Plasma clearance rates of zeranol, trenbolone and oestradiol in treated steers.

SHRIMANKER, K., SALTER, L.J. and PATTERSON, R.L.S.
ARC Meat Research Institute, Langford, Bristol BS18 7DY, U.K.

INTRODUCTION

Several anabolic agents currently used as growth promoters are steroids or resemble steroid hormones in many of their physiological properties. Like steroid and thyroid hormones, they circulate in the blood bound either by low affinity proteins (e.g. albumin) or by high affinity proteins, for example, sex-hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG).

The physiological effects as well as the metabolic fate of a hormone is undoubtedly influenced by the extent to which the hormone is bound to high-affinity plasma proteins. The percentage of hormone that is bound to high affinity hormone binding protein is inversely correlated to its plasma clearance rate[1],[2]. Thus, it is not difficult to understand why the binding studies have served as an important adjunct in the evaluation of the bioavailability and plasma clearance rates of various drugs and hormones.

Despite the importance of the binding studies and their relationship to the plasma clearance rates no studies have previously been reported on the specific binding of anabolic agents used in farm animals. We have therefore determined the SHBG-binding capacities of trenbolone, trenbolone acetate, zeranol and oestradiol. We have also developed RIA methods to assay concentrations of these agents in bovine serum and have used them to measure the concentration at regular intervals after implantation in order to assess their clearance rates.

EXPERIMENTAL

Reagents

[2,4,6,7 - ^{3}H] Oestradiol-17β (95 Ci/mmol), [1,2 - ^{3}H] 5α-dihydrotestosterone (60 Ci/mmol), [1,2,6,7 - ^{3}H] testosterone (107 Ci/mmole) and [1,2,6,7 - ^{3}H] cortisol (85 Ci/mmol) were obtained from Amersham International. [Undecyl-1,2 - ^{3}H] zeranol (50 Ci/mmol) and non-radioactive zeranol and its analogues were gifts from International Minerals and Chemical Corporation, U.S.A. [5,6 - ^{3}H] trenbolone (56 Ci/mmol), [5,6,-^{3}H] trenbolone acetate (56 Ci/mmol) and non-radioactive trenbolone, trenbolone acetate and their metabolites were gifts from Roussel-Uclaf, France.

Implants of Ralgro^R (36 mg zeranol), Finaplix^R (300 mg trenbolone acetate) and Compudose 365^R (45 mg oestradiol-17β) were gifts from Crown Chemical Company Ltd., Kent, Hoechst U.K. Ltd. and Elanco Products Ltd., Hants., respectively.

Instagel scintillation cocktail was obtained from Packard. All other reagents and chemicals were obtained from Sigma. 0.1M Phosphate buffered saline (PBS), pH 7.4, containing 0.9% NaCl and 0.05% NaN_3 was used. For radioimmunoassays (RIAs) the PBS contained 0.1% gelatin.

Equipment

The assay tubes for RIAs (LP3) were obtained from Luckham, Sussex. Borosilicate glass tubes (16 x 75 mm) for sex-hormone binding globulin studies were obtained from Radleys, Herts. Radioactivity was measured on a Packard Tri-Carb liquid scintillation spectrometer.

Animals

Three groups, each of six Friesian x Holstein steers were implanted in the left ear with either Ralgro, Finaplix or Compudose-365. Another three groups, each of six, served as controls. The steers were matched for similar weights and age. Blood samples were collected from all steers at weekly intervals from the left jugular vein and, after clotting, the blood was centrifuged to obtain serum which was stored at -20°C. Serum from a Friesian cow in late pregnancy was stored at -20°C until required for the SHBG studies. Young female New Zealand White rabbits were used for raising of antibodies.

METHODOLOGY

(i) Antisera to trenbolone, trenbolone acetate and zeranol.

Trenbolone and trenbolone acetate were converted to 3-O-carboxymethyloximes, and zeranol to the 6'-hemisuccinate. The oximes and the hemisuccinate were conjugated to bovine serum albumin (BSA) by the mixed anhydride method[3]. Three rabbits were immunised by intramuscular injection, each with one of the above BSA conjugates. They were bled from a marginal ear vein and the serum was stored at -20°C. An adequate titre of antibodies was detected in each rabbit five months after immunization. Antiserum to oestradiol-17β-6-CMO-BSA was a gift from I.N.R.A., Nouzilly, France.

