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A RIKILT yeast estrogen bioassay (REA) for estrogen residue detection in urine of calves experimentally treated with 17b-estradiol

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17/S-Estradiol is one of the most powerful sex steroids illegally used in bovine production. The objective of this study was to evaluate the application and the specificity of the RIKILT yeast estrogen bioassay (REA) for the detection of molecules with estrogenic activities in the urine of calves experimentally treated with anabolics. Four groups of six calves each received an injection of 17/3-estradiol intramuscularly (group B), androsterone and gliburide (group A), and testosterone (group C) molecules at different dosage for 40 days. Group D was the control. The ability of the REA test to detect estrogenic activity in urine samples from all animals was assessed. All estrogen-treated animals (group B) showed as being positive up to 7 days after administration of the highest dosage of 17/3-estradiol, while the other three groups showed as being negative. The identity of estrogenic molecules in the urine of group B (17/3-estradiol, 17a-estradiol) was confirmed by gas chromatography-mass spectrometry (GC/MS). This is the first time the REA test has been applied to detect 17/S-estradiol in the urine of calves treated with the hormone *in vivo.* The technique may offer an advantageous laboratory method for the veterinary surveillance of illegal steroid use.

Keywords: bioassay; drug residues; hormones

Introduction

European Union regulations ban the use of growth promoters in meat-producing animals (European Commission 1996a). However, their illegal use raises cause for concern. By acting on lipid and protein metabolism, anabolic steroids increase the growth rate, stimulate muscle growth, and reduce fat tissue in calves (Groot et al. 1998). One of the most efficient of these anabolics is 17/J-estradiol. Histologically, it induces hyperplasia and metaplasia of prostate epithelial cells and urethral epithelium (Ruitenberg et al. 1970; Kroes et al. 1971; Groot et al. 1998). Squamous metaplasia is a specific response to a variety of estrogenic substances such as estradiol, diethylstilbes-trol (DES) and zeranol, and it is considered to be a reliable histological marker to assess their estrogenic action in cattle.

But for surveillance and monitoring of the ban to be effective, validated analytic methods that can screen for and confirm hormone residues in urine and other sample matrices are needed (European Commission 1996b, 2002), as is knowledge of the absorption, biotransformation, and excretion kinetics of illegally administered hormones and the levels of endogenous hormones in these matrices. As current official techniques seem to be unsatisfactory (Groot et al. 2007), new simple methods need to be developed and implemented alongside conventional veterinary controls.

Recent scientific data have implicated estrogen in the tumour progression of human breast and uterine cancer, where it acts as a hormone-stimulating cell proliferator and as a procarcinogen agent that induces genetic damage (Liehr 2000; Foster 2008; Subramanian et al. 2008). Methods that can monitor estrogen residues in animal foods and derivates and reduce the risk of contamination are therefore urgently required.

Various techniques that can detect estrogenic substances include gas chromatography-mass spectrometry (GC-MS) (Daeseleire et al. 1992; Fotsis and Adlercreutz 1987), high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Sorensen and Elbaek 2005), and immunoassay (Dixon and Russell 1983; Chen et al. 1996; Jansen et al. 1986).

Despite the limited number of positives found in controls, findings and analysis of illegal preparations show that steroids, natural hormones and β-agonists are still being used (Courtheyn et al. 2002; Nielen et al. 2003). The analytical methods for measuring residues of steroids and β-agonists as used in control programmes are unable to detect very new or outdated compounds and might be one of the possible explanations for the limited positives found so far. Screening and confirmatory GC-MS, and even the multi-residue liquid chromatography tandem-mass spectrometry (LC/MS/MS) methods, are limited to a short list of a priori known hormone residues (Cai and Henion 1997; Hewitt et al. 2002; Van Poucke and Van Peteghem 2002). The multi-analyte screening ability of radio- and enzyme immunoassays depends on the limited degree of cross-reactivity of the antibody used (Vanoosthuyze et al. 1997; Haasnoot et al. 2002). Alternatively, receptor-based assays can be used to detect compounds having affinity for a given receptor (Mueller 2002); specifically, yeast-based assays feature several advantages, including robustness, low cost, a lack of known endogenous receptors, and the use of media devoid of steroids (Breithofer et al. 1998; Graumann et al. 1999; Witters et al. 2001).

