INVESTIGATION OF URINARY STEROID METABOLITES IN CALF URINE AFTER ORAL AND INTRAMUSCULAR ADMINISTRATION OF DHEA

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ABSTRACT

DHEA (3β-hydroxy-androst-5-en-17-one) is a natural steroid prohormone. Despite a lack of information on the effect, DHEA and other prohormones are frequently used as a food supplement by body-builders. DHEA is suspected for growth promoting abuse in cattle as well. Considering the latter, urine samples from a previous exposure study in which calves were exposed to 1 g DHEA per day during 7 days, were used. The calves were divided in three groups: one orally treated, one intramuscularly injected and a control group. The effect of this treatment on the urinary profile of several precursors and metabolites of DHEA was investigated. Urine samples were collected several days before and during the 7 days of administration and were submitted to a clean-up procedure consisting of a separation of the different conjugates (free, glucuronidated and sulphated forms) of each compound on a SAX column (Varian). An LC-MS/MS method was developed for the detection and quantification of several metabolites of the pathway of DHEA including 17α- and 17β-testosterone, 4-androstenedione, 5-androstenediol, pregnenolone and hydroxypregnenolone. Elevated levels of DHEA, 5-androstenediol and 17α-testosterone were observed in the free and sulphated fraction of the urine of the treated calves, thus indicating that the administered DHEA is metabolized mainly by the Δ^5-pathway with 5-androstenediol as the intermediate. Sulphoconjugates of DHEA and its metabolites were found to constitute the largest proportion of the urinary metabolites. The free form was also present, but in a lesser extent than the sulphated form, while glucuronides were negligible.

KEYWORDS: LC-MS/MS, DHEA, metabolites, 5-androstenediol, urine, prohormones

INTRODUCTION

The EC Directive 96/22 states that substances with hormonal activity are prohibited in cattle fattening[1]. Monitoring programs are required to live up to this Directive, thus requiring experience in analyzing feed, urine and tissue samples for screening and confirmation of hormone residues [2]. In addition, knowledge about absorption, biotransformation and excretion kinetics of illegally administered hormonal substances, as well as levels of endogenous hormones in livestock, is another requisite [1;3]. Besides steroids, there is a tendency in the livestock production towards misuse of feed supplements and preparations containing prohormones. The action of these prohormones is based on the conversion into more active hormones in target organs, after administration and uptake in the blood circulation. This may lead to anabolic action and subsequently improved lean/fat ratios in farm animals [4;5].

DHEA (Dehydroepiandrosterone, 3β-hydroxy-androst-5-en-17-one) is a natural steroid prohormone and is a key intermediate in the biosynthesis of biologically potent androgens and estrogens [6;7]. Endogenous steroids can be produced by the means of two alternative pathways, the Δ^4- and the Δ^5- pathway, corresponding to the metabolisation of cholesterol to pregnenolone and progesterone as the primary precursors and respectively 4-androstenedione and DHEA as their intermediates. Starting from DHEA, the conversion to 17β-testosterone (figure 1) can be catalyzed by hydroxysteroid dehydrogenases (HSDs) over 4-androstenedione (mainly in the gonads) or 5-androstenediol (mainly in the adrenal gland) [5;8-10]. However, abuse of DHEA, and also other prohormones, has been hard to prove due to the incomplete understanding of the DHEA metabolism as well as intra- and inter-individual variability in urinary steroid excretion [4;11]. Recently, a metabolomics based screening strategy has been conducted by Rijk et al.[12] in which several bovines where treated with prohormones such as DHEA and pregnenolone. Data were analyzed using multivariate statistics followed by identification of signals differential in urine of DHEA-treated versus control animals. This screening strategy is a useful tool to trace abuse with prohormones like DHEA and pregnenolone. However the concentration levels of this prohormones remains unknown and therefore a targeted analysis after this untargeted approach can be an added value.

The aim of our study was to focus on the excretion profile of DHEA and its metabolites in calf urine after an oral and intramuscular administration of DHEA. It was possible with our quantitative method to get an idea of the concentration levels of DHEA and several of its

metabolites after intake of DHEA, which was unknown in calves until now. Naturally, DHEA is mainly present in blood and urine of older animals in its sulphated form and to a much lesser extent in its free form or as a glucuronide conjugate [11]. The sample clean-up was set up such that each form, i.e. free, sulphated or glucuronidated form of DHEA and its metabolites could be investigated.

MATERIALS AND METHODS

Experimental Protocol

Urine samples were collected according to the study design presented in figure 2. Eight calves ranging in age from 6 till 10 months were used in this study, aiming to simulate the actual practice. These real-life bovines were housed in stables and normally fed. At two different time intervals several calves were allocated to a group and received either an oral treatment with DHEA or an intramuscular treatment with DHEA, while others served as controls. Treated calves were administered with 1 g of DHEA, orally or intramuscularly, every day for seven days. In the first period (june 2005), 1 calf was treated orally, 1 intramuscularly and 3 served as controls and did not receive any DHEA supplementation. In the second period (december 2006), 1 calf was treated orally, 1 intramuscularly and 1 served as a control. DHEA treatment was performed in the morning and urine sampling in the late afternoon for the first period and just before the next treatment in the second group (period 2). Urine samples were collected several days before treatment (5 days before treatment in period 1 and on days -20 and -5 before treatment in period 2) and during 7 days of administration in both periods. This study was undertaken after approval by the Ethical Committee of Ghent University. Samples were collected and frozen at -20°C until analysis.

