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Original Scientific Article

DETERMINATION OF SEX HORMONES IN RAT HAIR AFTER ADMINISTRATION OF TESTOSTERONE PROPIONATE AND ESTRADIOL VALERATE

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Summary: 17ß-testosterone (testosterone) and 17ß-estradiol (estradiol), together with their synthetic analogues, have been banned in the EU as growth promoters for fattening purposes. A simple, reliable method for their detection in animals is therefore required. Levels of testosterone and estradiol in hair and plasma of Wistar rats have been determined, following intramuscular (i.m.) administration of testosterone propionate and estradiol valerate, using commercial ELISA kits. Experiments were performed on prepubertal female rats for the detection of hair testosterone and on prepubertal male rats for the detection of hair estradiol. A significant increase of testosterone in hair was observed 3, 6 and 9 days after the administration of testosterone propionate, with no significant change in plasma content. Estradiol valerate administration also resulted in a significantly increased concentration in hair on the 3rd and 9th days, and in plasma on the 9th day. Thus, detection of testosterone and estradiol in hair after i.m. testosterone propionate and estradiol valerate administration can be achieved using commercial ELISA kits. Up to 0.80 % of the administered testosterone and 0.06 % of the administered estradiol were present in hair. This method could therefore serve as the basis for establishing a pre-screening tool for detecting abuse of anabolic steroids in farm animals.

Key words: testosterone; estradiol; hair; plasma; rats; ELISA

Introduction

17β-testosterone (testosterone) is the principal endogenous androgen synthesized by Leydig cells in the testes. Small amounts are also produced by the adrenal cortex. 17β-estradiol (estradiol) is produced mostly in the ovary and placenta. Sex steroids are transported in the blood from the steroidogenic tissues to target cells by blood where they are bound to sex hormone-binding globulin. They diffuse readily across the cell membrane and bind to intracellular receptors in the cytoplasm. Their activity is achieved through interaction of the receptor-hormone complexes to the hormone response elements on DNA that act as transcription factors, activating or inhibiting gene expression (1).

Testosterone has androgenic and anabolic activities. It regulates the male reproductive system by binding to androgen receptors present in particular in the reproductive organs, as well as interacting with receptors in muscles and fat tissue. Testosterone is also converted to estradiol and the more potent androgen dihydrotestosterone. In veterinary medicine testosterone and its synthetic analogues have been used for treating aplastic anaemia in dogs and cats, myeloproliferative disease and enhancing athletic performance in horses (1, 2).

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Estradiol plays an important role in the estrous cycle and in late pregnancy. Therapeutically it is used to prevent uterine infections and accelerate uterine involution in postpartum cows, for estrus synchronization in heifers and for induction of parturition in mares (1). Treatment of perianal gland adenoma in dogs with estradiol has also been described (3).

Although testosterone and estradiol, as well as their synthetic analogues, have been banned in the EU as growth promoters for fattening purposes (4, 5) they are still in use in many countries. According to 'The 2011 Prohibited List' of the World Anti-Doping Code (6), anabolic androgenic steroids belong to class S1.1 and are prohibited in and out of competition, including, notably, athletes and racing horses worldwide. For fattening and doping purposes steroids are usually administered as synthetic steroid esters. In the organism they are rapidly hydrolysed into natural steroids and free fatty acids. Therefore, exogenous steroid esters are difficult to detect in body fluids. Following oral intake of testosterone undecanoate, the unmetabolized ester could be detected in plasma of athletes only for 6 hours (7).

