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Control system for detection of the illegal use of naturally occurring steroids in calves

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ABSTRACT

Within the scope of the National Plan for Hormone Control in The Netherlands, a study was performed to develop a system for control of the illegal use of three naturally occurring hormones [oestradiol-17β (E_2-17β), testosterone (T), progesterone (P)] for fattening purposes in animal production. Using a specific high-performance liquid chromatographic–radioimmunoassay method, reference values were established for concentrations of E_2-17β, T and P and some of their metabolites in blood plasma and urine from untreated male and female veal calves. E_2-17β levels of both male and female calves were <0.01 μg/l in blood plasma and <0.2 μg/l in urine. For male veal calves levels of T and epitestosterone (epiT) in blood plasma and urine varied widely. The P levels were <0.1–0.3 μg/l in blood plasma and <0.6–10 μg/l in urine from both male and female calves. To investigate the effect of anabolic treatment on the hormone levels in plasma and excreta, male veal calves were injected, subcutaneously into the dewlap, with a solution containing 20 mg of E_2-17β benzoate and 200 mg of T propionate in 5 ml of arachis oil. Only the levels of E_2-17β and E_2-17α in blood plasma and excreta were elevated until about one week after injection, compared with the untreated control calves and the reference values. T and epiT levels were similar in plasma and excreta from both untreated and treated animals.

INTRODUCTION

Steroid hormones can be used to improve the growth of cattle reared for meat production. Many experimental studies have shown an increased body weight, an increase of protein deposition and a decrease of fat deposition as a result of steroid administration [1]. These hormones can be classified into two groups: the natural or endogenous steroids and the synthetic or exogenous steroids. The use of either category for fattening purposes has been prohibited in The Netherlands for many years and in all EC countries since 1 January 1988 (European Commis-

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sion Directive 86/469). One of the implications of this ban is the necessity of developing a system for monitoring for the presence of anabolic steroids in blood plasma or excreta of animals reared for meat production. The presence of exogenous steroids in, for example, urine, confirmed according to analytical criteria (European Commission Directive 87/410), is proof of illegal administration.

However, the situation is much more complex for endogenous steroids. The presence alone is no proof at all of illegal administration. The physiological levels of these steroids have to be taken into account. This means that the physiological concentrations of the three most important endogenous steroids, oestradiol-17β (E_2-17β), testosterone (T) and progesterone (P), and their quantitatively most important metabolites, oestradiol-17α (E_2-17α) and epitestosterone (epiT), in blood (plasma or serum), tissue, organs and excreta (urine, bile and faeces) have to be known in order to be able to distinguish between the physiological situation and the illegal administration of preparations containing one or more endogenous steroids.

The large variations found for the various steroids in the different matrices as reported in the literature led us to establish reference values of the naturally occurring steroids in blood plasma and excreta of untreated male and female veal calves of different age using a specific high-performance liquid chromatographic–radioimmunoassay (HPLC–RIA) technique. Furthermore, an experiment was performed, in which an oestradiol-17β–testosterone cocktail was injected into the dewlap of male veal calves, to investigate whether this injection results in elevated levels of the steroids. Hormone concentrations measured in plasma, urine and faeces were compared with concentrations in samples from untreated control and reference values.

EXPERIMENTAL

Equipment

HPLC was used as described previously [2]. In short, a Knauer stainless-steel column (125 mm × 4.6 mm I.D.) was packed with LiChrosorb-Diol (5 μm; Merck, Darmstadt, Germany). As mobile phase 5% (v/v) isopropyl alcohol (Rathburn Chemicals, Walkerburn, U.K.) in *n*-hexane (J. T. Baker, Phillipsburg, NJ, U.S.A.) was used at a flow-rate of 1.2 ml/min.

Reversed-phase C_{18} cartridges (J. T. Baker) were activated by washing with 2.5 ml of 100% methanol followed by 2.5 ml of distilled water.