Reagents and chemicals

Methanol was high-performance liquid chromatography grade and obtained from VWR International (Zaventem, Belgium). Ethyl acetate was purchased from Acros Organics (Geel, Belgium), diethylamine from Sigma-Aldrich and Helix pomatia digestive juice (Cat. No. 127 698; β-glucuronidase activity: 4.5 standard units; arylsulfatase activity: 14 standard units) from Boehringer Mannheim (Mannheim, Germany). Water was obtained from a Milli-Q Gradient System (Millipore, Bedford, MA, USA). Sodium acetate, glacial acetic acid, formic acid, fuming hydrochloric acid 37%, lithium chloride (pro analysis) were purchased from

Merck (Overijse, Belgium). Standards of dehydroepiandrosterone (DHEA), 4-androstene-3,17- dione (AED), 17α-testosterone (α-T), 17β-testosterone (β-T) and pregnenolone (Preg) were purchased from Sigma-Aldrich, while 17α-Hydroxypregnenolone (OH-Preg) and 5-androstene-3β,17β-diol (5-Andro) were obtained from Steraloids (Newport, RI, USA). 17β-19-Nortestosterone-D_3 (NT-D_3) and 17α-methyltestosterone-D_3 (MT-D_3) were supplied by RIVM (Bilthoven, The Netherlands). Miglyol 812 (Certa SA, Braine-l'Alleud, Belgium) was used for dissolving the DHEA for intramuscular injection.

Preparation of standard solutions

Stock standard solutions (1 $mg.mL^{-1}$) of α-T, β-T, AED, 5-Andro, Preg, OH-Preg and DHEA were prepared by dissolving 5.0 mg in 5.00 mL of methanol. Stock standard solutions of NT-D_3 and MT-D_3 were prepared from ampoules containing 0.1 mg of lyophilised powder by adding 1.00 mL of methanol to the ampoules, vortexing and transferring the methanol into a glass tube giving standard solutions of 0.1 $mg.mL^{-1}$. Stock solutions were stored at 4°C and had a shell life of at least 1 year. Working standard solutions are prepared by dilution with methanol to the appropiate concentrations of 10 and 1 $ng.\mu L^{-1}$ and stored at 4°C for maximum 3 months. From the individual stock standard solutions different standard mixtures were prepared and stored at 4°C for 3 months. These standard mixtures were used to create a matrix calibration curve.

Materials and apparatus

Octadecyl (C_{18}) (6 mL, 500 mg) and aminopropyl (NH_2) (3 mL, 500 mg) SPE columns were purchased from Grace Discovery Sciences (Lokeren, Belgium). The C_{18}-SPE column was conditioned by passing through 2 x 5 mL of methanol followed by 2 x 5 mL of water. The NH_2-SPE column was conditioned by passing through 2 x 3 mL ethylacetate. Bond Elut strong anion exchange (SAX) SPE columns were obtained from Varian (Sint-Katelijne Waver, Belgium). The SAX column was conditioned by subsequently passing 4 mL methanol, 4 mL water, 20 mL of 0.5 M acetic acid in water, 20 mL of water and 5 mL of methanol.

Analysis were performed on a Alliance 2695 HPLC system instrument coupled to a Quattro LCZ mass spectrometer (both from Waters, Milford, MA, USA) equipped with the Masslynx software for data processing. Chromatographic separation was achieved on a Symmetry C_{18} column (2.1 x 150 mm, 5 µm) preceded by a guard column Symmetry C_{18} (2.1 x 10 mm, 3.5 µm) (both from Waters, Milford, MA, USA). The column was kept at room temperature (20-

23°C). The mobile phase consisted of water/MeOH/ formic acid (FA) (89.7:10:0.3) and MeOH/FA (99.7/0.3) using the gradient elution program described in table 1. The injection volume was 100 µL.

The MS/MS operating parameters were obtained and optimized under positive-ion (ESI+) mode. Multiple reaction monitoring (MRM) transitions for each analyte were individually optimized, an overview of the precursor and product ions used in this MRM method, together with the cone voltages and collision energies, is given in table 2. Capillary voltage was set at 4.8 kV, the extractor at 3 V and high-purity nitrogen was used as spray gas. Source and desolvation temperatures were set at 150°C and 350°C respectively.

Separation of the free, glucuronide and sulphate fractions

The sample clean-up was based on the method descibed by Van Poucke *et al.* for the fractionation of free and conjugated steroids for the detection of boldenone metabolites in calf urine [13]. In short, the procedure is as follows: the pH of 10 mL of the urine samples were adjusted to 4.6 with 3 M acetate buffer (pH 4.6) and applied onto C_{18} cartridges. After washing with 2 x 5 mL of water and 2 x 5 mL 10% methanol, a preconditioned SAX column was placed under the C_{18} column. The free and conjugated fractions were then eluted with 2 x 5 mL methanol where only the the conjugated fractions were retained and the free form was collected. Next, the glucuronide fraction was eluted from the SAX column with 10 mL FA (0.5 M) in methanol. In the third step, a preconditioned C_{18} cartridge was placed underneath the SAX column and the sulphate fraction was eluted from the SAX column with 10 mL triethylamine (0.5 M) in water and was trapped onto the C_{18} column. After washing the C_{18} column with 2 x 5 mL water, the suphate fraction was finally eluted with 5 mL of methanol. All fractions were then evaporated to dryness at 40°C under nitrogen.

