylether. The fractions containing this activity were pooled and used for the sulfation of 0.5 mCi of [6,7-^{3}H]E_2. The [6,7-^{3}H]E_2-3-S obtained was purified by DEAE-A25 anion exchange chromatography. The elution was carried out by an NaCl linear gradient (0–0.8 M). The [6,7-^{3}H]E_2-3-S formed was extracted from the aqueous phase by 4 × 8 vol ethyl acetate.

Plasma samples

Blood samples were collected on ethylenediaminetetraacetic acid (EDTA) from the jugular veins of four normal adult stallions, then centrifuged and stored at −20°C. Prior to use, the plasma was mixed with charcoal (50 mg/ml) for 1 h to remove endogenous steroids, centrifuged (1 h at 10,000 *g*) and the supernatant was filtrated (micropore filter 0.22 μm). All these procedures were carried out at 4°C.

Sephacryl S-200 gel filtration and gel electrophoresis

The molecular weight of ESA was determined at room temperature by gel filtration on a Sephacryl S-200 column (1.5 × 84.5 cm). Elution by 10 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl (Buffer II) was carried out at a flow rate of 30 ml/h. The apparent distribution coefficient (Kav) and the molecular weight of ESA were calculated as described by Laurent and Killander [19].

SDS–PAGE electrophoresis was performed using 5% gel acrylamide according to the method of Weber *et al.* [20].

Removal of albumin by Affi-gel blue [21]

A 5 ml Affi-gel blue column was equilibrated with Buffer II at 4°C. An aliquot of 500 μl steroid-free plasma was applied. The elution was carried out using the same buffer and fractions of 500 μl were collected. Proteins were located by the method of Bradford [22] and albumin concentrations were determined by a direct spectrophotometric assay using bromocresol green reagent according to the method of Rodkey [23].

Removal of glycoproteins by Con-A Sepharose [21]

Con-A Sepharose gel (1.5 ml) was washed twice with Buffer II and the supernatants were discarded after centrifugation. Steroid-free plasma (500 μl) was added to the resulting gel and the mixture was incubated at 37°C for 30 min with mixing every 5 min. After centrifugation (1500 *g* for 5 min), the supernatant was recovered and centrifuged again (3000 *g* for 10 min). The supernatant was assessed for protein and albumin concentrations as described above.

Binding of steroid sulfates

E_1-3-S and E_2-3-S binding experiments were performed by equilibrium dialysis according to the method described by Silberzahn *et al.* [6]. 1 ml of diluted or undiluted plasma, albumin-free plasma, glycoprotein-free plasma or diluted ESA was dialyzed for 48 h at 4°C under continuous shaking against 3.5 ml Buffer II containing increasing amounts of E_1-3-S or E_2-3-S in the presence of 100,000 cpm of radioactive steroids. Aliquots of 0.5 ml were then taken from both inside and outside the bag for the radioactivity measurements. Data were

analyzed according to the Scatchard procedure [24] as described by Rosenthal *et al.* [25]. The concentration of bound steroid (B) was plotted against the ratio of bound to unbound steroid (B/U). The values were calculated as follows:

$$B = \frac{(\mathrm{cpm}_i - \mathrm{cpm}_0) \times S \text{ ng}}{(V_i \times \mathrm{cpm}_i) + (V_0 \times \mathrm{cpm}_0)}$$

$$B/U = \frac{\mathrm{cpm}_i - \mathrm{cpm}_0}{\mathrm{cpm}_0}$$

where cpm_i and cpm_0 are respectively the amounts of radioactivity present inside and outside the dialysis bag and the V_i and V_0 corresponding volumes. S is the quantity, in ng, of estrogen sulfate used in the assay.

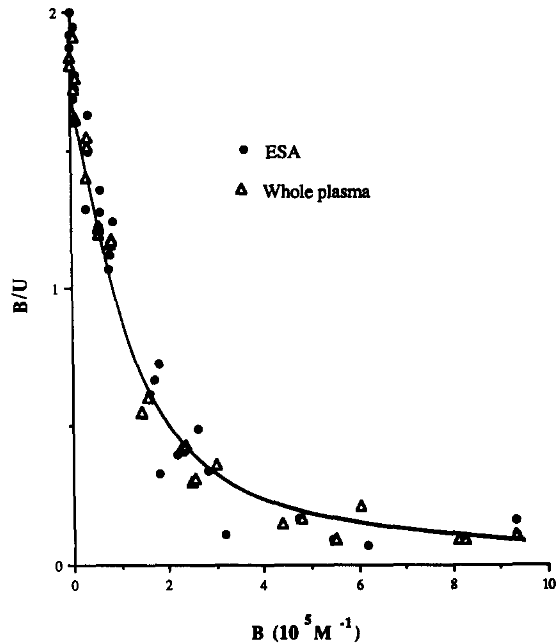

Fig. 1. Scatchard analysis of the interaction of E_1-3-S with 0.035% ESA or diluted whole stallion plasma (1/100).