Chemicals

All chemicals used were of HPLC grade or p.a. quality. Extrelut prepacked columns (Art. No. 15371) were obtained from Merck. Dextrane T70 was obtained from Pharmacia (Uppsala, Sweden). Bovine serum albumin (BSA) was obtained from Armour Pharmaceutical (Eastbourne, U.K.) and *Helix pomatia* juice from Réactifs IBF (Villeneuve-la-Garenne, France). Oestradiol-17β (E_2-

17β; Art. No. E-1132), oestradiol-17α (E_2-17α; Art. No. E-8750), testosterone (T; Art. No. T-1500), epitestosterone (epiT; Art. No. E.-5878) and progesterone (P; Art. No. P-9776) were obtained from Sigma (St. Louis, MO, U.S.A.). The tritiated steroids were purified by HPLC before use. [2,4,6,7-^{3}H(N)]Oestradiol-17β (specific activity 100 Ci/mmol), [1,2,6,7-^{3}H]testosterone (95 Ci/mmol) and [1,2,6,7-^{3}H]progesterone (115 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). [2,4-^{3}H(N)]Oestradiol-17α (43 Ci/mmol) was obtained from Amersham (Houten, The Netherlands), and [1,2-^{3}H(N)]epitestosterone (49 Ci/mmol) from Wien Labs. (Succasunna, NJ, U.S.A.).

The antiserum against oestradiol-17β (acquired within our Institute) was raised in rabbits with 17β-oestradiol–6-carboxymethyloxime (6-CMO)-bovine serum albumin (BSA) as the immunogen. Cross-reactions, tested according to Abraham [3], were as follows: oestradiol-17β, 100%; oestrone, 0.1%; oestriol, 0.5%; testosterone, 0.4%. The antiserum against oestradiol-17α (obtained from Biogenesis, Bournemouth, U.K.) was raised in rabbits against 17α-oestradiol–6-CMO-BSA, and cross-reacted with oestradiol-17α (100%), oestradiol-17β (0.4%), oestrone (0.2%) and oestriol (0.04%). The antiserum against testosterone, kindly donated by Dr. F. H. de Jong (Department of Biochemistry II, Medical Faculty, Erasmus University, Rotterdam, The Netherlands) was obtained after immunizing rabbits with testosterone–3-CMO-BSA. It cross-reacted with testosterone (100%), dihydrotestosterone (60%), 5α-androstane-3α,17β-diol (3%), 5α-androstane-3β,17β-diol (1.3%) and epitestosterone (0.5%). The antiserum against epitestosterone was obtained from Biogenesis. It was raised in rabbits against epitestosterone–3-CMO-BSA and cross-reacted with epitestosterone (100%), 5α-epitestosterone (79%), androstenedione (3.7%), testosterone (1.8%), 5α-androstane-3β,17β-diol (0.4%), 5α-dihydrotestosterone (<0.4%) and 17α-oestradiol (<0.3%). The antiserum against progesterone (acquired within our Institute) was raised in rabbits with 11α-hydroxyprogesterone-hemisuccinate-BSA as the immunogen. It cross-reacted with progesterone (100%), 17-hydroxyprogesterone (0.9%), cortisol (2.2%), corticosterone (4.5%), desoxycorticosterone (4%) and pregnenolone (4%).

Methods

The HPLC–RIA method was a modification of the method originally described by Jansen *et al.* [4]. A schematic diagram of the method is presented in Fig. 1. A detailed description of the method is given elsewhere [5]. Data concerning the recovery of spiked samples (Table I), intra-assay variation (Table II) and inter-assay variation (Table III) are presented.

Samples for reference values

Blood collected via the vena jugularis in heparinized tubes and urine samples were obtained from calves in the morning, 2–4 h after feeding. The calves not treated with anabolic steroids were reared by experimental farms of Dutch milk

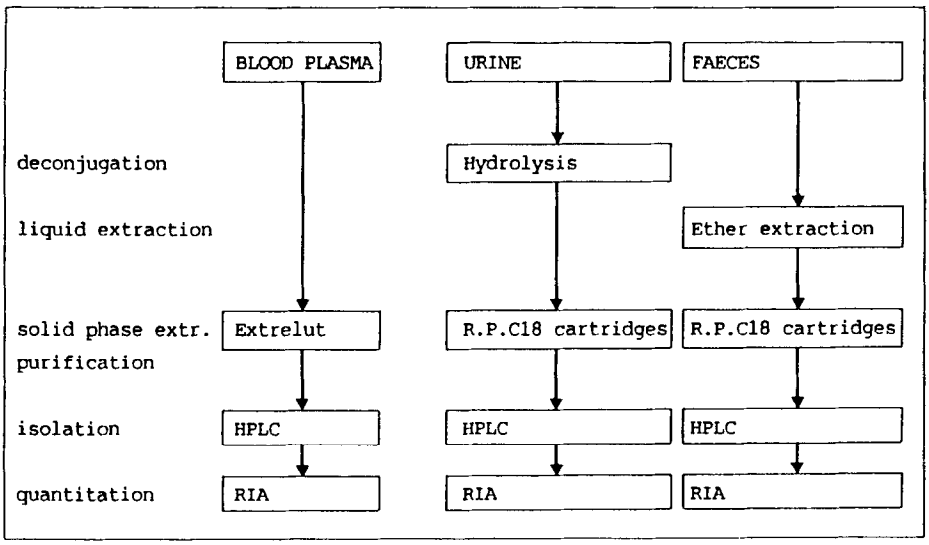

Fig. 1. General scheme of the HPLC–RIA procedure.

replacer industries. Samples were collected from calves of different ages (Table IV).