Recently, Bovee et al. (2004a, 2004b) developed a bioassay called RIKILT yeast estrogen bioassay (REA), which is fully validated according to the guidelines as prescribed in European Commission (2002) and accredited by ISO 17025. The assay consists of a recombinant yeast cell that stably expresses the human estrogen receptor *a* (ERa) and a yeast-enhanced green fluorescent protein (yEGFP) as a reporter protein in response to estrogens. GFP is a protein that exhibits green fluorescence that can be measured directly (Cormack et al. 1997). yEGFP can be measured much more easily than an extra-chromosomal reporter construct with β-galactosidase as a substrate-based reporter protein, a system frequently used by older yeast-based assays (Routledge and Sumpter 1997; Gaido et al. 1997; Rehmann et al. 1999; Morito et al. 2001; Le Guevel and Pakdel 2001).

The EC50 revealed by the REA test was 0.5 nM for 17y6-estradiol and comparable with reporter EC50 values for yeast estrogen bioassays that contain β-galactosidase as a reporter (Bovee et al. 2004c). The yEGFP assay can be performed completely in 96-well plates within 24 h and it does not need cell wall disruption or the addition of a substrate. Finally, the REA test is extremely robust and survives extracts from dirty sample matrices such as urine and feed and it is able to recognize estrogen molecules (natural and artificial hormones, aflatoxin and phytoestrogens) (Bovee et al. 2004b).

This bioassay is routinely applied as a qualitative screening method in both calf urine and feed sample matrices in Dutch laboratories; only sample extracts showing a fluorescence response beyond the decision limit CCa will be declared to be suspect for estrogen activity. Subsequently, the identity of substances causing estrogenicity must be determined by conventional residue analytical methods such as GC-MS or by using bioassay-directed quadrupole time-of-flight MS (QTOFMS) identification approaches (Nielen et al. 2004, 2006). Moreover, the bioassay could be a good tool to use alongside instrumental analysis for determining estradiol residues in bovine urine.

The aim of this study was to evaluate the ability of the REA to detect estradiol residues in the urine of experimentally treated veal calves, and to compare the results with those from official analytical methods (GC/MS).

Material and methods

Animals and experimental design

Twenty-four cross-bred Friesian male calves of 6 months of age were randomly assigned to four experimental groups: group A *(n* = 6) treated daily with 1 ml/animal of gliburide (0.2 mg I^{-1}) and androsterone (8mgl^{-1}) cocktail intramuscular (Krentz and Bailey 2005); group B *(n = 6)* treated weekly with *Hβ*-estradiol (diluted in 10 ml of benzylic alcohol and 1 ml of ethyl oleate) intramuscular six times until 1 week before slaughter, for a total of 190 mg/animal (Table 1); and group C *(n = 6)* treated weekly with testosterone propionate (diluted in sesame oil) intramuscular, for a total of 1.050 g/animal (Table 1). Group D *(n = 6)* was the control. The entire treatment phase lasted for a total of 44 days.

The average body weight of the animals at the start of the animal housed was approximately 140 kg; the calves were tethered and fed with liquid milk replacer twice a day (providing per kg: dry matter (DM) 950 g, crude protein (CP) 230 g, ether extract (EE) 210 g, ash 60 g, cellulose 1 g, retinol 75 mg, ascorbic acid 50 mg, Cu 5mg, cholecalciferol 0.125mg, and alphatoco-pherol 80 mg). The amount of fodder was increased gradually up to 8 litres/calf/day, then increased gradually up to 16 litres/calf/day; after 1 month, a quantity of 0.5 kg of barley straw (per kg: DM 900 g, CP 20 g, EE 10 g, ash 60 g, and crude fibre 410 g) was added to the diet according to the indications suggested by European Commission (97/182/EC). The experiment was authorized by the Italian Ministry of Health and the Ethical Committee of the University of Turin. The carcasses of the treated animals were destroyed (2003/74/CE - DL 16 March 2006, No. 158).