Hydrolysis

Next, the glucuronide and sulphate fractions were submitted to an hydrolysis step. The enzymatic hydrolysis of the glucuronide fractions was achieved by adding 5 mL of a 0.2 M acetate buffer (pH 4.6) and 25 µL of a tenfold dilution of Helix Pomatia juice in water. The samples were then kept for 2 h at 60°C. For the hydrolysis of the sulphate fraction, the dried residue was dissolved in 5 mL of a solvolysis solution consisting of 1 M lithium chloride / hydrochloric acid. This samples were kept 1 h at 80°C and afterwards 15 mL of water was added and the samples were centrifuged at 2800 *g*.

Final sample clean-up

The dried residue of the free fraction was redissolved in 10 mL of water. The free fractions and the supernatant of the glucuronide and sulphate fractions were then applied onto a C_{18} column. Afer washing with 2 x 5 mL of water and 2 x 5 mL 10% methanol, the column was dried and placed underneath a preconditioned NH_2 column. The columns were then eluted with 5 mL of ethyl acetate. The eluates were evaporated to dryness at 40°C under nitrogen. The dried residues were each dissolved in 150 µL of mobile phase (methanol/water/FA (60/39.7/0.3)).

Quantification and identification

Matrix calibration curves were performed daily by analyzing 5 blank urines (10 mL) spiked at 5 different levels in the 1-80 $ng.mL^{-1}$ range for α-T, β-T, AED, OH-Preg and Preg, the 1-200 $ng.mL^{-1}$ range for 5-Andro, and the 1-1000 $ng.mL^{-1}$ range for DHEA. All urine samples were spiked with MT-D_3 at a concentration level of 2 $ng.mL^{-1}$ as an internal standard. The final extracts from treated calves were diluted 10 times with the mobile phase and the external NT-D_3 standard was added at the end of the analysis at a concentration of 2 $ng.mL^{-1}$. Diluted extracts of urine samples were only used to quantify the samples that did not fit in the linear range of the calibration curve.

Calibration plots were constructed by applying the least-squares regression model and by plotting the response against the hormone concentration. Compounds were only used in the data analysis when the criteria of the Commission Decision 2002/657/EC were fulfilled [14] . Several urines were analyzed on two different time intervals. The concentrations of all target compounds in the entire concentration range differed maximally 30%.

Data analysis

All results are reported as the mean ± SE (standard deviation) and the median. Data were analyzed using a two-way analysis of variance. Non-parametric data were analyzed by means of Kruskal-Wallis test. When significant effects were revealed or only two groups were examined, an independent t-test or Mann-Whitney test taking account of the Bonferroni correction was used to locate the pair wise differences between groups. Spearman's correlations coefficients were calculated to determine significant correlations between the concentration of the several hormones and the day of urine collection. $P < 0.05$ was considered as statistically significant.

All calculations were executed in Excel® or in SPSS®.

RESULTS AND DISCUSSION

Urinary concentrations of DHEA, AED, 5-Andro, α-T, β-T, Preg and OH-Preg in calves were investigated before starting the treatment and during the DHEA treatment. Levels of the precursors Preg and OH-preg in urines during the entire study were found too low to be confirmed and/or quantified. Therefore it is not possible to draw any conclusion about the levels of Preg and OH-Preg before and after treatment with DHEA and as a consequence the data and statistical processing of these compounds are left out of the discussion. Table 3a shows the mean basal concentrations, the median, minimum and maximum value of the target analytes in the urines from the 5 calves of group 1 (June 2005) before starting the treatment. This was evaluated in order to show whether the treated calves were not calves which already excreted higher concentrations of these hormones and which statistically did not differ from the control calves at the beginning of the treatment. No significant differences were observed in group 1 in the baseline levels of DHEA and its metabolites 5-Andro, α-T, β-T, AED in the free or sulphated form. Group 2 (December 2006) did not yield enough data to prove this statistically. However, the assumption was made that there was also no significant difference in the basal urinary concentrations of these hormones in the 3 calves of group 2, shown in table 3b. Despite the large inter- and intravariability in all hormones in free and sulphated form, initial basal levels were not statistically different and within the normal range. Therefore, the biological variability is not expected to give any problems when comparing the control group with the treatment group during the study. However, when comparing the mean concentrations and corresponding standard deviations of the two groups (table 3a and 3b), slightly higher concentrations of the sulphated form of 5-Andro, α-T and β-T were observed in group 2. This was expected as the age of the calves differs between the two groups and was part of the general idea of using real-life bovine animals. In group 1 the calves were only 6 months old in contrast with the calves of group 2 that were already 9 till 10 months of age. These observed differences were checked statistically. Therefore basal urinary concentrations of all the hormones in free and sulphated form before treatment were compared between the group 1 and 2. A statistically significant difference in the levels of sulphated α-T ($p=0.027$) and β-T ($p=0.012$) was found between group 1 and 2. These results, higher levels of α-T and β-T in group 2, are in accordance with results earlier obtained by analyzing urine samples of calves and young bovines [3;15]. These studies pointed out that when the two isomers of testosterone (α and β) were followed, α-T was the first that appeared in the urine of calves.

When the calves got older and turned into young bovines, the concentration of α-T increased and low amounts of β-T appeared.

In a next step baseline levels of the control animals before and during the treatment were compared. Because of the proven differences between the two groups for α-T and β-T, data of α-T and β-T obtained in group 2 where excluded when comparing the baseline levels of the control animals before and during treatment. Table 4 shows the mean concentrations, standard deviations, median, minimum and maximum value of the urinary concentrations of the free and sulphated forms of DHEA and its metabolites of all control animals before the start of and during the study. A small but statistically significant difference in the concentration of the free and sulphated form of DHEA ($p=0.021$) and 5-Andro ($p=0.026$) was found for which we have no explanation.

