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Toine EH. *Bovee*[a'*], Gerrit *Bor*[a], Use *Becue*[h], *Frieda E.J. Daamen*[c], *Majorie BM. van Duursen*[c], Sylui *Lehmann*[d], *Gvnter Vollmer*[d], *Raffaella* De *Maria*[e], *Jennifer* E. Fox-f, Hilda Witters[9], Silfee *Bernh6ft*[h], *Karl-Werner Schramm*, Ron* L.A.P. *Hoogenboom*[a], Michel W.E Nielen[a"]

[a] RIKILT-Institute of Food Safety, *Wageningen University and* Research Centre, *P.O.* Box 230, 6700 AE *Wageningen, The* Netherlands
[b] Gent University, Laboratory of Food Analysis, Harelbefeestraat 72, *B-9000 Gent,* Belgium
[c] IRAS, Institute *for* Risk Assessment Sciences, Utrecht University, *P.O.* Box *80177,* 3508 TD Utrecht, The Netherlands
[d] Institute of Zoology, Department of Molecular Cell Physiology and Endocrinology, TU Dresden, Dresden, *Germany*
[e] University of Turin, Department of Animal Pathology, Faculty of Veterinary Medicine, Via Leonardo da Vinci 44, 10095 Grugliasco, TO, Italy
[f] Center/or Ecology and Evolutionary Biology, University of Oregon, Eugene, OR 97403, USA
s Vito-Flemish Institute *for* Technological Research, Boeretang 200, BE-2400 Mol, Belgium
[h] Helmholtz Zentrum Mlinchen, Research Center *for* Environmental Health, Institute o/Ecological Chemistry, Ingolstaedter Landstr. 1, 85764 Neuherberg, *Germany*
[1] *Wageningen* University, Laboratory of *Organic* Chemistry, Dreijenplein 8, 6703 HB *Wageningen, The* Netherlands

A B S T R A C T

An inter-laboratory exercise was performed with a yeast estrogen bioassay, based on the expression of yeast enhanced green fluorescent protein (yEGFP), for the determination of estrogenic activity in extracts of calf urine samples. Urine samples were spiked with 1 and 5ngmL^{-1} 17p-estradiol and 17a-ethynylestradiol, 10 and 50ngmL^{-1} mestranol, and lOOngmL^{-1} testosterone and progesterone. Sample extracts of blank and spiked urine samples were prepared at our laboratory and sent to seven laboratories together with a reagent blank, a DMSO blank, and eight 17p-estradiol stock solutions in DMSO ranging in concentration from 0 to 545ngmL~^{1}. Sample extracts and standards were coded and tested blindly. A decision limit (CCa) was determined based on the response of seven blank urine samples. Signals of the negative controls, e.g. urine samples spiked with lOOngmL^{-1} testosterone or progesterone, were all below the determined CCa and were thus screened as compliant. Positive controls, i.e. the urine samples spiked at two levels with 17p-estradiol, 17a-ethynylestradiol and mestranol, were almost all screened as suspect, i.e. gave signals above the determined CCa. Determined EC50 values calculated from the 17p-estradiol dose-response curves obtained by the seven laboratories ranged from 0.59 to 0.95 nM.