Sex steroids of endogenous and exogenous origin are inactivated in the liver and excreted mostly in the bile and urine. Estimation of reproductive status in various species by measuring faecal androgens, estrogens and progestins has been described (8, 9, 10, 11). Besides their presence in urine and faeces, steroids are also present in saliva, milk, different tissues, and hair (12, 13, 14, 15). Steroids and steroid esters can be incorporated into the hair fibre from blood via the hair follicles (15, 16, 17) and via sweat and sebum excretion at the surface of the skin, followed by diffusion into the hair fibres (15, 18). Some individual differences in steroid metabolism rates also occur (18). Hair steroids can be detected and quantified by gas or liquid chromatography coupled to mass spectrometry (17, 18, 19, 20, 21), desorption electrospray ionization mass spectrometry (22) and enzyme-immunoassay (23). Although commercial ELISA kits are useful for determining steroids in various matrices, their detection in hair by this means has not been described. The aim of the present study was therefore to explore whether commercial ELISA kits can be used for detecting testosterone and estradiol in hair. The study was performed by measuring testosterone and estradiol concentrations in rat hair after intramuscular administration of testosterone propionate and estradiol valerate. Testosterone and estradiol concentrations were also determined in plasma. The differences in hair steroid content between treated and non-treated rats could serve as a base for establishing the use of ELISA for detecting steroids in farm animals, where abuse of anabolic steroids can occur. To avoid the influence of endogenous testosterone and estradiol, experiments were performed on prepubertal female rats for the detection of hair testosterone and on prepubertal male rats for the detection of hair estradiol. The cross-reactivity of testosterone propionate and estradiol valerate with commercial ELISA kits was also checked.

Materials and methods

Experimental design for testosterone detection in hair

15 female Wistar rats aged 46-48 days at the beginning of the experiment were used in the study. The animals were divided into a control group of 7 and an experimental group of 8 animals. The environmental conditions were: 21°C, 12h light-dark regime, standard rat feed and water *ad libitum*. Seven days after housing, the animals from the experimental group were treated i.m. with 20 mg of testosterone propionate (T1875, Sigma – Aldrich, St.Louis, USA) per kilogram of body weight. Testosterone propionate injection solution was prepared by dissolving testosterone propionate in sterile olive oil (20 mg/mL) one day before treatment. Rats from the control group were treated with placebo (0.2 mL of olive oil i.m.).

Experimental design for estradiol detection in hair

15 male Wistar rats aged 47-48 days at the beginning of the experiment were used in the study. The animals were divided into a control group of 7 and an experimental group of 8 animals. The environmental conditions were the same as in the testosterone experiment. Seven days after housing, the animals from the experimental group were treated i.m. with 10 mg of estradiol valerate (E 1631, Sigma – Aldrich, St.Louis, USA) per kilogram of body weight. Solutions were prepared by dissolving estradiol valerate in sterile olive oil (10 mg/mL) one day before treatment. Rats from the

control group were treated with placebo (0.2 mL of olive oil i.m.).

The study protocol was approved by the Ministry of Agriculture, Forestry and Food, Veterinary Administration of the Republic of Slovenia; license number 34401-11/2011/4.

Sampling regime

Hair samples were collected by cutting with electric scissors from the rat's back. They were collected before treatment and on the 3rd, 6th and 9th day after treatment and stored at -20 ^{0}C until extraction.

Blood samples were collected from the ophthalmic plexus using heparinized glass micro-haematocrit tubes. Plasma was prepared by centrifugation at 2.500 g for 30 minutes at room temperature and stored at -20 ^{0}C until analysis. Blood samples were collected before and on the 9th day after the treatment. At the time of blood sampling the rats were anaesthetized with carbon dioxide.

Steroid extraction from hair

Approximately 0.15 g of hair was put in a mortar and 20 mL of liquid nitrogen was added to freeze the hair. The sample was pulverized by grinding for two minutes. 0.1 g of hair powder was weighed and placed into a plastic tube. 0.6 mL absolute methanol (Cat. No.1.06007., Merck, New Jersey, USA) and 0.5 mL bi-distilled water were added (24). The tube contents were shaken at 500 RPM for 30 minutes, centrifuged at 2500 g for 20 minutes at 4 ^{0}C and the supernatant decanted. The extract (supernatant) was stored in plastic tubes at -20 ^{0}C until analysis.

ELISA procedure

Testosterone and estradiol concentrations in intact plasma and in hair extracts were determined using commercial Testosterone ELISA and 17beta – estradiol ELISA kits (both IBL, Hamburg, Germany) following the instruction manuals. Absorbances were measured with a microtiter plate photometer Multiskan FC (Thermo Fisher Scientific, Waltham, USA). The concentrations of testosterone and estradiol in hair extracts were expressed as ng per g of hair.