Urine samples of experimental group B were collected at 5, 24, 48 and 168 h after the injection of estradiol (I, III, and VI doses), analysed by REA, and then confirmed by GC-MS. Urine samples from the experimental groups (A, C) were analysed with the REA test at 15 and 44 days into the study. All urine samples from each experimental group were compared with the fluorescence data obtained from the control animals.

Table 1. Treatment of calves from groups B and C.

Dose	Group B 17*β*-estradiol (mg/animal)	Group C testosterone (mg/animal)
I	20	150
II	20	150
III	30	150
IV	40	200
V	40	200
VI	40	200

Steroid extraction of calf urine samples

Aliquots of 2 ml of calf urine of each experimental group were adjusted to pH 4.8 and 6ml β-glucuroni-dase/arylsulfatase (300 and 22.5 U ml^{-1}, respectively) were added. Enzymatic deconjugation was carried out overnight in a water bath at 37°C. Next, 2ml of 0.25 M sodium acetate buffer, pH 4.8, were added and the hydrolysed sample was subjected to solid-phase extraction (SPE) on a C18 column fitted on a vacuum manifold (Supelco, Sigma-Aldrich, St. Louis, MO, USA). The column was previously conditioned with 2.5 ml methanol and 2.5 ml sodium acetate buffer. The column was then washed with 1.5ml 10% (w/v) sodium carbonate solution, 3.0 ml water, 1.5ml sodium acetate buffer, pH 4.8, 3.0 ml water, and finally with 2 ml methanol/water (50:50, v/v). The column was air-dried and eluted with 4 ml acetonitrile, which was applied to an NH_2-column (Supelco) previously conditioned with 3.0 ml acetonitrile. The acetonitrile eluate was evaporated to 2 ml under a stream of nitrogen gas. A 100-ml aliquot of this extract (equivalent to 100 ml urine) was transferred to a 96-well plate in triplicate and 50 ml water and 2 ml DMSO were added to each well. To remove the acetonitrile from this mixture, the plate was dried overnight in a fume cupboard and was then ready to be screened for estrogenic activities with the yeast estrogen bioassay.

REA

The yeast cytosensor expressing the human estrogen receptor *a* (hERa) and yeast-enhanced green fluorescent protein (yEGFP) in response to estrogens has been described previously (Bovee et al. 2004a, 2004b). The yeast growth was performed according to published data (Bovee et al. 2004a). For exposure in 96-well plates, 200-ml aliquots of this yeast culture were pipetted into each well already containing the extracts of the urine samples. A 17y6-estradiol dose-response curve in DMSO was included in each exposure experiment (Figure 1). Each urine sample extract and each 17y6-estradiol standard was assayed in triplicate. Exposure of the yeast to the sample was performed 24 h and the yeast fluorescence at zero hours and 24 h was measured directly in a Victor 31420 Multilabel Counter (PerkinElmer, Waltham, MA, USA) using excitation at 485 nm and emission measurement at 530 nm. Differences of the fluorescence measurement at 24 h and zero hours of yeast exposed to samples *(t_24 - t_0)* were calculated and corrected for the reagent blank data, thus providing the final estrogenic activity data for each sample. The samples are reported 'suspect' when the t_24 - t0 fluorescence measurement exceeds the CCa (fluorescence of 4409) obtained in the ring test in our laboratory at the University of Turin (Bovee et al. 2008); otherwise, the samples are reported 'compliant'. The bioassay is a qualitative screening method, i.e. no concentrations are reported. We analysed a total of 114 samples.