Free (unconjugated) E_2-17β, T, epiT and P were determined in blood plasma

TABLE I

STANDARD RECOVERY OF THE HPLC–RIA PROCEDURE

Steroid	Blood plasma (n = 3)			Urine (n = 3)		
	Added (μg/l)	Measured (μg/l)	%	Added (μg/l)	Measured (μg/l)	%
Oestradiol-17β	0.025	0.029	116	0.8	0.92	115
	0.05	0.056	112	1.6	1.5	94
	0.10	0.087	87	3.2	3.3	103
Oestradiol-17α	–	–	–	5	4.8	96
			–	10	10.3	103
	–	–	–	20	18	90
Testosterone	0.4	0.4	100	1.6	1.57	98
	0.8	0.91	113	3.2	3.04	95
	1.6	1.55	97	4	4.02	101
Epitestosterone	1.0	1.0	100	40	43	108
	5.0	5.8	116	50	46	92
	10.0	10.8	108	100	112	112
Progesterone	0.1	0.11	110	–	–	–
	0.2	0.19	95	–	–	–
	0.4	0.47	117	–	–	–

TABLE II

INTRA-ASSAY VARIATION OF THE HPLC–RIA PROCEDURE AT VARIOUS STEROID CONCENTRATIONS

Steroid	Blood plasma (n = 10)		Urine (n = 10)	
	Mean ± S.D. (μg/l)	C.V. (%)	Mean ± S.D. (μg/l)	C.V. (%)
Oestradiol-17β	0.04 ± 0.004	10	–	
Oestradiol-17α	–		5.3 ± 0.2	3.8
	–		1.4 ± 0.09	6.4
Epitestosterone	4.8 ± 0.3	6.3	5.6 + 0.9	16
Progesterone	0.21 ± 0.03	14	1.3 ± 0.3	23

samples. Total (conjugated and unconjugated) E_2-17β, E_2-17α, T, epiT and P were determined in urine samples collected at 15 and 28 weeks of age.

Animal experiment

The experiment involved 40 male veal calves of the Friesian-Dutch and Holstein-Friesian breeds, which were individually identified with a plastic earmark. The calves were purchased at an age of 0–1 week and a weight of *ca.* 40 kg. They were individually housed in wooden boxes on a slatted floor in a stable optimized for ventilation and temperature. The animals were fed twice daily in agreement with normal practice. Until the age of 2 months the calves were fed milk replacer

TABLE III

INTER-ASSAY VARIATION OF THE HPLC–RIA PROCEDURE AT VARIOUS STEROID CONCENTRATIONS

Steroid	Blood plasma			Urine		
	Mean ± S.D. (μg/l)	C.V. (%)	n	Mean ± S.D. (μg/l)	C.V. (%)	n
Oestradiol-17β	0.02 ± 0.006	30	8	0.12 ± 0.04	33	16
	0.03 ± 0.009	30	10	0.07 ± 0.02	29	17
	0.20 ± 0.029	15	10			
Oestradiol-17α	–	–	–	6.5 ± 1.4	22	9
	–	–	–	3.7 ± 0.7	19	9
Testosterone	4.4 ± 0.83	19	10	13.8 ± 2.5	18	9
	7.5 ± 1.1	15	10	3.7 ± 0.5	14	9
Epitestosterone	6.9 ± 0.4	5.8	7	75 ± 15	20	7
	3.6 ± 0.6	17	7	13 ± 2.4	18	5
Progesterone	0.4 ± 0.1	25	5	5.8 ± 0.7	12	4
	1.3 ± 0.3	23	6	4.7 ± 0.7	15	4
	9.1 ± 1.0	11	6	2.8 ± 0.6	21	4

TABLE IV

SAMPLE COLLECTION FOR RECORDING REFERENCE VALUES

Sample	Age (weeks)	Number
Males		
Plasma and urine	15	50
Plasma	22	61
Plasma and urine	28	47
Females		
Plasma and urine	15	33
Plasma	24–25	34
Plasma and urine	28	50

Purple, after that time milk replacer Yellow (DMV Campina, Veghel, The Netherlands), both suspended in warm water; the amount and concentration of the solution were age-dependent.