Partial validation of the ELISA kits, which included determination of intra-assay and inter-assay coefficients of variation (CV) for hair testosterone and estradiol, was performed. Samples with low (control group) and high (experimental group) concentrations of testosterone and estradiol in hair extracts were run in triplicate in each ELISA test and repeated in the next ELISA tests.

Intra-assay CVs for hair testosterone were determined as 4.7 % and 4.3 %. Inter-assay CVs for testosterone were 8.2 % and 7.7 %. Intra- and inter-assay CVs for hair estradiol were 5.1 % and 9.3 % (intra-assay CVs) and 6.7 % and 11.4 % (inter-assay CVs).

Commercial testosterone and estradiol ELISA kits were also tested for cross-reactivity with testosterone propionate and estradiol valerate. 100 µg of testosterone propionate (T1875, Sigma – Aldrich, St.Louis, USA) or estradiol valerate (E 1631, Sigma – Aldrich, St.Louis, USA) were dissolved in 1 ml of absolute methanol (Cat. No. 1.06007., Merck, New Jersey, USA). Serial dilutions with phosphate buffer were used for preparing 10 ng/mL and 1 ng/mL of testosterone propionate and estradiol valerate solutions respectively, which were used for testing the commercial testosterone ELISA kit for cross-reactivity with testosterone propionate and the commercial estradiol ELISA kit for cross-reactivity with estradiol valerate.

Statistical analysis

Data were analysed using SPSS 17.0 (Chicago, USA) commercial software. Means and standard errors of the mean (mean ± SE) were calculated for plasma and hair testosterone and estradiol concentrations. Repeated measures ANOVA with Bonferroni correction was used for testing for statistically significant differences of testosterone and estradiol concentrations in hair between different sampling times in the control and experimental groups of animals. At each sampling time, hair testosterone and estradiol concentrations between control and experimental group were compared by independent t-test, using Bonferroni correction for multiple tests. In the control and experimental groups, paired t-test was used to compare plasma testosterone and estradiol concentrations before and after treatment. Independent t-test was used to compare plasma estradiol concentrations in the control and experimental groups before and after treatment. Pearson's correlation coefficient analysis was performed to determine if there is a statistically significant correlation between plasma and hair testosterone and estradiol levels. A value of $P < 0.05$ was considered significant.

Results

Testosterone concentrations in hair and plasma

Hair testosterone concentrations in the control and experimental group are presented in Figure 1. Before treatment the mean hair testosterone concentration (± SE) did not differ significantly between the control and experimental groups, 7.11 ± 1.09 and 10.17 ± 2.82 ng/g, respectively. In the control group testosterone concentrations remained unchanged throughout the experiment, while in the experimental group, mean hair testosterone concentration increased, reaching the highest value (182.47 ± 32.92 ng/g) three days after treatment. On the 6th day of the experiment, mean testosterone concentration (150.01 ± 23.78 ng/g) was lower than that on day 3, although the difference was not significant. At the end of experiment (on the 9th day) , the mean testosterone concentration (76.76 ± 14.33 ng/g) was significantly lower than on days 3 and 6 ($P < 0.05$); however, the value was significantly higher ($P < 0.05$) than that before treatment (10.17 ± 2.82 ng/g).

The differences in hair testosterone concentrations between the control and experimental groups were statistically significant ($P < 0.01$) on days 3, 6 and 9 of the experiment.