Subsequently the influence of the oral and intramuscular treatment with DHEA was investigated, looking at DHEA and its metabolites in their sulphated and free form. The urinary baseline levels of the sulphated form of DHEA and its metabolites from the control group (control) as well as the influence of 1 g DHEA orally (oral) or intramuscularly (IM) administered on urinary DHEA metabolites are illustrated in figure 3. Compared to the control group the oral treated group differed significantly for the sulphated forms of DHEA ($p=0.000$) and 5-Andro ($p=0.000$). The intramuscularly treated group differed significantly from the control group for the sulphated forms of DHEA ($p=0.000$), 5-Andro ($p=0.000$) and α-T ($p=0.006$). These statistically proven differences can clearly be seen in figure 3, but when looking at the graphs of α-T, although not proven, a clear increase of α-T after the oral treatment can be observed as well. The highest increase was obtained in the concentration of the sulphoconjugates of DHEA (DHEAS) (>2000 $ng.mL^{-1}$), followed by the sulphated forms of 5-Andro and α-T (both around 400 $ng.mL^{-1}$). The 5-Andro metabolite is the intermediate in the adrenal gland in the conversion of DHEA to 17β-testosterone immediately derived by the action of 17β-hydroxysteroid dehydrogenase [16], while α-T is considered as the main metabolite of β-T from the action of 3β-hydroxysteroid dehydrogenase. There was no increase in the sulphated form of β-T (figure 3D) and no statistical difference between the orally and intramuscularly treated group for these sulphated conjugates could be detected. Figure 4 summarizes the results of the concentrations of DHEA and its metabolites in the free form of the three different treatments: control group (control), orally treated group (oral) and intramuscularly treated group (IM). The comparison of the concentrations of DHEA and its metabolites of the different groups in the free form differed slightly from the sulphated form.

Here there was a statistically significant difference between the control group and the orally treated group for DHEA (p=0.000) and α-T (p=0.000), and when comparing the control group with the intramuscularly treated group for DHEA (p=0.000), 5-Andro (p=0.001) and α-T (p=0.000). The concentration of AED (the metabolite of the Δ^4-pathway) was a bit higher in the orally treated group but could not be statistically proven. The difference for the intermediate of the Δ^5-pathway (5-Andro) between the oral group and the control group could also not be proven statistically in contrast to the comparison of the IM group with the control group, but figure 4B shows that this difference is very small. Thus it can be concluded that there was no statistical difference between the orally and intramuscularly treated group for these free target compounds. Comparing the data from figure 3 and figure 4, it becomes clear that the amounts of the free forms are relatively low compared to their sulphated forms.

Searching for a trend in the concentration levels of DHEA and its metabolites of several days, the correlation (Spearman's correlation coefficient R) was tested between the day of collection of the urine sample and the concentration of the hormone. This was only performed for the sulphated forms of DHEA and 5-Andro for the treated calves, as for the other hormones and the free fraction concentration levels were too low or the linearity of the correlation could not be demonstrated by means of a scatterplot. Figure 5 shows the urinary levels of the sulphated forms of DHEA and 5-Andro of the separate calves of the two groups during the 7 consecutive days of treatment. The concentrations of the first day of urine collection of the second group have been left out, because the urine was taken after 8 hours instead of 24 hours like the other days and therefore showed much higher concentrations. The concentration of DHEA of the intramuscularly treated calf of group 2 showed a positive correlation ($p<0.05$ and $R=0.86$) with the days of treatment, meaning that the concentration increased as the calf was treated more days. The intramuscularly treated calf of group 1 did not show this correlation. We have no straightforward explanation, but the calf in group 2 was treated in the winter in contrast with the calf in group 1 that received the treatment in the summertime. Therefore the second calf had more adipose tissue, possibly retaining the DHEA longer and releasing more DHEA after a few days, resulting in higher DHEAS concentration in the urine. A positive correlation, but to a lesser extent, was also seen in this IM treated calf of group 2 for 5-Andro ($p=0.05$ and $R=0.75$). In contrast with the intramuscularly treated group, the orally treated group showed a steady-state condition.

CONCLUSIONS

Sulphates, glucuronides and free forms were analyzed separately in order to gather information about the distribution of DHEA and its metabolites over these three forms. In general our results show that DHEAS constitute the largest proportion of urinary levels of DHEA. This was also found in humans, where DHEA is rapidly converted into DHEAS, which can be converted back to DHEA by peripheral sulphatases [17]. In this way DHEAS serves as a large precursor reservoir for the production of androgens and estrogens in non-reproductive tissues [18]. The free form of DHEA was present to a much lesser extent than the sulphated form and DHEA-glucuronides were negligible and in most cases not quantifiable because concentrations were below the limit of detection. The same is valid for the determined DHEA metabolites: mainly present as sulphoconjugates, hardly in their free form, and negligible as glucuronides. There was no statistically significant difference between the levels of all target hormones in sulphated and free forms when comparing the orally treated with the intramuscularly treated group. When comparing the control group with the treated groups a statistically significant difference was obtained for sulphated DHEA and 5-Andro and the free form of DHEA and α-T for the orally treated group and sulphated and free form of DHEA, 5-Andro and α-T for the intramuscularly treated group. These findings confirm that administered DHEA metabolizes mainly by the Δ^5-pathway with 5-Andro as the intermediate and that the metabolisation by the Δ^4-pathway with AED as the intermediate is hardly increased.

No elevated levels of sulphated or free β-T were observed after administered DHEA, either oral or IM. This seems strange, as levels of α-T were increasing, while α-T is considered as the main metabolite of β-T from the action of 3β-hydroxysteroid dehydrogenase. However, these results are in accordance with the findings obtained by administration of DHEA to the gelding and the mare where there was a high conversion to 5-Andro and a much lower conversion to α- and β-T [19]. These results are also confirmed in humans where ingested DHEA was rapidly metabolized to 5-Andro [20].