1. Introduction

Use of growth promoters for fattening purposes in cattle is banned in the European Union since 1988 [1]. This ban prohibits all substances having hormonal action but does not provide a list of forbidden hormones. Despite the limited number of positives found in control programs, findings and analyses of illegal preparations show that steroids, natural hormones and β-agonists are being used [2,3]. Analytical methods for residues of steroids and β-agonists as used in the control programs are focused on target compounds and not able to detect very new or outdated compounds. The screening and confirmatory gas chromatography/mass spectrometry (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 [4-6]. However, especially the latter methods are developing fast and become suitable to detect unknown anabolic steroids as well [7]. The multi-analyte screening ability of radio- and enzyme immuno-assays is dependent on the limited degree of cross-reactivity of the antibody used [8,9]. Alternatively, receptor-based assays can be used to detect all compounds having affinity for a given receptor [10]. This feature is very helpful in detecting known and unknown compounds, as receptor stimulation plays a key role in the mechanism of action of growth promoters. In contrast to competitive ligand binding receptor assays, that cannot distinguish a receptor agonist from an antagonist, transcription activation assays can be used to identify antagonists as well, by giving them in combination with a dose of a known agonist that gives a half-maximal or near-maximal response [11]. Compared to bioassays based on mammalian cell lines, yeast-based assays have several advantages. These include robustness, low cost, lack of known endogenous receptors and the use of media that are devoid of steroids [12,13]. Yeast estrogen bioassays have been useful for the rapid determination of estrogenic activity in environmental samples [14,15]. Most of these assays use an extra-chromosomal reporter construct with β-galactosidase as a substrate based reporter protein. We developed a yeast estrogen bioassay, stably expressing human estrogen receptor *a* (hERa) and an estrogen response element-driven yeast enhanced green fluorescent protein (yEGFP) [10]. This assay is completely performed in a 96-well plate and both fluorescence and absorbance (OD) are measured directly from intact living yeast cells. This yeast estrogen assay is relatively simple and sensitive, as shown by an EC50 value for ^β-estradiol of 0.5 nM corresponding to 27 pg ^β-estradiol in a well. Furthermore, a large number of chemically different compounds with known estrogenic properties were tested, all of them causing a dose-related increase in the production of green fluorescent protein. The gestagens progesterone and medroxyprogesterone-acetate showed no response and the androgen testosterone only showed a very weak response [10]. This yeast estrogen assay was validated as a qualitative screening method for the determination of estrogenic activity in calf urine and animal feed and turned out to be very robust [16]. These validations were performed according to EC Decision 2002/657, which prescribes the determination of the decision limit (CCa), detection capability ^Cβ), the specificity/selectivity and the stability/ruggedness/applicability [17]. The percentage of false positives and false negatives was 0% and the method was proven to be sensitive, specific, and robust and samples were stable for at least 60 days when stored at -20°C. The method acquired an ISO 17025 accreditation status in the Netherlands in 2005 for both matrices and is routinely used. Actually, this was the first successful example of a developed, fully validated and applied bioassay for the screening of hormonal substances in urine and feed. Although the test is used by different laboratories in Europe and the US, no inter-laboratory exercise was performed to demonstrate its validated status, robustness and applicability all over the world. In the present study this yeast estrogen bioassay was used in an inter-laboratory study for the determination of estrogenic activity in extracts of calf urine samples.

2. Experimental

2.1. Chemicals

^β-estradiol, 17a-ethynylestradiol, mestranol, and progesterone were obtained from Sigma (St. Louis, MO, USA) and ^β-testosterone was obtained from Steraloids (Newport, RI, USA). Acetic acid, dimethyl sulfoxide (DMSO), sodium carbonate and sodium acetate trihydrate were obtained from Merck (Darmstadt, Germany). Acetonitrile and methanol were obtained from Biosolve (Valkenswaard, The Netherlands). Chemicals to prepare the growth media for yeast and the preparation of standard stock solutions in DMSO were as described previously [16].

2.2. Selection and preparation *of* test samples

A quick scan was performed at the end of 2006 and over 200 calf urine samples were sent to our Institute and screened with the yeast estrogen bioassay. Based on the highest volume amounts that were still available we selected eight samples that were screened as compliant. Six samples showed a normal response and two samples showed a slightly elevated response, but the responses were all well below the decision limit. These eight compliant samples were analysed with GC/MS/MS as described elsewhere [18] and one sample with a normal and average response was chosen and used to prepare positive and negative controls. The other seven samples were used as blank urine samples only. The extraction procedure of blank and spiked urine samples is as described in [16]. In short: aliquots of 2 mL blank calf urine and spiked calf urine samples were adjusted to pH 4.8 and 20 µL β-glucuronidasezarylsulfatase (3 U mL^{-1}) was added. Deconju-gation was carried out overnight at 37 °C, 2 mL sodium acetate buffer pH 4.8 was added, samples were subjected to solid phase extraction using a C18 and a NH2 column. This latter column was eluted with acetonitrile. All samples were replicated 5-fold and all five acetonitrile extracts were pooled together, dried and redissolved in 200 µL DMSO (2 µL of the DMSO extract is equivalent to 100 µL urine). In the same way a reagent blank was prepared, using 2 mL of the 0.25 M sodium acetate buffer pH 4.8 instead of urine. Aliquots of 20 µL of the

DMSO extracts were transferred in total recovery vials with a screw cap from Waters (product number 186000384 C) and sent to each participant together with the protocol. The DMSO extracts and 17p-estradiol stock solutions in DMSO were numbered 1-26 and analysed blindly by the participants using the protocol as described below. All participants sent their raw data to RIKILT where all the calculations were carried out, in order to keep it a blindly performed inter-laboratory comparison.