At the age of 19 weeks the calves were distributed among three treatment groups and one control group of ten animals each on the basis of body weight, weight gain between 0 and 19 weeks, haemoglobin content and health.

The animals of the treatment groups were injected, subcutaneously in the right part of the dewlap, with 5 ml of arachis oil containing 20 mg of oestradiol-17β benzoate, 200 mg of testosterone propionate and 0.25 ml of benzyl alcohol (Intervet International, Boxmeer, The Netherlands). The concentration of steroids in the cocktail was checked by HPLC with UV detection. The animals of the control group were injected with a placebo (5 ml of arachis oil containing 0.25 ml of benzyl alcohol). Injection was performed as follows: group I: at the age of 19 weeks with the placebo; group II: at the age of 19 weeks with the hormone solution; group III: at the age of 19 and 23 weeks with the hormone solution; group IV: at the age of 23 weeks with the hormone solution.

Blood (heparinized) from the left vena jugularis, urine and faeces were collected weekly and 2, 3 and 5 days after injection. Blood plasma was analysed for unconjugated E_2-17β, T and epiT, urine for total E_2-17β, E_2-17α, T and epiT, and faeces for unconjugated E_2-17β, E_2-17α, T and epiT.

Statistics

Reference values were taken to be those concentrations between P2.5 and P97.5 according to Rümke and Bezemer [6]. From the reference values, decision levels (limits at which one can decide whether or not an animal has been treated with a naturally occurring hormone) were calculated by fitting a truncated log–normal distribution to the steroid concentrations. Decision levels were fixed at the 99th percentile of this distribution. Possible age-related differences in concentration were analysed statistically by the test of Wilcoxon.

Data from the animal experiment were analysed statistically by the Wilcoxon

test. Because a non-Gaussian distribution of the results within a group was found in most cases, the median and range are presented instead of mean and standard deviation.

RESULTS

Reference values

The reference values obtained after analysis of the natural anabolics and their quantitatively most important metabolites in plasma and urine samples are summarized in Tables V and VI, respectively.

TABLE V

REFERENCE VALUES OF NATURALLY OCCURRING UNCONJUGATED STEROIDS IN BLOOD PLASMA OF MALE AND FEMALE VEAL CALVES

Steroid		Reference value (μg/l)		
		15 weeks	22 weeks	28 weeks
Male calves				
Oestradiol-17β (detection limit: 0.01 μg/l)		<0.01	<0.01	<0.01
Testosterone (detection limit: 0.1 μg/l)		0.4–2.3	0.3–7.5	0.5–5.0
	Median	0.8	1.5	1.3
Epitestosterone (detection limit: 0.2 μg/l)		2.9–11	–	0.3–2.3
	Median	7.1	–	0.8
Progesterone (detection limit: 0.1 μg/l)		<0.1–0.2	<0.1	<0.1
	Median	<0.1		
Epi/T ratio		3–13	–	0.3–1.4
	Median	8	–	0.5
		15 weeks	24–25 weeks	28 weeks
Female calves				
Oestradiol-17β (detection limit: 0.01 μg/l)		<0.01	<0.01	<0.01
Testosterone (detection limit: 0.1 μg/l)		<0.1	<0.1	<0.1
Epitestosterone (detection limit: 0.2 μg/l)		<0.2–0.3	–	<0.2–0.4
	Median	<0.2	–	<0.2
Progesterone (detection limit: 0.1 μg/l)		<0.1–0.2	<0.1	<0.1–1.0
	Median	0.15		0.1

TABLE VI

REFERENCE VALUES OF NATURALLY OCCURRING TOTAL (CONJUGATED AND UNCONJUGATED) STEROIDS IN URINE FROM MALE AND FEMALE VEAL CALVES

Steroid		Reference value (μg/l)			
		Male calves		Female calves	
		15 weeks	28 weeks	15 weeks	28 weeks
Oestradiol-17β (detection limit: 0.05 μg/l)	Median	<0.05–0.1 <0.05	<0.05–0.1 <0.05	<0.05	<0.05
Oestradiol-17α (detection limit: 0.3 μg/l)	Median	0.3–5.0 1.2	0.6–5.0 2.0	<0.3–2.5 0.8	0.4–3.8 1.5
Testosterone (detection limit: 0.5 μg/l)	Median	0.5–4.0 1.0	1.1–28 3.7	<0.5–1.0 <0.5	<0.5–2.2 1.1
Epitestosterone (detection limit: 0.8 μg/l)	Median	15–180 40	20–125 41	2.1–19 6	3.5–28 17
Progesterone (detection limit: 0.6 μg/l)	Median	<0.6–2.0 1.0	0.8–5.5 2.2	<0.6–2.5 0.9	1.4–7.0 3.2
EpiT/T ratio	Median	21–60 45	3–25 9.5	– –	– –

All E_2-17β levels, except two (0.02 μg/l), of the 280 blood plasma samples from male and female calves were below the detection limit of the assay (<0.01 μg/l).