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Fig. 1 : Biosynthesis of 17β-Testosterone

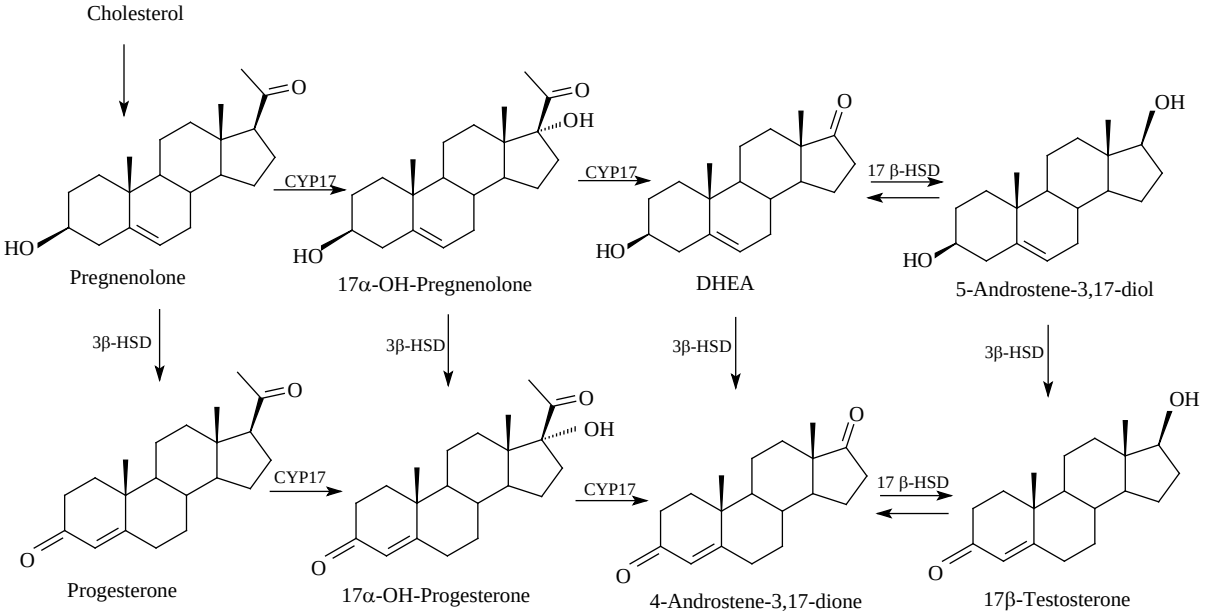


Fig. 2 : Study design

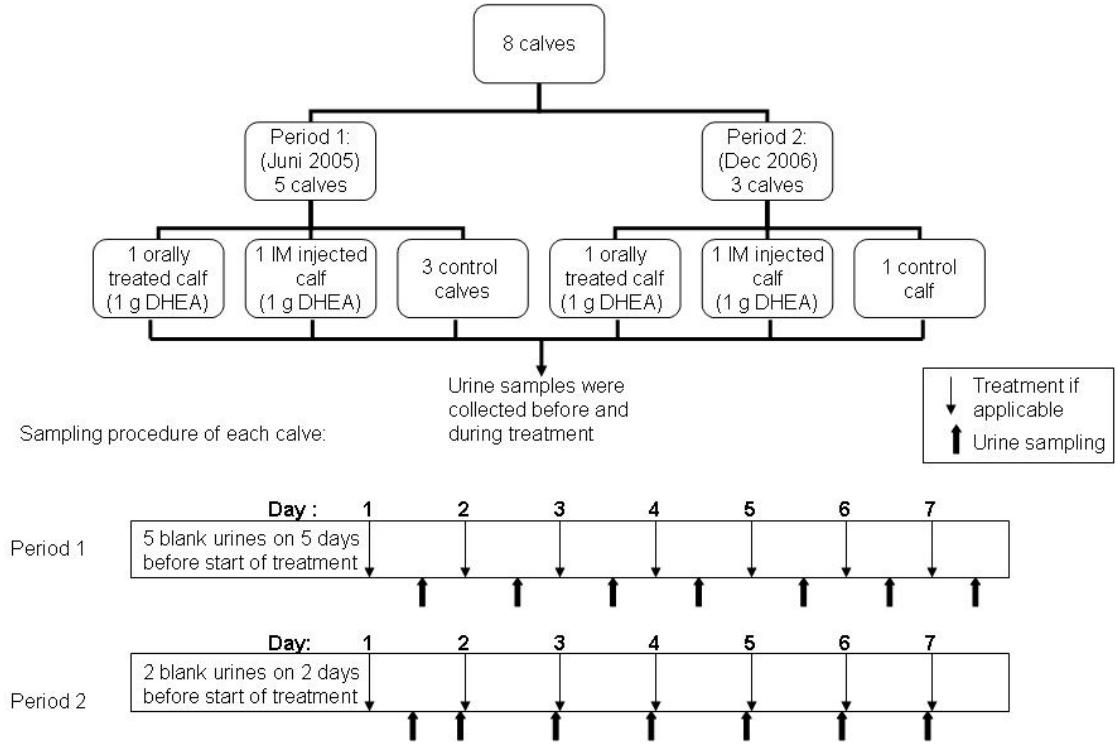
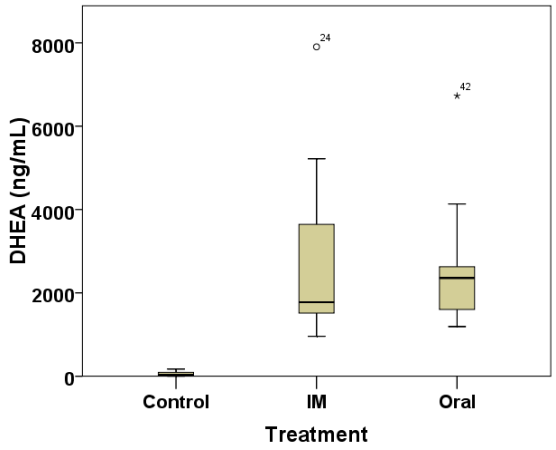