RESULTS

ESA

The molecular weight of ESA was 70,000. The albumin concentration in the stallion plasma samples varied from 34 to 38 g/l.

Binding of estrogen sulfates to plasma proteins and ESA

The experiments with plasma showed the presence of two binding sites with moderate affinity ($K_1 = 1.3 \times 10^5\,M^{-1}$) for E_1-3-S, four binding sites with moderate affinity ($K_1 = 1 \times 10^5\,M^{-1}$) for E_2-3-S, and several binding sites (5 and 13–16 sites for E_1-3-S and E_2-3-S, respectively) with weaker affinity for these two steroids ($K_2 \approx 4 \times 10^3\,M^{-1}$ and $2.4 \times 10^3\,M^{-1}$ for E_1-3-S and E_2-3-S, respectively) (Figs 1 and 2). Similar results were obtained with ESA (Figs 1 and 2) and fatty acid-free ESA (data not shown).

Binding of estrogen sulfates to albumin-free plasma

Affi-gel blue chromatography of plasma removed 99.2% of the albumin. When albumin-free plasma was dialysed against increasing concentrations of E_1-3-S and E_2-3-S, the

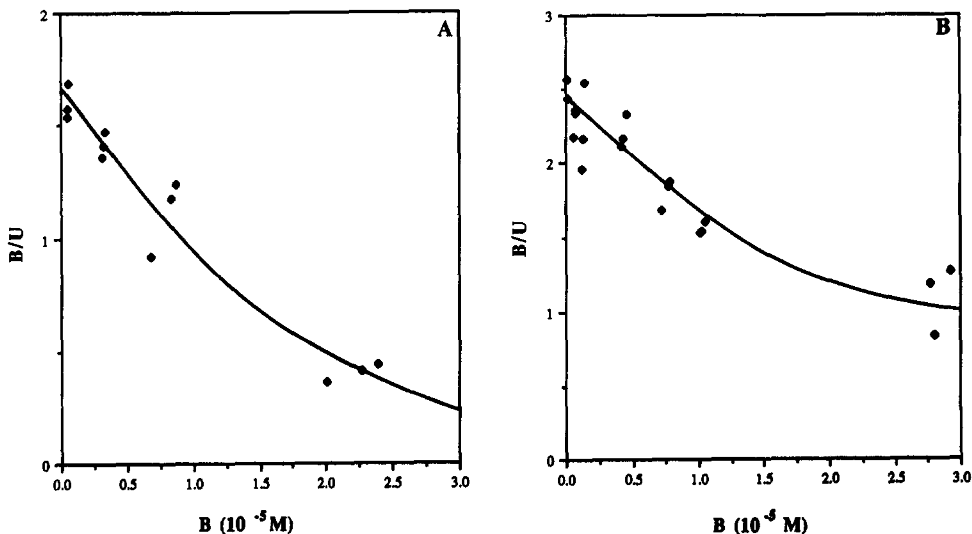

Fig. 2. (A) The plot obtained after dialysis of diluted glycoprotein free-plasma (1/100) against E_1-3-S. (B) The Scatchard plot of the interaciton of E_2-3-S with ESA (0.035%).

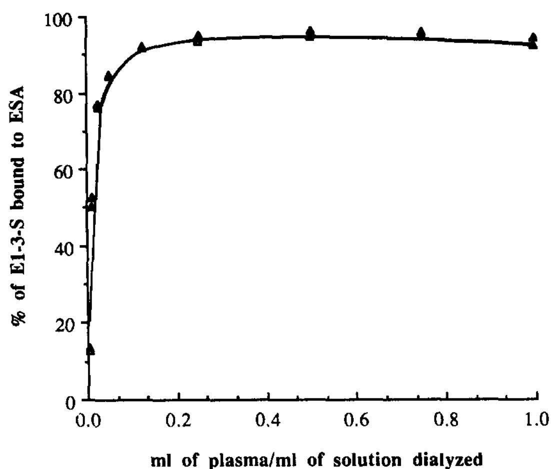

Fig. 3. Effect of plasma dilution on the percentage of E_1-3-S bound to ESA (0.2 μM).

amounts of radioactivity measured inside the dialysis bag were equal to those measured outside the bag. This demonstrates the absence of any binding of estrogen sulfates after the removal of albumin from plasma.