In blood plasma samples from male calves an increase ($p < 0.01$) of the T levels was found at 28 weeks of age. On the other hand, epiT concentrations in blood plasma samples collected at 28 weeks of age were lower ($p < 0.001$) than in samples collected at 15 weeks of age, resulting in a significant change of the epiT/T ratio between the two ages.

T and epiT levels in blood plasma from female calves were found to be near or below the detection limit of the assay (0.1 and 0.2 μg/l, respectively). The P concentration in blood plasma from female calves was significantly higher than in blood plasma from male calves. In female calves at 28 weeks of age a wider range of P levels was found than at 15 weeks of age.

In urine samples from both male and female calves the median of total E_2-17β concentration was below the detection limit of the assay (<0.05 μg/l). Levels of about one third of the samples analysed ranged between 0.05 and 0.2 μg/l. Almost all E_2-17α values were higher than the detection limit of the assay (>0.03 μg/l), as was found for T and epiT in urine from male calves. An age effect for epiT that

was found in blood plasma was not observed in urine. However, the epiT/T ratio in urine of male calves was age-dependent, owing to the higher mean excretion of T at 28 weeks of age.

Animal experiment

Steroids in blood plasma, urine and faeces from animals of groups II and IV were analysed only in samples collected 1, 2 and 3 weeks after injection. The concentrations of steroids in the matrices from animals of these groups were comparable with those from animals receiving two injections (group III). For the sake of brevity, the steroid concentrations in samples from groups II and IV have been omitted.

Blood plasma

The median and range for E_2-17β, T and epiT levels in blood plasma from animals of groups I and III are summarized in Table VII.

The unconjugated E_2-17β levels in blood plasma collected from calves of the untreated group were consistently below the detection limit of the assay (<0.01 μg/l). In the treated groups, E_2-17β levels were above the detection limit for *ca.* 1 week after injection.

T levels in the control animals were comparable with those in the treated calves at all sampling times, except for two days after injection when T concentrations were higher ($p < 0.05$) in the treated animals.

The unconjugated epiT levels in blood plasma from animals of all three treated groups were similar to those of the control animals throughout the experimental period.

Urine

The steroid levels found in urine samples from calves of groups I and III are summarized in Table VIII.

E_2-17β levels in samples from treated animals 2, 5 and 7 days after the first injection and 7 days after the second injection were higher ($p < 0.05$) than in those from the control animals.

E_2-17α levels in urine samples from control calves varied between 0.8 and 18 μg/l. At 2, 5 and 7 days after injection an increase ($p < 0.05$) of E_2-17α excretion was found in samples from treated animals.

After injection of the hormone cocktail, urinary T excretion for the treated animals (0.7–21 μg/l) was comparable with that for untreated animals (0.7–22 μg/l).

No differences in epiT levels of urine samples were found between controls and treated calves.

Faeces

The results of the analysis of faecal samples are summarized in Table IX. In

TABLE VII

LEVELS OF NATURALLY OCCURRING STEROIDS IN BLOOD PLASMA FROM THE UNTREATED GROUP (I) AND THE GROUP (III) TREATED AT 19 AND 23 WEEKS OF AGE

Values are in μg/l.

Steroid			Days after first injection								
			0[a]	2	5	7	14	28[b]	30	32	35
Oestradiol-17β											
Group I:	median		<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
	range	(H[c])	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Group III:	median		<DL	0.23	0.06	0.04	<DL	0.25	0.13	0.04	0.03
	range	(L[c])	<DL	0.15	0.02	0.01	<DL	0.15	0.05	0.01	<DL
		(H)	<DL	0.41	0.09	0.07	0.02	0.97	0.19	0.08	0.05