Fig. 3 : Urinary levels of the sulphated form of DHEA (A), 5-Andro (B), α-T (C) and β-T (D) of the control group (control, n=29), intramuscularly treated group (IM, n=14) and orally treated group (oral, n=13)

o Outlier, * extreme value

A)



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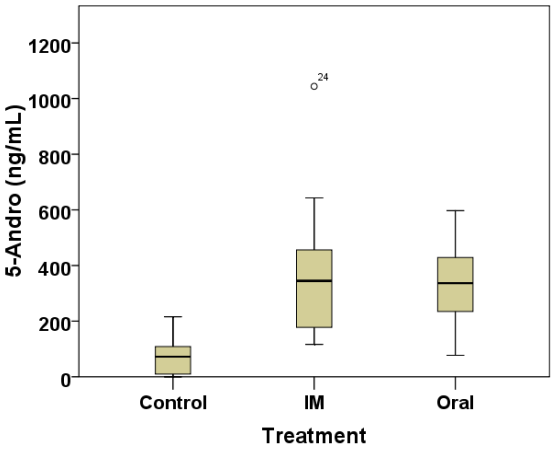

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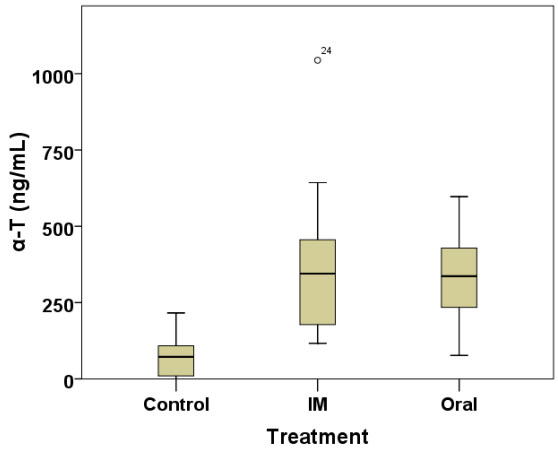

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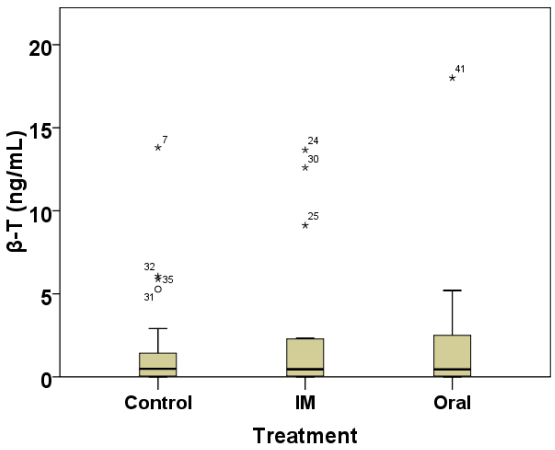


Fig. 4 : Urinary levels of the free form of DHEA (A), 5-Andro (B), α-T (C), β-T (D) and AED (E) of the control group (control, n=29), intramuscularly treated group (IM, n=14) and orally treated group (oral, n=13)