GC/MS analysis

The deconjugated estrogens were extracted from 2 ml of aqueous samples, derivatized and analysed with an Agilent Technologies 6890 N GC connected to an Agilent 5975 MSD (Akre et al. 2004; Mouatassim-Souali et al. 2003; Daeseleire et al. 1998). The internal standard was methyl testosterone at a final concentration of 75ngml^{-1}. In order to hydrolyse the glucur-ono-conjugate of 17a- and 17y6-estradiol, the pH was adjusted between 6 and 7 with HCl 1 M; 50 ml of enzyme /^-glucuronidase (441 IU) were added and hydrolysed for 1 h at 42° C. We extracted 17a- and *Hβ*-estradiol at pH 9 for NaOH 1 M by liquid-liquid extraction with 10 ml of tert-butyl-methyl-ether. The organic phase was dried under nitrogen stream at 50° C. The analytes were derivatized with 50 ml of tetramethylsilane and heated to 70° C for 30min in thermoblock.

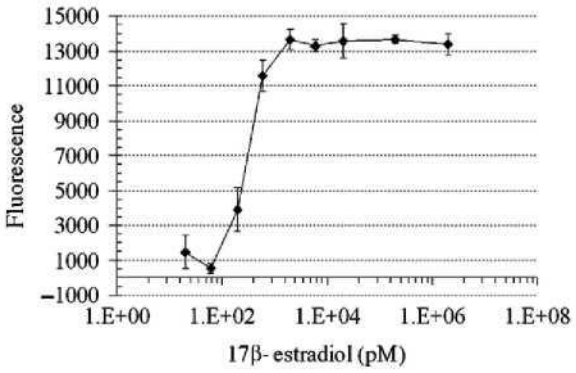

Figure 1. Response of REA after 24-h exposure to 17/S-estradiol. Exposure was started by adding 2ml of a 17/S-estradiol stock solution in DMSO to 200 ml of a yeast culture. Fluorescence was determined after zero and 24 h, as described in the Materials and Methods section (see 'Yeast estrogen bioassay'). Fluorescence signals are the means ± standard deviation (SD) of a triplicate experiment and are corrected for the signals obtained at zero hours and a reagent blank.

Compounds of interest were separated on a HP-5 column (17 m x 0.20 mm x 0.33 mm film thickness). The temperature programme was 90° C for 1.50min, ramped at 25°Cmin^{-1} to 260°C and held for 3min, ramped at 25°Cmin^{-1} to 320°C and held for 1.30 min. A 2^1 aliquot was injected into the GC-MS system in splitless mode. The injector port temperature was 280°C, the transfer line from the GC system to the mass-selective detector was held at 280° C. Helium was the carrier gas at 2.1 ml min^{-1} initial flow. The pressure was constant. The mass-selective detector was operated in electronic impact mode using selected ion monitoring (SIM).

Selection of diagnostic ions was carried out after acquisition of complete mass spectra for analyte, both alone and in the working standard, under the same chromatographic conditions as for the samples. The masses for 17a- and 17/?-estradiol were *m/z = 416*, 401, 326, 285, 232, 446 and 301 for methyl testosterone. The dwell time was 80 ms. The retention times were 11.80min for 17a-estradiol, 12.20min for 17^-estra-diol, and 12.80 min for internal standard. Calibration curves were constructed by extracting urine spiked with 17a- and 17/S-estradiol at a final concentration of 5, 10, 30, 50 and lOOngml^{-1}. The calibration graph for each analyte is described by the equation:

$$Y = mX + b$$

where *Y* is the ratio between the response value of the analyte (peak area) and the response value of the internal standard (peak area); and *X* is the concentration inngml^{-1} of analyte in the sample. We analysed a total of 104 samples.

Statistical analysis

The validity of the REA test was evaluated by comparing the test results with those of the GC-MS method which represents the 'golden test' or 'gold standard' (Petrie and Watson 2006). Validity criteria were sensitivity, specificity and concordance index or efficiency, which is the concordance degree of the test. Evaluation was easily done using a 2 x 2 contingency table and the sensitivity (%) was calculated as 100 times the number of true positives/ (number of true positives plus number of false-negatives), the specificity (%) as 100 times the number of true negatives/(number of false positives plus number of true negatives), and the concordance index (%) as 100 times (number of true positives plus number of true negatives)/total number of samples.