2.3. Protocol sent to participants

The Rikilt Estrogen bioAssay (REA) is based on a yeast cell that expresses yeast enhanced green fluorescent protein in response to estrogens. Compared with other reporters, the yEGFP reporter protein is very convenient because it is directly measurable in intact living cells, i.e. cell wall disruption or the addition of a substrate are not needed. When exposed to *Uβ*-estradiol, the concentration where half-maximal activation is reached is between 0.5 and lnM. This ring test intends to investigate the applicability of the REA for detecting estrogenic activity in extracts of calf urine samples.

1 Chemicals
- 1.1 Demineralised, deionised and pyrogen free water (e.g. from a Millipore system or an Ultra Purelab system from Elga).
- 1.2 Yeast Nitrogen Base without amino acids and without ammonium sulphate (e.g. Becton, Dickinson and Com pany (BD) Difco™ 233520).
- 1.3 Ammonium sulphate (e.g. Merck 12019).
- 1.4 Dextrose (e.g. Becton, Dickinson and Company (BD) Difco™ 215530).
- 1.5 L-Leucine (e.g. Sigma L-1512).
- 1.6 17p-Estradiol (e.g. Sigma E-8875).
- 1.7 DMSO (e.g. Merck 2950).
- 1.8 Bacto-agar (e.g. Becton, Dickinson and Company (BD) Difco™ 214010).
- 1.9 Sodium hydroxide (e.g. pellets Merck 6498).

2 Reagents and media
- 2.1 L-Leucine stock solution (6mg/mL). Weigh 180mg L- leucine (1.5) and dissolve in 30mL demi water (1.1). Filter sterilise the solution using a 0.2 µm filter (e.g acrodisc 4192).
- 2.2 Minimal Medium with L-leucine (MM/L). Weigh 1.7 g Yeast Nitrogen Base (1.2), 5 g ammonium sulphate (1.3) and 20 g dextrose (1.4) and dissolve in 1 L demi water (1.1). Autoclave the solution 15min. at 121 °C. Cool down to room temperature. Just before use: add 20 mL of the L-leucine stock solution to 1 L Minimal Medium and mix (often less medium is needed, adjust volumes in same ratio).
- 2.3 Minimal Medium Agar Plates with L-leucine. Weigh 1.7 g Yeast Nitrogen Base (1.2), 5 g ammonium sulphate (1.3), 20 g dextrose (1.4) and 20 gbacto-agar (1.8), add one pellet of NaOH (1.9) and dissolve in 1L demi water (1.1). Autoclave the solution 15min. at 121 °C. Cool down to 60 °C and add 20 ml of the L-leucine stock solution (2.1), mix and pore plates (approximately 30 mL per plate having a diameter of 10 cm). (Adjust the amounts and the volumes in the same ratio if you only need a few plates.).
- 2.4 17p-estradiol stock solution (20mM in DMSO). Weigh 5mg 17p-estradiol (1.6) and dissolve in 918 µL DMSO (1.7). Make dilutions until the low nM range, e.g.: 2mM, 200 µM, 20 µM, 2 µM, 600 nM, 200 nM, 60 nM, 20 nM, 6 nM and2nM.
- 2.5 DMSO extracts supplied by RIKILT. Twenty-six vials coded as sample 1 to sample 26, each one contains a calf urine extract or a reference compound in DMSO (20-30 µL).

3 Apparatus and equipment
- 3.1 Fluorometer for fluorescent measurement (e.g. Cytofluor-Multi Well Plate Reader-Perseptive Biosys- tems Series 4000). Use excitation at 485 nm and measure emission at 530 nm.
- 3.2 Microplate-reader for measurement of the absorbance or optical density (OD) at 610,620 or 630 nm (e.g. Bio-Tek Instruments Inc. Elx 808).
- 3.3 Incubator with orbital shaking (e.g. from New Brunswick Scientific).
- 3.4 Incubator without orbital shaking (e.g. New Brunswick scientific).
- 3.5 Laminar flow cabinet (e.g. from Clean Air).
- 3.6 Autoclave.
- 3.7 Sterile flasks or tubes (e.g. Greiner 227261).
- 3.8 96-Multi Well Plates (e.g. Costar 3595 or Greiner 651201).
- 3.9 Petri dishes with a diameter of approximately 10 cm (e.g. Greiner 633171).