o Outlier, * extreme value

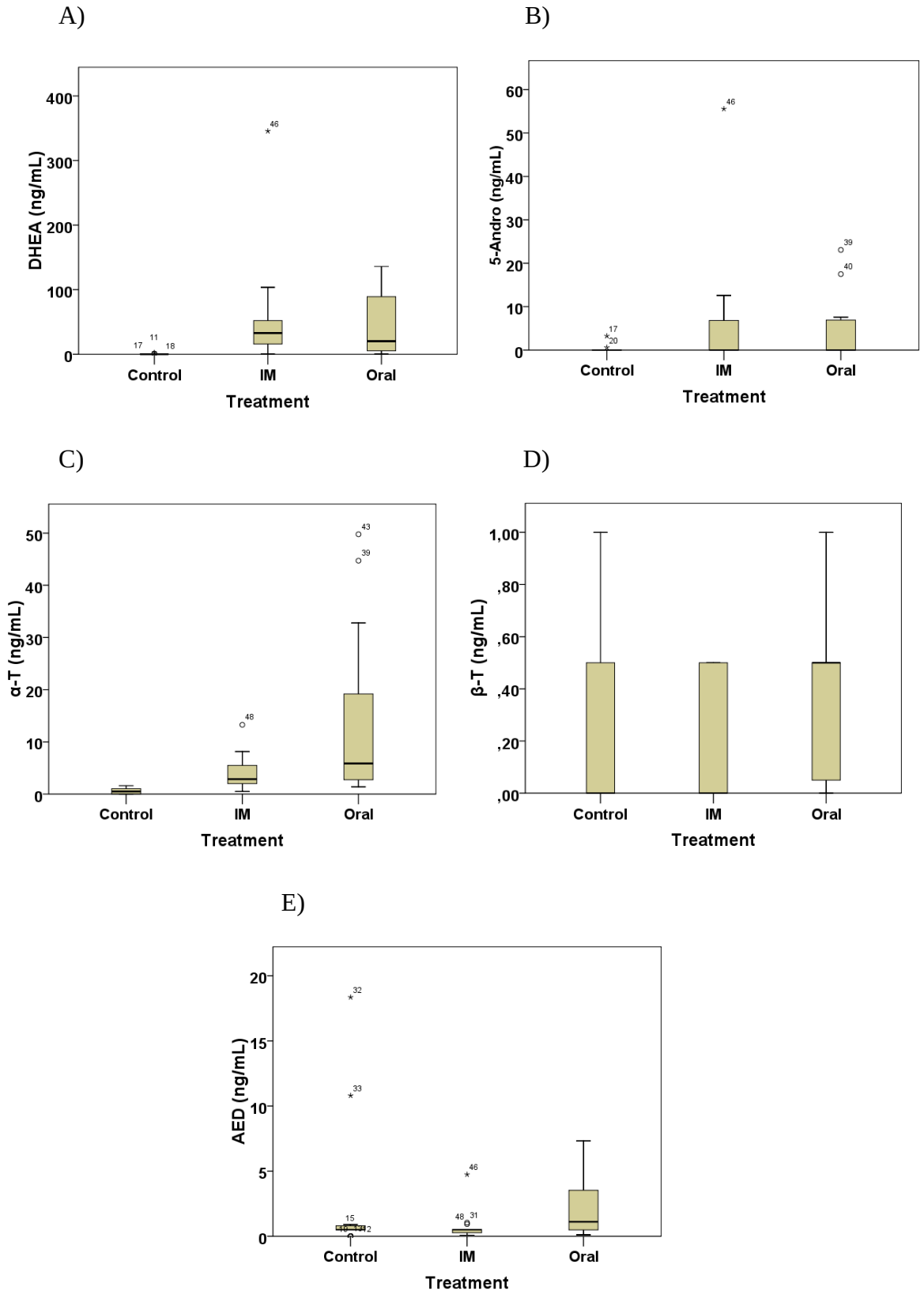
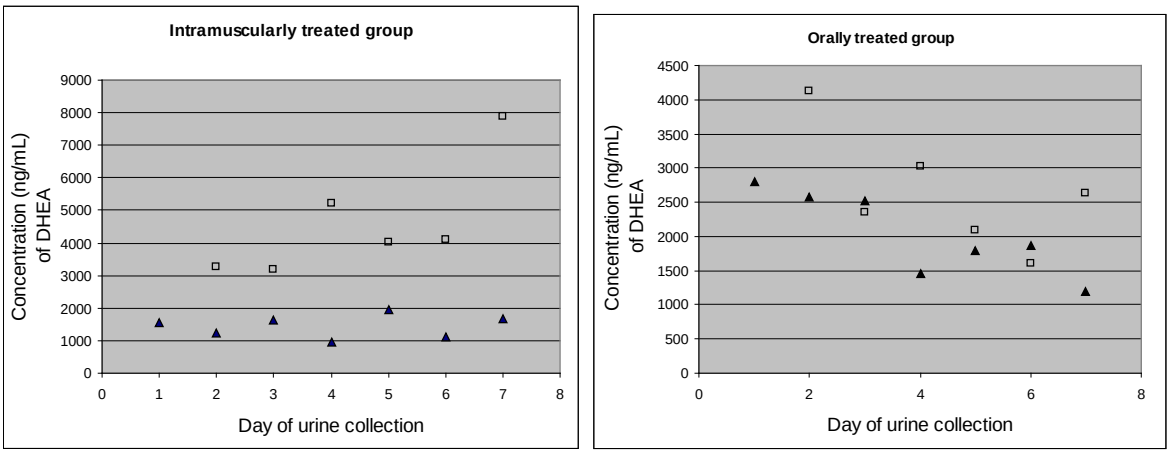


Fig. 5 : Urinary levels of DHEA (A) and 5-Andro (B) of the 7 consecutive days of treatment from two treated calves of group 1 (filled triangles) and two treated calves of group 2 (open squares)

A)



B)

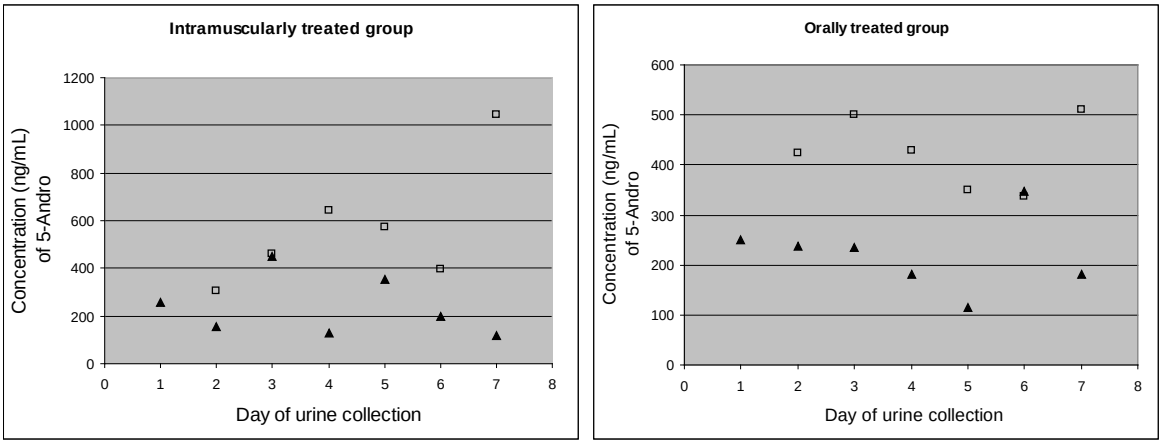


Table 1 : Gradient elution program for the separation of the precursors and metabolites of DHEA