			0[a]	2	7	21	28[b]	35	49
Testosterone									
Group I:	median		1.7	1.6	1.7	6.0	–	6.5	3.9
	range	(L)	0.8	0.5	0.4	0.5	–	0.7	1.0
		(H)	3.9	4.4	8.3	9.4	–	23	27
Group III:	median		2.5	7.7	3.1	0.7	–	1.7	1.8
	range	(L)	0.6	4.6	1.3	0.2	–	0.7	0.2
		(H)	10	14	6.4	5.8	–	13	14
Epitestosterone									
Group I:	median		2.1	2.1	1.8	1.9	–	2.3	1.9
	range	(L)	0.8	0.7	1.0	0.4	–	0.3	0.4
		(H)	20	11	12	17	–	5.1	11
Group III:	median		2.6	2.1	1.2	0.6	–	0.6	0.8
	range	(L)	0.6	0.6	0.4	0.3	–	0.4	0.2
		(H)	4.1	5.6	4.4	3.4		2.5	5.6

[a] Time of first injection with the hormone cocktail or placebo.
[b] Time of second injection with the hormone cocktail.
[c] H = highest value; L = lowest value.

faecal samples from the untreated group, E_2-17β levels varied between 0.1 and 2.3 μg/kg. In samples from the treated animals a significant increase of the mean E_2-17β concentration was still found 14 days after administration of the cocktail. However, levels in samples from the control animals and those from treated animals were found to overlap.

Levels of E_2-17α were higher ($p < 0.05$) in faeces from the treated group 5 days after the first injection and 7 days after the second injection than in faecal samples from the control animals. In samples from the latter group a range of < 1.5 μg/kg (detection limit) to 12 μg/kg was found. At 5 days after the first administration

TABLE VIII

LEVELS OF NATURALLY OCCURRING STEROIDS IN URINE FROM THE UNTREATED GROUP (I) AND THE GROUP (III) TREATED AT 19 AND 23 WEEKS OF AGE

Values are in μg/l.

Steroid			Days after first injection								
			0[a]	2	5	7	14	28[b]	30	32	35
Oestradiol-17β											
Group I:	median		<DL	0.16	0.15	<DL	<DL	<DL	0.12	0.06	<DL
	range	(L[c])	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
		(H[c])	0.17	0.32	0.66	0.28	0.34	0.24	0.39	0.51	0.16
Group III:	median		<DL	6.0	3.0	0.72	0.31	15	5.8	3.6	1.5
	range	(L)	<DL	1.7	1.9	0.28	0.14	4.5	2.9	1.2	0.33
		(H)	<DL	15	6.0	6.4	3.7	27	26	6.8	3.8

			0[a]	2	5	7	21	28[b]	35	49
Oestradiol-17α										
Group I:	median		2.7	5.8		3.6	2.5	–	2.8	5.5
	range	(L)	1.0	2.0		0.8	1.4	–	2.0	1.3
		(H)	8.2	18		12	16	–	6.9	17
Group III:	median		2.7	78	30	15	4.3	–	–	14
	range	(L)	0.83	25	10	7.6	1.8	–		9.1
		(H)	4.6	311	141	33	26			25
Testosterone										
Group I:	median		2.0	1.6	–	2.7	8.6	–	8.8	5.0
	range	(L)	<DL	0.7	–	0.9	2.7	–	8.0	3.6
		(H)	13	3.8	–	5.8	18	–	21	18
Group III:	median		1.2	4.2	–	3.0	2.9	–	7.7	3.9
	range	(L)	<DL	0.7	–	1.1	1.1	–	4.1	2.9
		(H)	2.3	22	–	10	11	–	11	8.9
Epitestosterone										
Group I:	median		42	35	–	32	58	–	45	25
	range	(L)	6.0	13	–	15	5.7	–	28	18
		(H)	420	86	–	263	291	–	144	455
Group III:	median		20	44	–	24	33	–	33	23
	range	(L)	5.6	14	–	14	13	–	19	15
		(H)	71	279	–	78	90	–	69	46

[a] Time of first injection with the hormone cocktail or placebo.
[b] Time of second injection with the hormone cocktail.
[c] L = lowest value; H = highest value.

and at 7 days after the second administration, levels were 29–228 and 12–77 μg/kg, respectively.

T and epiT levels in faecal samples from both the control animals and the

calves treated with the oestradiol–testosterone cocktail consistently overlapped. Mean epiT excretion was *ca.* ten times higher than mean T excretion, and varied between 6 and 79 μg/kg for untreated calves and between 4 and 101 μg/kg for treated calves.

TABLE IX

LEVELS OF NATURALLY OCCURRING STEROIDS IN FAECES FROM THE UNTREATED GROUP (I) AND THE GROUP (III) TREATED AT 19 AND 23 WEEKS OF AGE

Values are in μg/kg.