2.3.1. *Procedure*

Before starting the experiment, inoculate the REA yeast in 10 mL fresh Minimal Medium with L-leucine (2.2) from either a -80 °C glycerol stock or an MM/L agar plate. Grow the cells 24h (overnight) at 30°C with 200rpm in the incubator (3.3). The next day, take 100 µL from this fresh yeast overnight culture to inoculate a Minimal Medium Agar Plate with L-leucine (2.3). Incubate this plate for 2 days at 30°C (3.4). Store the plate at 4°C. The yeast colonies from this plate can be used for 3 months to prepare fresh yeast overnight cultures.

2.3.1.1. Day 1. Pick a colony of the REA yeast from the MM/L plate and use it to inoculate 10 mL Minimal Medium with L-leucine (Section 2.2: pipette 10 mL Minimal Medium in a sterile flask or tube (3.7) and add 200 µL of the L-leucine stock (2.1).) Grow the cells overnight at 30 °C with 200 rpm in the incubator (3.3).

2.3.1.2. Day 2. Dilute the REA yeast overnight culture in fresh Minimal Medium with L-leucine (Section 2.2) until an absorbance (OD) at 630 nm is reached between 0.07 and 0.13 (cuvet of lcm). Measure against MM/L medium without the yeast. If it is not possible to measure the OD, dilute the overnight culture as follows: take a sterile flask or tube (3.7) and add 41 mL Minimal Medium, 900 µL L-leucine stock solution (see 2.2) and 3.1 mL of the yeast overnight culture. Mix the diluted yeast culture and transfer 200 µL in every single well of two 96-Multi Well Plates (3.8).

Before opening the 26 vials, first spin them carefully in a centrifuge at 500 rpm in order to get the complete DMSO

MW 1	1	2	3	4	5	6	7	8	9	10	11	12
A	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only
B	yeast only	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8	sample 9	sample 10	yeast only
C	yeast only	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8	sample 9	sample 10	yeast only
D	yeast only	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8	sample 9	sample 10	yeast only
E	yeast only	sample 11	sample 12	sample 13	sample 14	sample 15	sample 16	sample 17	sample 18	yeast only	yeast only	yeast only
F	yeast only	sample 11	sample 12	sample 13	sample 14	sample 15	sample 16	sample 17	sample 18	yeast only	yeast only	yeast only
G	yeast only	sample 11	sample 12	sample 13	sample 14	sample 15	sample 16	sample 17	sample 18	yeast only	yeast only	yeast only
H	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only

MW 2	1	2	3	4	5	6	7	8	9	10	11	12
A	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only
B	yeast only	sample 19	sample 20	sample 21	sample 22	sample 23	sample 24	sample 25	sample 26	yeast only	yeast only	yeast only
C	yeast only	sample 19	sample 20	sample 21	sample 22	sample 23	sample 24	sample 25	sample 26	yeast only	yeast only	yeast only
D	yeast only	sample 19	sample 20	sample 21	sample 22	sample 23	sample 24	sample 25	sample 26	yeast only	yeast only	yeast only
E	yeast only											yeast only
F	yeast only	room	for	your	own	17b-estradiol	standard	stock	solutions	in	DMSO	yeast only
G	yeast only	0	2 nM>20 pM	6 nM > 60 pM	20 nM > 200 pM	60 nM > 600 pM	200 nM > 2 nM	600 nM > 6 nM	2 µM > 20 nM	20 µM > 200 nM	200 µM > 2 µM	yeast only
H	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only	yeast only

Fig. 1 - Scheme for both 96-MW plates. Send your results, the OD and fluorescent measurements of both 96-well plates, to Toine F.H. Bovee by mail.

extract at the bottom of the vial. Then add 2 µL amounts of all supplied samples (2.5) in triplicate to the yeast according to the scheme as presented in Fig. 1. Add 2µL amounts of your own 17p-estradiol stock solutions (2.4) in triplicate to the yeast and according to the scheme as presented in Fig. 1.

Transfer these 2 µL sample extracts and your 17p-estradiol stocks directly into the 200 µL yeast suspension in the well in triplicate and use clean tips for every single well. Check by eye whether you transferred the 2 µL into the well. Measure the fluorescence with a fluorometer (3.1) at t = Oh and use excitation at 485 nm and measure emission at 530 nm. Close both 96-MW plates with a lid and use tape to fasten the lid. Incubate both plates in the incubator (3.3) at 30°C with 125 rpm.