Binding of estrogen sulfates to glycoprotein-free plasma

After removal of glycoproteins by con-A sepharose chromatography, the binding curves of E_1-3-S and E_2-3-S were the same as those obtained with whole plasma or ESA (Figs 1 and 2). The association constant was $1.1 \times 10^5\,M^{-1}$ for E_1-3-S which corresponds to the value obtained with plasma and ESA.

Influence of plasma dilution on binding of E_1-3-S

1 ml of stallion plasma at various dilutions was dialysed against 3.5 ml Buffer II containing 0.2 μM E_1-3-S (assumed to be the physiological concentration) and 100,000 cpm of [6,7-^{3}H]E_1-3-S. Figure 3 shows that under physiological conditions, 95% of this estrogen sulfate was bound to undiluted equine plasma.

DISCUSSION

The binding of steroid hormones to serum protein plays an important role in the transport and distribution of the hormone. Estrogen sulfates were found to bind very similarly to whole equine plasma, glycoprotein-free plasma or ESA. In contrast, the removal of albumin by affi-gel blue from the plasma resulted in the absence of binding of E_1-3-S or E_2-3-S. Consequently, it appears that ESA is the only estrogen sulfate-binding protein in the plasma of the adult stallion. The data for estrogen sulfate binding to ESA show the existence of two and four binding sites with moderate affinity for E_1-3-S and E_2-3-S, respectively and of several binding sites with low affinity for these two sulfoconjugated steroids. The results are different from those obtained wtih 1% HSA [12, 21] which show only one binding site with moderate affinity ($K_1 = 1.1 \times 10^5\,M^{-1}$ for E_1-3-S and $5 \times 10^5\,M^{-1}$ for E_2-3-S) and seven binding sites with low affinity for both E_1-3-S and E_2-3-S ($K_2 = 0.5 \times 10^4\,M^{-1}$ and $0.14 \times 10^4\,M^{-1}$, respectively).

Under physiological conditions, estrogen sulfates were largely bound to serum albumin. The percentage of E_1-3-S bound to ESA is comparable to that observed with HSA (98.5% for HSA vs 95% for ESA). The presence of a greater number of binding sites on the ESA molecule might account for the high level of circulating estrogen sulfates in the stallion (E_1-3-S: 40–100 ng/ml) compared to the low levels in men (E_1-3-S: 0.4–1 ng/ml) [26].

Twombly and Levitz (1960) [27] have shown that estrone sulfate has a long half-life in human blood. In man, the metabolic clearance rate (MCR) of E_1-3-S is considerably less (7.8%) than that of the unconjugated E_1 [28, 29] indicating that E_1-3-S is cleared much more slowly than E_1. The high binding of estrogen sulfates to serum albumin may be an important factor in the low MCRs of these steroids.

Interstitial tissue, testicular microsomal aromatase activity and blood estrogen sulfate content increase greatly up to 20 years of age in the stallion while blood testosterone and unconjugated estrogens levels rise only slightly during aging (unpublished data). In the absence of a specific binding protein in the horse, the increasing amounts of androgens may be regulated by a testicular aromatization-conjugation mechanism. However, the high proportion of estrogen sulfates bound to ESA rules out the possibility that conjugation would only serve to facilitate their transport in the blood by converting them to more water-soluble metabolites which would be rapidly excreted in urine or bile. On the contrary, estrogen sulfates are almost entirely bound to serum albumin which slows down their excretion rate and raises the question as to their real function. Sulfoconjugation may be a possible form of inactivation of estrogens until they reach their target cells where they could be activated by hydrolysis. Indeed, estrogen sulfates are thought to be possible precursors to the

free estrogens in breast cancer cells [30], the accessory sex organs of the mature domestic boar [31] and in tissue minces of anterior pituitary, hypothalamus, and cerebral cortex of female sheep [32]. Furthermore, the capacity of human endometrium to hydrolyze steroid sulfates has been proved [33]. A significant increase in the ability of human chorion and decidua to hydrolyse steroid sulfates was observed around the time of onset labor [34]. It is also possible that estrogen sulfates may act as such without hydrolysis on target cells not yet identified in the horse.

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