Results

Figure 1 shows the dose-response curve obtained with the REA test after 24-h exposure to n^-estradiol. Exposure was started by adding 2 ml of a 17/S-estradiol stock solution in DMSO to 200 ml of a yeast culture. The signals are the responses obtained after 24 h of exposure and are corrected with the responses obtained at zero hours ($t_2$4 - t0) and with the response ($t_2$4 - t0) obtained with a reagent blank (DMSO). All the responses are reported as the means of a triplicate.

After 24 h of yeast exposure with the different *Hβ*-estradiol concentration (standards), we did not find any differences in the yeast growth, meaning that no toxic effects on the yeast could be observed (data not shown).

Figure 2 illustrates the trends of REA response in calf urine samples collected at different times and after administration of different doses. As shown in Figure 2a and b, all urine samples collected at 5 and 24 h after the first and third dose of 17/S-estradiol showed a higher fluorescence than the decision limit CCa (fluorescence 4409) (Bovee et al. 2008). Five out of six urine samples collected after the first dose and four out of six samples collected 48 h after the third dose showed fluorescence over 4409 (Figure 2a and b). After 1 week, all urine samples from animals that had received the first and third dose showed a fluorescence lower than the CCa. Instead, at the sixth dose all the 17/S-estradiol-treated animals (Figure 2c) had fluorescence data higher than 4409 also after 168 h.

Table 2 compares the data from the REA test and the GC-MS analysis. The GC-MS results of all urine samples after administration of dose I at T0 demonstrate that endogen 17a-estradiol and 17/?-estradiol were absent (sensitivity of method was 1 ppb). At 5 h after the first dose a small quantity of 17/?-estradiol and a significant increase in its metabolite (17a-estradiol) was detectable in the animals. After 24 h, the 17/S-estradiol level began to decrease, whereas the 17a-estradiol level continued to rise. At 48 h after injection, 17/S-estradiol had been completely metabolized and converted into 17a-estradiol.

At 1 week (168 h) after the administration of the first dose, 17/S-estradiol was absent, except in animal 301095 where traces of 17a-estradiol were found. At T0 of the third dose no analytes were found, but an increase in both 17a-estradiol and 17/?-estradiol was detectable after treatment.

REA test specificity was evaluated by comparing the urine samples from calves in groups A (cocktail treatment), C (testosterone treatment) and D (control animal); no animals were found positive to estrogens (Table 3).

Table 4 illustrates the 2 x 2 contingency table for evaluating the validity of the REA method as a qualitative

screening test versus the results obtained with GC/MS. The REA test was found to have:

Sensitivity: 100 x (83/(83 + 5)) = 94.3% Specificity: 100 x (16/(0 + 16)) = 100% Concordance index = 100 x (83 + 16)/104 = 95.2%