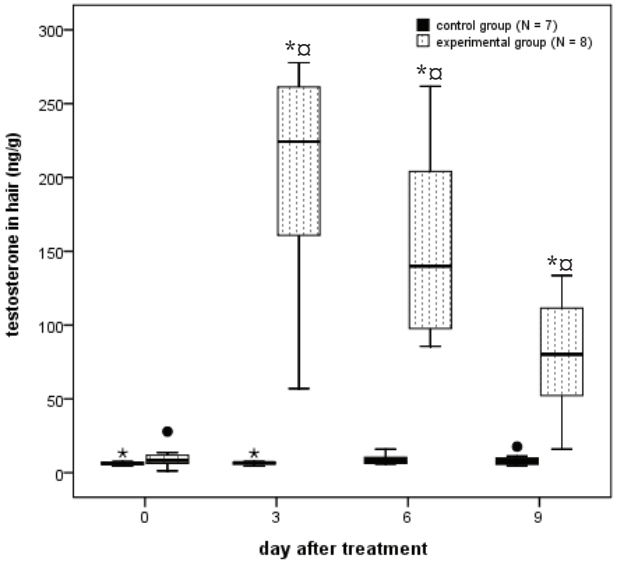

Figure 1: Testosterone concentrations in hair of the control and experimental groups

*P<0.05 (comparison of control and experimental groups)
¤P<0.05 (comparison with value before treatment)

Mean plasma testosterone concentrations (Table 1) before and on the 9th day after testosterone propionate or placebo administration were low, with no significant differences between and within the control and experimental group.

Table 1: Plasma testosterone concentrations (mean ± SE) in the control and experimental groups

plasma testosterone (ng/mL)		
	before treatment	after treatment
control group (N = 7)	0.77 ± 0.18	0.48 ± 0.09
experimental group (N = 8)	0.65 ± 0.26	0.56 ± 0.14

Pearson's correlation coefficients between hair and plasma testosterone were r = 0.10 (control group) and r = 0.24 (experimental group) and were not found significant.

Determination of cross-reactivity of the testosterone ELISA kit to testosterone propionate showed that measurements of testosterone propionate solutions in concentrations of 10 and 1 ng/mL had not achieved the detection limit (0.1 ng/mL).

Estradiol concentrations in hair and plasma

Hair estradiol concentrations in the control and experimental groups are presented in Figure 2. Before estradiol valerate or placebo administration, mean hair estradiol concentrations did not differ significantly between the control and experimental groups (0.40 ± 0.05 and 0.49 ± 0.09 ng/g, respectively). In the control group, estradiol concentrations remained unchanged throughout the experiment. In the experimental group, mean hair estradiol concentration increased significantly ($P < 0.05$) from 0.49 ± 0.09 ng/g to 2.75 ± 0.53 ng/g on the 3rd day after estradiol valerate administration. The peak value (6.07 ± 1.53 ng/g) was observed on the 6th day after treatment. On the 9th day after treatment there was a drop of mean hair estradiol (4.46 ± 0.75 ng/g). In the experimental group, no significant differences in estradiol concentrations between those measured at days 3, 6 and 9 were observed. Hair estradiol concentrations in the treated animals were significantly higher ($P < 0.01$) than on the control group on days 3, 6 and 9 after treatment.

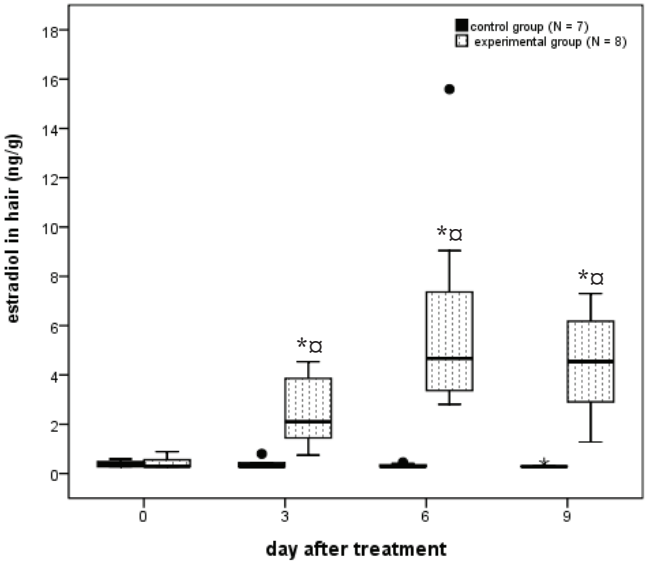

Figure 2: Estradiol concentrations in hair of the control and experimental groups

* P < 0.05 (comparison of control and experimental group)
¤ P < 0.05 (comparison with value before treatment)
β P = 0.055 (comparison with value before treatment)

In plasma, before treatment, there were no significant differences in estradiol concentrations (Table 2) between the two groups. Nine days after estradiol valerate administration, mean plasma estradiol concentration was significantly higher (P < 0.01) than the mean value before treatment, as well as than the control group value (P < 0.01).