Time(min)	Flow rate (mL min^{-1})	%A	%B	Curve
0	0.3	45	55	Initial
1	0.3	45	55	1
8	0.3	40	60	9
13	0.3	40	60	1
14	0.3	0	100	6
22	0.3	0	100	1
23	0.3	45	55	6
30	0.3	45	55	1

A : water/MeOH/FA (89.7/10/0.3); B : MeOH/FA (99.7/0.3)

Table 2 : Precursor and product ions. cone voltages and collision energies for each transition monitored in MRM (ESI+) analysis

Compound	Precursor ion (m/z)	Molecular Ion	Cone voltage (V)	Product ion (m/z)	Collision energy (eV)
T (α and β)	289.4	$[M+H]^+$	18	109.1*	33
				97.1	
DHEA	253.4	$[M+H-2H_2O]^+$	25	225.0	15
				197.0*	17
AED	287.2	$[M+H]^+$	36	109.3*	18
				97.2	
5-Andro	273.4	$[M+H-H_2O]^+$	27	255.3*	10
				159.2	12
Preg	317.3	$[M+H]^+$	24	281.4*	15
				255.3	13
OH-Preg	333.2	$[M+H]^+$	22	297.6*	10
				133.2	18
MT-D_3	306.0	$[M+H]^+$	50	109.0*	30
				97.0	
NT-D_3	278.0	$[M+H]^+$	45	109.0*	28
				83.0	

*Most abundant product ion

Table 3a : Basal urinary concentrations (free and sulfated form) of DHEA and its metabolites of the calves included in group 1 before treatment

	n	Mean ± SE ($ng.mL^{-1}$)	Median ($ng.mL^{-1}$)	Minimum ($ng.mL^{-1}$)	Maximum ($ng.mL^{-1}$)
Free form of					
DHEA	24	0.0 ± 0.0	0.0	0.0	0.0
5-Andro	24	0.3 ± 0.9	0.0	0.0	3.9
α-T	24	0.7 ± 0.9	0.5	0.0	4.9
β-T	24	0.1 ± 0.2	0.0	0.0	0.5
AED	24	0.4 ± 0.3	0.5	0.0	1.3
Sulfated form of					
DHEA	26	45.6 ± 40.7	40.8	4.2	131.4
5-Andro	26	29.9 ± 32.6	21.3	0.0	132.9
α-T	26	9.1 ± 11.9	3.2	0.0	39.6
β-T	26	2.5 ± 4.3	0.4	0.0	16.5

Table 3b : Basal urinary concentrations (free and sulfated form) of DHEA and its metabolites of the calves included in group 2 before treatment

	n	Mean ± SE ($ng.mL^{-1}$)	Median ($ng.mL^{-1}$)	Minimum ($ng.mL^{-1}$)	Maximum ($ng.mL^{-1}$)
Free form of					
DHEA	7	0.0 ± 0.0	0.0	0.0	0.0
5-Andro	7	0.0 ± 0.0	0.0	0.0	0.0
α-T	7	0.3 ± 0.4	0.0	0.0	0.8
β-T	7	0.2 ± 0.3	0.0	0.0	0.5
AED	7	0.4 ± 0.2	0.5	0.0	0.5
Sulfated form of					
DHEA	6	43.9 ± 49.1	23.1	11.8	138.2
5-Andro	6	70.2 ± 63.9	69.8	0.0	158.7
α-T	6	72.2 ± 49.5	57.6	15.5	134.0
β-T	6	8.4 ± 7.2	6.9	1.5	22.2

Table 4 : Urinary concentrations (free and sulfated form) of DHEA and its metabolites of the control animals before and during the period of treatment

	Before or during treatment	n	Mean ± SE ($ng.mL^{-1}$)	Median ($ng.mL^{-1}$)	Minimum ($ng.mL^{-1}$)	Maximum ($ng.mL^{-1}$)
Free form of						
DHEA	before	17	0.0 ± 00	0.0	0.0	0,0
	during	29	0.3 ± 0.6	0.0	0.0	2.4
5-Andro	before	17	0.4 ± 1.1	0.0	0.0	3.9
	during	29	0.1 ± 0.6	0.0	0.0	3.2
α-T*	before	14	1.0 ± 1.2	0.6	0.0	4.9
	during	29	0.6 ± 0.6	0.5	0.0	1.6
β-T*	before	14	0.2 ± 0.2	0.0	0.0	0.5
	during	29	0.3 ± 0.4	0.0	0.0	1.0
AED	before	17	0.5 ± 0.3	0.5	0.0	1.3
	during	29	1.4 ± 2.9	0.5	0.0	18.4
Sulfated form of						
DHEA	before	17	43.2 ± 36.1	42.5	6.2	131.4
	during	29	51.1 ± 46.7	34.7	0,0	171.4
5-Andro	before	17	28.1 ± 31.2	15.7	0,0	118.8
	during	29	75.8 ± 63.7	72.4	0,0	215.9
α-T*	before	15	7.6 ± 10.0	2.6	0,0	32.1
	during	29	12.7 ± 15.4	4.8	0,0	47.1
β-T*	before	15	1.5 ± 2.6	0.3	0,0	9.9
	during	29	1.1 ± 2.9	0.1	0,0	13.8

*: includes only data from group 1