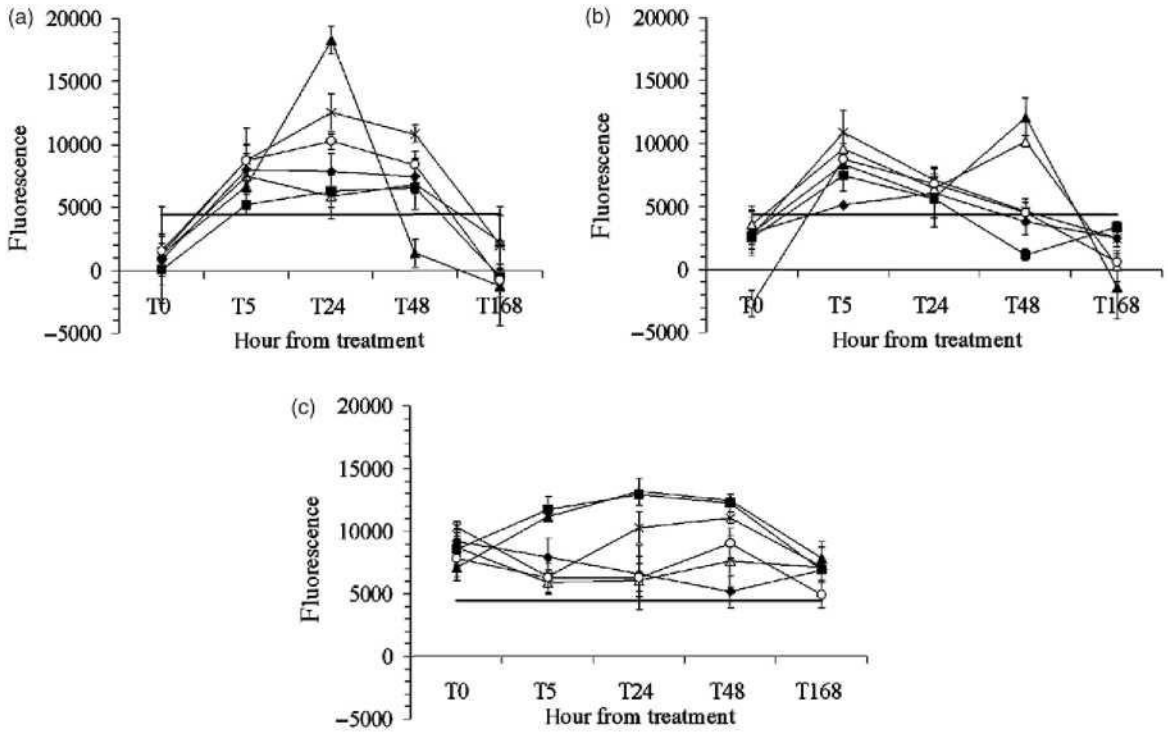

Figure 2. Results obtained by REA to 17/S-estradiol and metabolites from an extract of urine samples from each animal of group B treated with (a) 20 mg/animal of 17 ^-estradiol (I dose), (b) 30 mg/animal of 17 ^-estradiol **(III** dose), and (c) 40 mg/animal of 17 ^-estradiol (VI dose). ♦, Calf number 297627; g, 290403; m, 301095; x, 301096; 4, 252732; O, 300408; and —, decision limit CCa (4409).

Table 2. 17a- and 17/3-Estradiol screening of urine samples by REA and GC-MS methods.

Animal ID	Dose	Time from injection (h)	17α-Estradiol (ng ml^{-1})	17β-estradiol (ng ml^{-1})	REA response
301096	I	0	<1	<1	−
		5	247.2	30.53	+
		24	464.74	<1	+
		48	98.68	<1	+
		168	<1	<1	−
	III	0	<1	<1	−
		5	568	76	+
		24	609	13	+
		48	248	4.5	+
		168	n.t	n.t	−
	VI	0	117	3.1	+
		5	418	96	+
		24	1356	80	+
		48	582	23	+
		72	239	8.7	+
		96	23	<1	+
		120	187.59	9.26	+
		144	82.97	4.72	+
		168	216.14	10.37	+
300408	I	0	<1	<1	−
		5	255.57	5.92	+
		24	294.91	22.02	+
		48	20.63	<1	+
		168	<1	<1	−

(*continued*)

Table 2. Continued.

Animal ID	Dose	Time from injection (h)	17α-Estradiol (ng ml^{-1})	17β-estradiol (ng ml^{-1})	REA response
	III	0	233.67	28.83	−
		5	411.	34	+
		24	399.65	20.84	+
		48	155.85	7.36	+
		168	n.t.	n.t.	−
	VI	0	20	<1	+
		5	2202	273	+
		24	525	19	+
		48	111	4.8	+
		72	129	3.6	+
		96	183	8.7	+
		120	96.03	4.29	+
		144	26.54	<1	+
		168	n.t.	n.t.	+
252732	I	0	<1	<1	−
		5	156.04	1.45	+
		24	238.25	<1	+
		48	4.57	<1	+
		168	<1	<1	−
	III	0	<1	<1	−
		5	401.35	62.5	+
		24	704.99	23.28	+
		48	180.67	5.09	+
		168	n.t.	n.t.	−
	VI	0	148	5.8	+
		5	820	183	+
		24	2022	112	+
		48	420	20	+
		72	72	1.8	−
		96	100	4.6	+
		120	n.t.	n.t.	+
		144	100	2.3	+
		168	67	3.8	+
301095	I	0	<1	<1	−
		5	341.62	67.74	+
		24	865.94	29.63	+
		48	6.29	<1	−
		168	2.52	<1	−
	III	0	<1	<1	−
		5	606.53	126.04	+
		24	297.43	8.16	+
		48	104.19	<1	+
		168	n.t.	n.t.	−
	VI	0	45.3	<1	+
		5	1142.03	369.12	+
		24	765.91	59.08	+
		48	141.34	<1	+
		72	58	2	+
		96	36	1.1	+
		120	70.46	<1	+
		144	16.11	<1	+
		168	64	2.8	+
290403	I	0	<1	<1	−
		5	353	13	+
		24	1601	41	+
		48	74	<1	+