Table 2: Plasma estradiol concentrations (mean ± SE) in the control and experimental groups

plasma estradiol (ng/mL)		
	before treatment	after treatment
control group (N = 7)	0.053 ± 0.018	0.062 ± 0.022
experimental group (N = 8)	0.091 ± 0.023	1.132 ± 0.133*¤

* P < 0.01 (comparison of control and experimental group)
¤ P < 0.01 (comparison with value before treatment)

Pearson's correlation coefficient between hair and plasma estradiol in control group (r = 0.20) was not found significant. In contrast, a significant positive correlation (r = 0.56; P < 0.05) was found between hair and plasma estradiol in the experimental group.

Determination of cross-reactivity of the estradiol ELISA kit to estradiol valerate showed that measurement of estradiol valerate solutions in concentrations of 10 and 1 ng/mL had not reached the detection limit (0.025 ng/mL).

Discussion

Commercial ELISA kits are used for routine and research purposes in endocrinology; they are accurate and sensitive, relatively cheap and easy to handle. Although mass spectrometry is considered to offer more selectivity than ELISA, mainly due to variable extents of cross-reactivity of steroids against the antibody (21), our results suggest that ELISA could serve as a pre-screening or alternative method for detecting testosterone and estradiol in hair. Our results have demonstrated for the first time that testosterone and estradiol can be detected in hair extracts after intramuscular administration of testosterone propionate and estradiol valerate using commercial ELISA kits. In Wistar rats treated with testosterone propionate or estradiol valerate, testosterone and estradiol concentrations in hair were significantly higher than in control groups from the 3rd to the 9th day after treatment. The results indicate that testosterone propionate and estradiol valerate are hydrolysed (7) in the rat's body and that, during distribution and excretion, some amounts of free testosterone and estradiol are incorporated into rat hair.

In animals treated with testosterone propionate, the content of testosterone in hair was, from the 3rd to the 9th day after treatment, significantly higher than in the control group. High testosterone values could result from hydrolysis of testosterone propionate and excretion of free testosterone onto the hair (15, 16, 18). Higher testosterone hair level on the 3rd day than on days 6 and 9 after testosterone propionate administration indicates intensive testosterone excretion via sweat, sebum and saliva immediately after administration. The slight decrease in testosterone concentrations on the 6th and 9th days after treatment could be caused by less intensive excretion onto the hair surface and eventual incorporation of previously excreted testosterone into hair fibre (15, 18).

Blood plasma testosterone concentrations before treatment and on the 9th day of the experiment were low, with no significant differences between control and experimental groups. These results indicate that, by the 9th day of the experiment, all

administered testosterone propionate has been hydrolyzed and all free testosterone excreted from the blood, which could also explain the gradual decrease of testosterone levels in hair from the 3rd to the 9th day after treatment. Non-significant correlation between hair and plasma testosterone might also be caused by the same reasons.

Similarly to testosterone, estradiol concentrations in hair increased but reached their highest mean value on the 6th day after administration although the value was not significantly higher (P = 0.055) than that before treatment. Some individual differences in metabolism and excretion rate of steroids are possible (18), which could be the reason for the wide dispersion of the hair estradiol measurements on the 6th day following treatment and consequent high standard deviation that resulted in a P value close to statistical significance.

In contrast to plasma testosterone concentrations, plasma estradiol increased significantly after estradiol valerate administration, resulting in a significant difference in estradiol concentration between the control and experimental groups. This suggests slower hydrolysis of estradiol valerate and prolonged persistence of estradiol in blood than of testosterone in the corresponding experiment. Estradiol is thus excreted onto the hair fibre over a longer period than that for testosterone, which could be the reason for the highest concentration being reached not earlier than on the 6th day after estradiol valerate administration. Additionally, the significant positive correlation between hair and plasma estradiol suggests that increased hair estradiol levels are the consequence of high plasma estradiol concentration.