(ii) Radioimmunoassays of trenbolone (TBOH), trenbolone acetate (TBA), zeranol (Z) and oestradiol (E_2)

Identical procedures were used for all the RIAs. Serum (1.0 ml) was pipetted into an extraction tube and extracted with diethylether (10 ml). The ether extracts were evaporated to dryness and reconstituted in 2.0 ml PBS. Duplicate aliquots (0.5 ml) were pipetted into assay tubes. For the standard curve, 0.5 ml PBS containing various concentrations of the appropriate anabolic agent (range 20 - 1000 pg/ml) were pipetted into assay tubes. Appropriately diluted antiserum in PBS (0.1 ml of a 1:7000 (TBOH), 1:7000 (TBA), 1:10,000 (Z) and 1:40,000 (E_2)) and 0.1 ml PBS containing 15000 dpm of the appropriate tritiated anabolic agent were pipetted into all the assay tubes and the contents vortex-mixed. Following incubation overnight at 4°C the free and bound anabolic agent were separated by addition of 0.5 ml 1% Charcoal in 0.1% dextran T-70 in PBS. The tubes were vortexed and allowed to stand in ice for 10 min before being centrifuged at 1500 **g** for 10 min at 4°C. The bound fraction contained in the clear supernatant was decanted directly into a polypropylene counting vial containing scintillation cocktail (7 ml) and the radioactivity counted. Samples of pooled serum not containing the anabolic agent, as well as samples from this pool containing known amounts of the anabolic agent were analyzed with each batch of assays.

(iii) Sex-Hormone Binding Globulin binding capacities of anabolic agents:

The SHBG-binding capacities of these compounds have been evaluated using an ammonium sulphate precipitation method employing 5α-dihydrotestosterone (DHT) as a reference hormone[4].

a). Precipitation of SHBG with saturated aqueous solution of ammonium sulphate.

The concentration of saturated ammonium sulphate required to precipitate SHBG exclusive of any other DHT-binding proteins was determined using tritiated DHT, cortisol and testosterone to investigate the precipitation of SHBG, CBG and BSA respectively, following the method of Rosner[4]. At both 4°C and 37°C, 50% saturated ammonium sulphate precipitated less than 5% of either the tritiated cortisol or the tritiated testosterone (and thus of CBG or BSA), whilst 85% of the tritiated DHT was precipitated. A higher concentration of saturated ammonium sulphate (55%) increased slightly the percentage of tritiated DHT in the precipitate (and hence SHBG) but a significant amount of BSA was also precipitated. Therefore, precipitation of SHBG was carried out using the 50% saturated solution.

b). Optimum dilution of cow pregnancy serum.

The cow pregnancy serum was diluted with PBS to give a range of dilution from 1 in 20 to 1 in 100 in 500 µl, each tube also containing 45000 dpm of ^{3}H-DHT. The tubes were equilibrated for 1 h at 37°C, and then 500 µl of cold saturated ammonium sulphate added. The tubes were vortex-mixed and placed on ice. After 2-5 min the tubes were centrifuged at 1500 **g** for 20 min and the radioactivity in the supernatant was counted. SHBG bound radioactivity was estimated by subtracting radioactivity in the supernatant from the total added radioactivity. A 1:40 dilution of the serum was used in the subsequent binding studies as this was found to bind approximately 50% of the added [^{3}H]-DHT.

c). Binding Studies.

The binding of each hormone to SHBG was studied by calculating the amount of hormone required to displace 50% of a standard amount of [^{3}H]-DHT from a standard quantity of SHBG. For each anabolic agent a series of tubes were prepared in duplicate containing a range of suitable concentrations between 0.5 and 10000 ng/tube. The amount delivered to each tube was in 300 µl of PBS. Minimum (0%) and maximum binding (100%) were established by preparing tubes containing 1000 ng and zero amount of DHT respectively. 100 µl of [^{3}H]-DHT in PBS was added to each tube and the contents vortex-mixed, followed by addition of 100 µl of 1 in 40 diluted cow pregnancy serum. The tubes were vortex mixed again and incubated at 37°C for 1h.

500 µl of saturated ammonium sulphate at 4°C was added to each tube which was vortex-mixed immediately to prevent localised high concentrations of ammonium sulphate and placed on ice. After 2-5 min., the tubes were centrifuged at 1500 **g** for 20 min at 4°C., then the radioactivity in the supernatant counted. The difference between the total radioactivity added and the radioactivity in the supernatant gave the amount of SHBG-bound radioactivity.

Direct comparison of the clearance rates of the three implants is not possible as they are prepared in different doses and formulation. However, it is clear that all preparations have rapid clearance rates from plasma consistent with their low SHBG-binding capacity. We plan to carry out further studies on the clearance rates of the anabolic agents prepared in the same dose and formulation.