(*continued*)

Table 2. Continued.

Animal ID	Dose	Time from injection (h)	17α-Estradiol (ng ml^{-1})	17β-estradiol (ng ml^{-1})	REA response
		168	<1	<1	−
	III	0	<1	<1	−
		5	637	137	+
		24	684	18	+
		48	176	6.0	−
		168	n.t.	n.t.	−
	VI	0	38.46	0	+
		5	296.93	74.77	+
		24	664.62	57.28	+
		48	365.52	20.72	+
		72	137	5.4	+
		96	100	4.7	+
		120	179.56	8.02	+
		144	111.14	<1	+
		168	61.15	<1	+
297627	I	0	<1	<1	−
		5	601	108	+
		24	435	8.9	+
		48	61	<1	+
		168	<1	<1	−
	III	0	<1	<1	−
		5	351	39	+
		24	560	15	+
		48	n.t.	n.t.	−
		168	n.t.	n.t.	−
	VI	0	73	1.4	+
		5	1618	158	+
		24	2761	110	+
		48	319	7.9	+
		72	87	1.7	+
		96	60	1.2	+
		120	n.t	n.t.	+
		144	44	<1	+
		168	71	1.0	+

Note: n.t., Not tested.

Table 3. Fluorescence data of REA test in groups D, A and C (CCa = 4409; Bovee et al. 2008).

Group	Treatment day 15	Treatment day 44
D	1213 ± 1714	−117 ± 2569
A	3348 ± 1223	1346 ± 1340
C	376 ± 330	1818 ± 1860

Table 4. The 2 x 2 contingency table for the evaluation of REA validity as a qualitative screening test (GC-MS method considered the golden test).

REA results	GC-MS estrogens positive	GC-MS estrogens negative
+	83[a]	0[b]
−	5[c]	16[d]

Notes: [a]Number of true positives.
[b]Number of false positives.
[c]Number of false negatives.
[d]Number of true negatives.

Discussion

Estrogens are among the most important growth promoters used illegally in animal production and their use is prohibited in the European Union (European Commission 1996a). An alternative approach to chemical analysis (Courtheyn et al. 2002) for the identification of treated animals might be the use of a bioassay. Bovee et al. (2004a, 2004b) developed a bioassay (REA) fully validated according to the guidelines as prescribed in European Commission (2002) and accredited by ISO 17025. In the Netherlands the test is routinely applied as a screening test for the detection of illegal estrogen treatment in calves.

In 2006, Nielen et al. analysed more than 120 calf urine samples randomly collected from different Dutch calves and compared the urine data obtained with the REA test with data derived from GC/MS/MS. The data for REA validation were obtained from aliquots of blank calf urine and calf urine samples spiked with 17y6-estradiol and other estrogen molecules. Currently, there are no data on REA application in animals experimentally treated with estrogenic hormones.

In this study we evaluated the ability of REA to detect estradiol residues in the urine of experimentally treated veal calves and compared the results with an official analytical method (GC/MS).

In hormone treatment of cattle, the urinary profile usually contains a mixture of metabolites of the anabolic drug that was used. Since the bioassay provides an additive measurement of all estrogenic residues and metabolites (having different relative estrogenic potencies), the chance of overlooking illegal treatment is relatively low as compared with other screening assays such as highly specific immunoassays. In a previous study (Bovee et al. 2004b), it was shown that 17y6-estradiol metabolites (17a-estradiol, estrone, estriol and hydroxylated estrogens) give a response in the bioassay.