Steroid			Days after first injection						
			0[a]	5	14	21	28[b]	35	49
Oestradiol-17β									
Group I:	median		–	0.27	–	0.10	–	–	0.23
	range	(L[c])	–	0.14	–	0.07	–	–	0.07
		(H[c])	–	2.3	–	0.85	–	–	0.55
Group III:	median		–	6.8	2.1	0.43	–	2.6	0.50
	range	(L)	–	3.6	0.08	0.12	–	0.27	0.19
		(H)	–	23	5.2	1.3	–	9.6	1.4
Oestradiol-17α									
Group I:	median		–	3.1	–	1.9	–	–	3.1
	range	(L)	–	<DL	–	<DL	–	–	<DL
		(H)	–	12	–	12	–	–	7.5
Group III:	median		–	104	16	4.8	–	33	7.4
	range	(L)	–	29	1.8	1.8	–	12	1.8
		(H)	–	228	50	9.2	–	77	28
Testosterone									
Group I:	median		–	2.8	–	1.6	–	–	1.1
	range	(L)	–	1.1	–	<DL	–	–	0.6
		(H)	–	6.9	–	3.6	–	–	2.2
Group III:	median		–	2.9	1.7	1.0	–	1.5	0.8
	range	(L)	–	1.4	0.6	0.7	–	0.5	0.5
		(H)	–	5.9	2.7	2.2	–	3.6	1.0
Epitestosterone									
Group I:	median		–	20	–	29	–	–	35
	range	(L)	–	5.5	–	6.0	–	–	18
		(H)	–	43	–	66	–	–	79
Group III:	median		–	47	19	14	–	24	14
	range	(L)	–	20	8.0	5.4	–	3.5	5.9
		(H)	–	101	29	32	–	89	32

[a] Time of first injection with the hormone cocktail or placebo.

[b] Time of second injection with the hormone cocktail.

[c] L = lowest value; H = highest value.

DISCUSSION

The detection limits of the assays (indicated in the tables as DL) were calculated at a mean relative binding (B/B_0) of 90% and corrected for recovery. These relative bindings were obtained during the successive assays performed.

The physiological levels of steroids in blood plasma and excreta of cattle can depend on nutrition, breed, age, sex, housing, photoperiod, stress, season and circadian rhythm [7]. Nutrition, especially the dietary fibre component, can influence steroid excretion in faeces and urine and the levels in blood plasma through the enterohepatic circulation, as has been shown in vegetarian and omnivorous humans [8] and in rats fed a high- or low-fibre diet [9]. As nutrition is standardized for most veal calves, this parameter may be negligible. A second variable may be the breed in case of different starting ages for puberty [10]. Steroid levels may depend on age, and hence the development of the sex hormone-producing organs. An increase ($p < 0.01$) of T concentration was found in blood plasma of male calves at 28 weeks of age as compared with plasma collected at 15 weeks of age. Conversely, a marked decrease with time was observed for epiT levels ($p < 0.001$). The increased T concentration at the higher age may be caused by increased endogenous production or, more likely, by decreased epimerase activity, resulting in lower epiT levels.

The concentrations of most of the steroids measured in urine from both male and female calves were age-dependent. Only the levels of epiT in urine from male calves were comparable at both sampling times. Female calves reach oestrus from *ca.* 30 weeks of age. This results in a cyclic rise of E_2-17β and P levels in blood and urine.

Anabolic steroids used as growth promotors have to be administered to cattle in a way that enables a slow release of the hormones. For practical reasons, arachis oil was chosen as a solvent, to which 5% benzyl alcohol was added as a preservative. The amount of the steroids injected was equal to the dose administered to calves through an implant in the base of the ear (20 mg of oestradiol-17β benzoate and 200 mg of testosterone propionate per calf and per injection) [11]. As only minimal growth effects were found in this experiment (results not shown) and the enhanced steroid concentrations were short-lasting, it can be expected that, in field conditions, when naturally occurring steroids are administered illegally, steroid levels would be higher and longer-lasting. Administration of the anabolics via an implant in the base of the ear resulted in plasma E_2-17β levels above the decision limit for at least 4 weeks [11].

In blood plasma of untreated animals, almost all E_2-17β levels were below the detecton limit of 0.01 μg/l and could not be determined with the HPLC–RIA method used. This finding agrees with previous studies [11–14]. Higher concentrations of E_2-17β in blood plasma of veal calves have been reported by Schopper [15] and Richou-Bac *et al.* [16], which may be explained by insufficient prepurification of the samples or inadequate specificity of the assay.