The transport of steroids into hair is mediated via the hair follicle or by diffusion into the hair fibre from sweat, sebum and saliva (16, 17, 18, 25). Significantly higher hair testosterone and estradiol values on the 3rd, 6th and 9th days than in control groups confirmed that it is possible to detect prior use of testosterone and estradiol esters by measuring testosterone or estradiol concentrations in hair. According to our results of cross-reactivity testing, testosterone propionate and estradiol valerate cannot be detected by the commercial testosterone and estradiol ELISA kits used in the present study, which indicates the reliability of the chosen method, since possible contamination of the hair during administration is not detectable. Additionally, the low intra- and inter-assay CVs in testosterone and estradiol measurements, the significant correlation between hair and plasma estradiol, and the fact that the concentrations of testosterone and estradiol measured in the samples of hair extracts were above the detection limit indicate the repeatability, sensitivity and accuracy of the described method.

Comparison of the intramuscularly administered doses of testosterone propionate and estradiol valerate and the testosterone and estradiol hair concentrations indicates that up to 0.80 % of the former and up to 0.06 % of the latter is incorporated in hair. Due to their prolonged presence in hair, high levels could indicate previous use of testosterone and estradiol esters. For fattening purposes in farm animals, steroids are used in lower doses (26) than those used in our study; the described method therefore cannot be applied directly to detect hair testosterone and estradiol in farm animals. Because species-specific kinetic characteristics must also be considered, the present study can only serve as a base for establishing the ELISA method for the detection of steroids in hair in farm animals. Although detection of steroids and steroid esters is widely performed by the more selective gas and liquid chromatography coupled to mass spectrometry (16, 17, 19, 20), commercial ELISA kits could potentially serve as a pre-screening tool for the detection of testosterone and estradiol in hair after parenteral administration of testosterone and estradiol esters.

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UGOTAVLJANJE SPOLNIH HORMONOV V DLAKI PODGAN PO APLIKACIJI TESTOSTERON PROPIONATA ALI ESTRADIOL VALERATA

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Povzetek: Uporaba 17 ß-testosterona (testosteron) in 17 ß-estradiola (estradiol) ter podobnih sintetičnih snovi z namenom, da bi se izboljšal prirast pri farmskih živalih, je v EU prepovedana. Za določanje teh snovi pri živalih bi bila uporabna enostavna in zanesljiva metoda. Z uporabo komercialnih ELISA kompletov smo v raziskavi ugotavljali vsebnost testosterona in estradiola v dlaki in plazmi podgan pasme wistar po predhodni intramuskularni (i.m.) aplikaciji testosteron propionata in estradiol valerata. Poskus ugotavljanja vsebnosti testosterona v dlaki je bil izveden na mladih samicah, estradiol v dlaki pa se je ugotavljal pri mladih samcih. Statistično značilno višjo koncentracijo testosterona v dlaki smo ugotovili tretji, šesti in deveti dan po aplikaciji testosteron propionata, medtem ko statistično značilnih razlik v krvni plazmi nismo ugotovili. Po aplikaciji estradiol valerata smo ugotovili statično značilno višjo koncentracijo estradiola v dlaki tretji in deveti dan, višja vrednost pa je bila deveti dan ugotovljena tudi v plazmi. Na podlagi rezultatov sklepamo, da je z uporabo komercialnih ELISA kompletov možno ugotavljati vsebnost testosterona in estradiola v dlaki po predhodni i.m. aplikaciji testosteron propionata in estradiol valerata. V dlaki je do 0,80 odstotka apliciranega testosterona in do 0,06 odstotka apliciranega estradiola. Opisana metoda bi se lahko uporabila kot osnova za uvedbo presejalne metode ugotavljanja zlorabe anaboličnih steroidov pri rejnih živalih.

Ključne besede: testosteron; estradiol; dlaka; plazma; podgane; test ELISA