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SUMMARY

Three groups, each of six Friesian x Holstein steers, were implanted with the anabolic agents zeranol, trenbolone acetate or oestradiol. Another three groups, each of six, served as controls. The steers were matched for similar weights and age.

Serum levels of zeranol, trenbolone, and oestradiol were monitored by radioimmunoassay methods at weekly intervals for at least twelve weeks. Percent relative sex-hormone binding globulin (SHBG) binding capacity of these anabolic agents were estimated using a chemical method involving selective precipitation of SHBG with ammonium sulphate and using 5α-dihydrotestosterone as a reference hormone with maximum binding capacity for SHBG. Rapid plasma clearance rates of these three anabolic agents are attributed to their relatively low SHBG binding capacity.

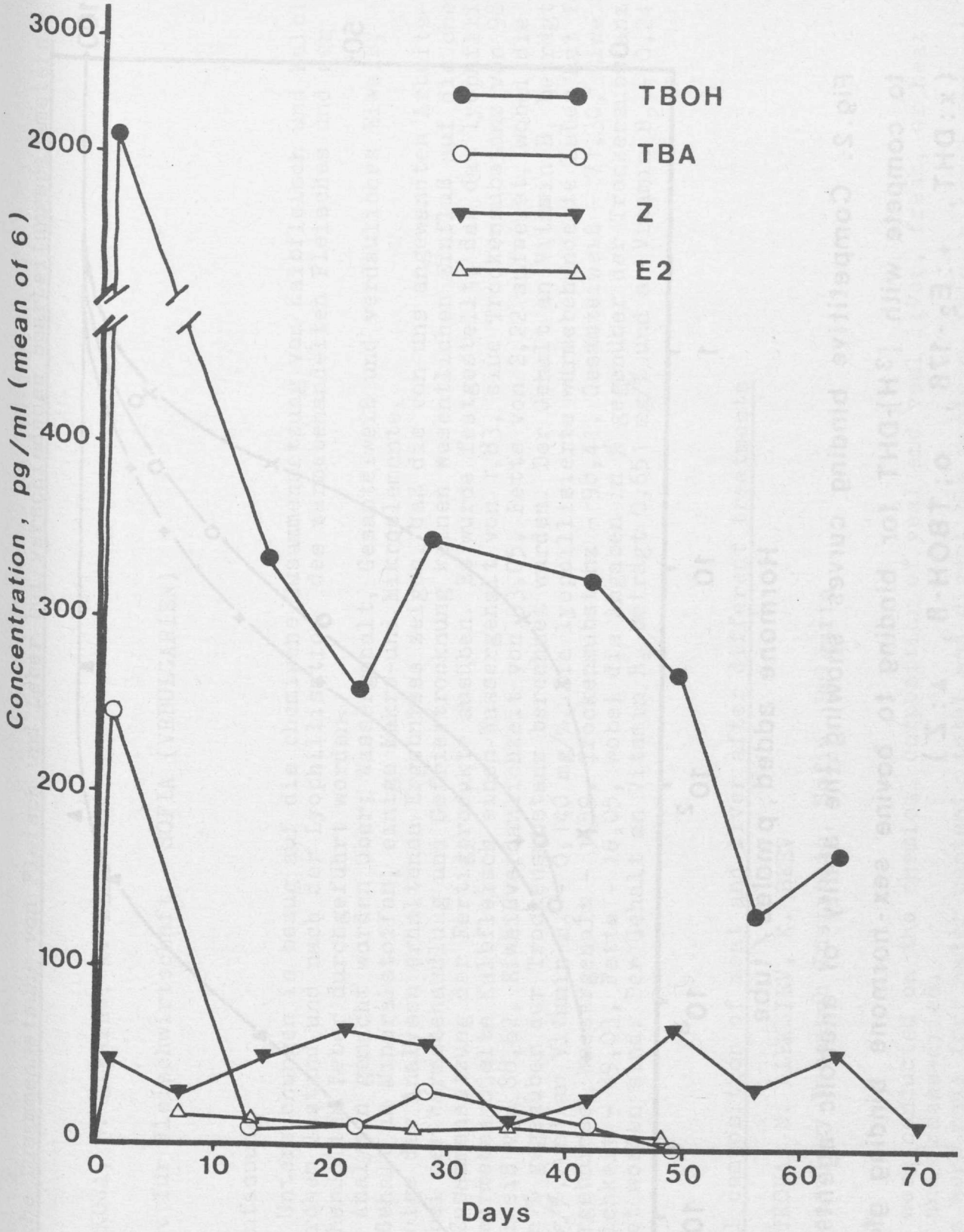

Fig.1. Serum concentration of anabolic agents following implantation.