We demonstrated that GC/MS analysis of urine samples confirmed the results obtained with the REA method, except for five samples of group B. Three real false-negatives were: the sample taken at 48 h after injection III in animal 290403, the sample taken at 72 h after injection VI in animal 252732, and the sample taken at zero hours after injection III in animal 300408, resulting in a percentage of false-negatives that is below the accepted level of 5%.

The other two samples corresponded to animal 301095 collected at 48 and 168 h after the first treatment; they did not contain 17y6-estradiol and contained small quantities of 17a-estradiol (6.29 and 2.52 ng ml^{-1}, respectively) and resulted in a negative REA. However, the 17a-estradiol metabolite is about ten times less potent than 17y6-estradiol; the relative estrogenic potency (REP) of 17a-estradiol is 0.093 compared with 17y6-estradiol (Bovee et al. 2004b). Moreover, the assay was validated on the 1 ppb *Hβ*-estradiol level, which is generally accepted as the limit in urine, and the assay was then shown to produce less than 5% of false-negatives. This Ing *Hβ*-estradiolml^{-1} urine level corresponds to an activity of 10 ng 17a-estradiolml^{l}. Thus, it can be discussed whether or not these screening results are real false-negatives. In addition, Bovee et al. (2005a) calculated that the limit for an increase in fluorescence of the REA test was about 5.5ng of 17a-estradiolml^{-1} urine, demonstrating that the negative screening outcome of the sample containing 2.52 ng 17a-estradiolml^{-1} was as expected, while the 6.29 ng 17a-estradiolml^{-1} sample is just above this limit and was screened as negative as well. However, the results (Figure 2a) showed an increased fluorescence response of the latter sample, but it was below the determined CCa value of 4409 (Bovee et al. 2008).

Finally, the outcome of the urine sample of animal 252732 at 48 h after the first dose of H^-estradiol contained no 17y6-estradiol and 4.47 ng 17a-estradiolml^{-1} and was screened as a suspect in the estrogen bioassay. Once

more it was demonstrated that the limit of detection is about 5.5ng 17a-estradiolml^{-1}, but this study also shows that all samples containing 1ng 17y6-estradiol or more (equal to 10 ng 17a-estradiolml^{-1} or more) are screened as being suspect. On the basis of these considerations, the last two samples could not be considered the real false-negative. Unlike the REA test, which is a qualitative method, GC-MS showed fluctuations in estrogen levels of urine samples from different calves under the same experimental conditions, the same time and dose; this fluctuation is likely due to variability in an animal's metabolism. Animal 301095 was noted to maintain a small quantity of 17a-estradiol also at 1 week after the first dose, probably because of greater difficulty in eliminating the metabolite. In calf 252732, on the other hand, 17a-estradiol concentration rapidly peaked and was then quickly eliminated (Table 2).

In conclusion, the animals can metabolize the first two treatments in 1 week, while subsequent stronger doses leave significant residues of 17a-estradiol and less 17y6-estradiol after 1 week of treatment. The 17a-estradiol is systematically present in a concentration ten to 50 times higher than the 17y6-estradiol, which is an index of quick conversion of one form in the other.

Statistical analysis confirmed that the bioassay is a valid method that can discriminate between estrogen-treated and non-treated calves when compared against the results of an official chemical test like GC-MS. However, screening with this bioassay is limited to calf urine because older bovines produce large amounts of endogenous 17a-estradiol and estrone, which would cause too many false-suspect urine values.

Sample preparation is quite similar in both methods, but the cost of this test and the easier interpretation of the REA results procedure make it a more practical method for large-scale screening to support the chromatography method. Currently, it is not possible to discriminate natural estrogen molecules from synthetic estrogenic hormones illegally injected into animals. Furthermore, the REA method can be applied only to samples from prepuberal veal calves fed regular milk replacer. Nonetheless, the REA method is a fast and simple assay for routine surveillance and monitoring and it can significantly reduce the GC-MS sample workload.

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