The T levels found in blood plasma of untreated male calves at 15 and 28 weeks of age correspond quite well to the levels reported in the literature. In male calves, (unconjugated) T concentrations have been reported of < 1 μg/l (younger than 4 months), up to 4 μg/l (4-7 months) and up to 10 μg/l (older than 7 months) [17–21]. Owing to the large variation, it is not possible to give an indication of the normal ranges of T concentrations.

In faecal samples the highest E_2-17β values found were *ca.* 20 μg/kg. In faeces one has to reckon with a possible intestinal microbial conversion of oestrone (an important metabolite of oestradiol-17β) into oestradiol-17β, which may be dependent on the type of feed [22].

Administration of T to male veal calves may result in a decrease of endogenous T production, probably via a hypophysial negative feedback mechanism. Consequently, the mean levels of T and of epiT, its main metabolite, in blood plasma and excreta from treated animals are equal to or even lower than those from control animals. This was also observed in an experiment in which male veal calves were treated with an oestradiol-17β benzoate–testosterone propionate implant [11].

The epiT/T ratio, which is used for doping control in sports, is not a suitable parameter for control in cattle. The ratio is dependent on the age of the calves, and the ratios in samples from the control animals and those from treated animals are found to overlap. This results in a low sensitivity (number of true positives divided by the total number of treated animals) or, at high decision levels, in a low specificity (number of true negatives divided by the total number of untreated animals).

The calculated decision levels (P99) obtained after log-transformation of values of the reference samples are summarized in Table X. The decision level for E_2-17β in blood plasma was calculated to be 0.01 μg/l (Table X); the sensitivity of this assay will then be 99%. To exclude false-positive results, the decision level for E_2-17β in blood plasma was fixed at 0.02 μg/l (Table XI). This means that, with a certainty of $> 99\%$, all blood plasma samples with an E_2-17β content higher than 0.02 μg/l derive from animals treated with this anabolic, provided that the animals are clinically healthy and have no cysts or tumours in organs producing sex hormones. In the animal experiment, the specificity is 100% until 5 days after injection. After that time specificity decreases rapidly.

For urine samples, the calculated decision level for E_2-17β for male calves was age-dependent: 0.1 and 0.2 μg/l at 15 and 28 weeks of age, respectively. The calculated decision levels for E_2-17α were 9.2 and 9.5 μg/l, respectively. As higher levels of E_2-17β and E_2-17α were found in urine samples from untreated calves in the animal experiment, and to avoid false-positive results, the decision level for E_2-17β and E_2-17α in urine from male veal calves was set at 1 and 20 μg/l, respectively (Table XI). In the experiment, this level resulted in a sensitivity of 100%, and a specificity of 60% was calculated at 5 days after injection.

In practice, only the decision levels of E_2-17β in blood plasma and of E_2-17β

TABLE X

CALCULATED DECISION LEVELS FOR STEROIDS IN BLOOD PLASMA AND URINE FROM MALE AND FEMALE VEAL CALVES AT 15 AND 28 WEEKS OF AGE

Values are in μg/l.

Steroid	Decision level (μg/l).			
	Male calves		Female calves	
	15 weeks	28 weeks	15 weeks	28 weeks
Blood plasma				
Oestradiol-17β	0.01	0.01	0.01	0.01
Testosterone	3.3	8.3	0.1	0.1
Epitestosterone	17	4.2	0.4	0.5
Progesterone	0.2	0.1	0.3	1.4[a]
Urine				
Oestradiol-17β	0.1	0.2	0.05	0.1
Oestradiol-17α	9.2	9.5	6.8	6.5
Testosterone	6.0	55	1.8	3.5
Epitestosterone	283	290	32	65
Progesterone	2.9	8.4	3.9	9.2

[a] A Gaussian distribution was not obtained, even not after log-transformation. This decision level (1.4 μg/l) is equal to the highest reference value obtained.

and E_2-17α in urine will be of interest. The T and epiT levels in blood plasma and excreta of male calves treated with testosterone will be equal to or even lower than the decision levels stated.

TABLE XI

FINAL DECISION LEVELS FOR OESTRADIOL-17β IN BLOOD PLASMA AND OF OESTRADIOL-17β AND -17α IN URINE OF MALE VEAL CALVES

Steroid	Decision level (μg/l)
Blood plasma	
Oestradiol-17β	0.02
Urine	
Oestradiol-17β	1
Oestradiol-17α	20

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