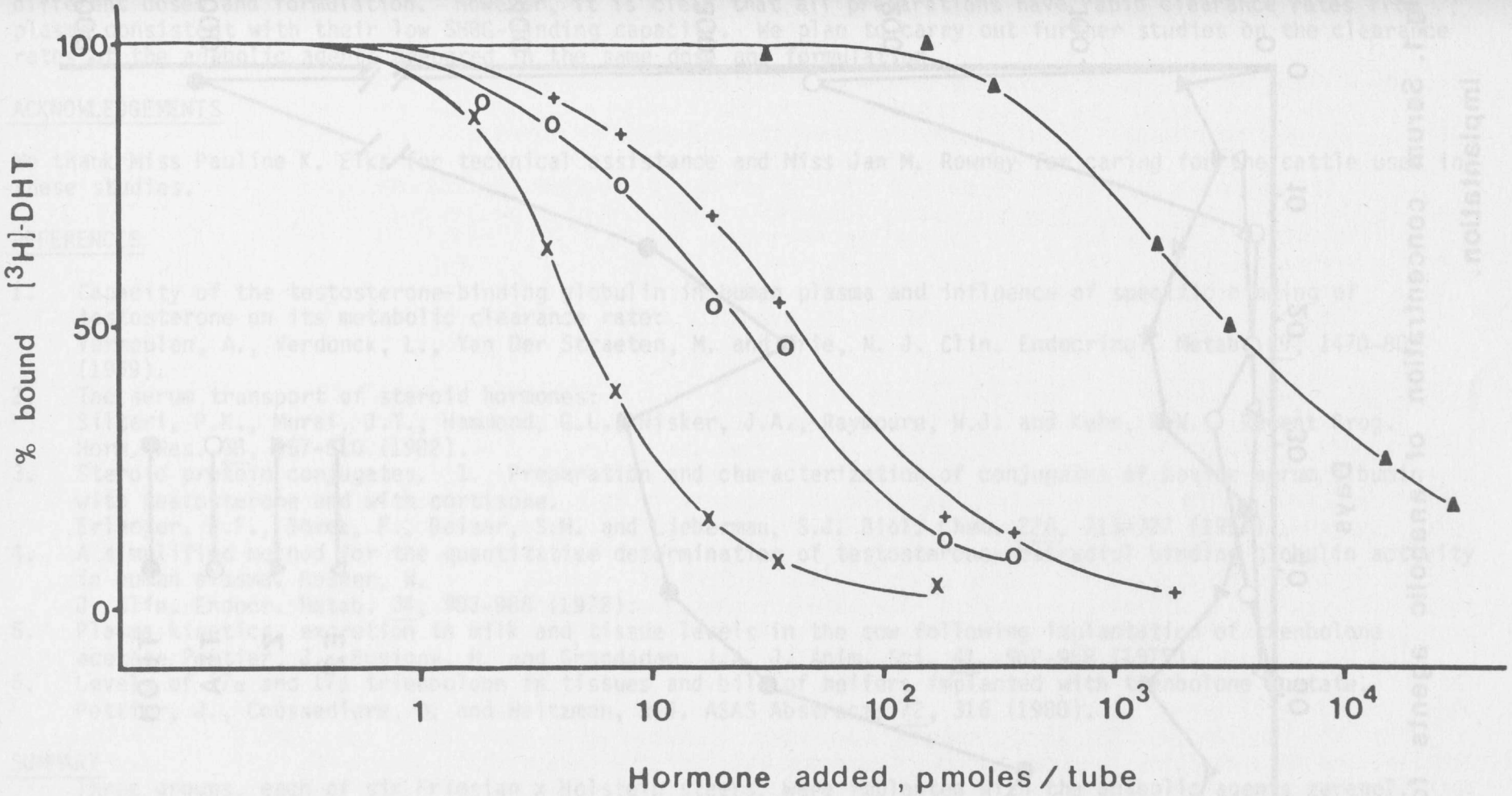

Fig.2. Competitive binding curves showing the ability of anabolic agents to compete with [^{3}H]-DHT for binding to bovine sex-hormone binding globulin (x: DHT, +: E_2-17ß, o: TBOH-ß, ▲: Z)