2.3.1.3. Day 3. Remove the tape carefully, open the lid and measure the fluorescence with a fluorometer (3.1) at t = 24 h and use excitation at 485 nm and measure emission at 530 nm (without lid!). Measure the absorbance OD_5io, $OD_6$2o or $OD_6$3o at t = 24h with a plate reader (3.2). If it is not possible to measure the absorbance, please check the density in the wells by the eye and see if there are differences in the density compared to the wells without added samples or 17p-estradiol stocks.

2.4. Determination *of the* decision limit CCa and *curve fitting*

Signal corrections and all calculations were carried out at RIKILT A decision limit CCa, defined as the mean of the blank plus three times its corresponding standard deviation, was calculated from the corrected fluorescence signals of seven blank urine samples. Dose-response curves obtained with eight 17p-estradiol stock solutions that were sent to the seven laboratories and dose-response curves obtained with 17p-estradiol stock solutions that were prepared by the participants themselves were fitted using the equation y= aO/(l+(x/al))a2) (Slide write Plus, Version 6.00). Equal to: response = (max. response-min. response) divided by (1 + ([agonist]/EC5o))width of transition. Where response is the measured fluorescence signal, [agonist] is the concentration
of 17p-estradiol and the EC50 is the concentration of Uβ-estradiol giving a half-maximum response.

3. Results and discussion

The yeast estrogen bioassay was validated previously in-house according to EC Decision 2002/657 and calf urine samples containing "1 ngmL^{-1}17p-estradiol equivalents" or more were all screened as suspect, while samples containing no estrogenic compounds were screened as compliant or negative [16].

Table 1 shows the results of the seven laboratories obtained with the 26 sample extracts that were analysed blind according to the procedure as described in the supplied protocol. All raw data were sent to RIKILT where all the calculations were carried out, in order to keep it a blindly performed inter-laboratory comparison. Samples #l-#7 were extracts of blank urine samples. Sample #16 was an extract of the blank urine that was used to prepare the spiked urine samples. Samples #8-#13 contained extracts of this blank urine spiked with respectively 1 and 5ngmL^{-1} 17p-estradiol, 1 and 5ngmL^{-1} 17a-ethynylestradiol and 10 and 50ngmL^{-1} mes-tranol. The 17a-ethynylestradiol was spiked at the same level as 17p-estradiol as this compound is at least as potent as *Uβ*-estradiol. Mestranol was spiked at a 10-fold higher level as this compound is about ten times less potent than 17p-estradiol [10,16]. Samples #14-#18 contained negative controls. Samples # 14 and #15 were the blank urine #16 that was spiked with respectively lOOngmL^{-1} 17p-testosterone or progesterone. Sample #17 was a reagent blank and sample #18 was a DMSO

blank. Samples #19-#26 were 17p-estradiol standards, ranging in concentration from 0 to 2000nM (0-545 $ngmL^{-1}$, and thus finally in the well from 0 to 5.5 $ngmL^{-1}$).

There were no differences between samples in the fluorescence measurement at t= 0 h and none of the laboratories observed a difference in the absorbance (OD) after 24 h of exposure (data not shown), demonstrating that none of the extracts was coloured or toxic for the yeast cells. The raw data were sent to RIKILT where fluorescence signals of samples #1-#18 were corrected for the fluorescence signal obtained with the

Table 1 - Results of an inter-laboratory study with 7 participants and 26 sample extracts that were analysed blindly.

Sample number	Sample type	RIKILT	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
1	Blank urine	-35	4.00	24	-2657	-266	-122	-0.050	-77
2	Blank urine	-25	2.33	56	-991	-354	-111	0.013	-109
3	Blank urine	35	5.33	1686	4589	890	682	-0.007	-136
4	Blank urine	-8	3.00	472	160	118	69	0.056	-159
5	Blank urine	26	3.00	-134	-9871	-682	4	0.011	-207
6	Blank urine	237	6.00	2940	9368	3605	1061	0.111	-76
7	Blank urine	122	4.67	2377	13235	2085	542	0.090	63
	CCα	**343**	**8.11**	**4837**	**25283**	**5459**	**1685**	**0.201**	**156**
8	Urine 1 ppb E2	768	13.00	10153	72899	15375	2227	0.338	566
9	Urine 5 ppb E2	897	14.00	11585	87469	17047	2058	0.475	1689
10	Urine 1 ppb EE2	692	14.33	10122	87127	13678	1570	0.302	622
11	Urine 5 ppb EE2	852	10.33	12184	74531	16796	1764	0.398	1554
12	Urine 10 ppb Mes	398	8.33	5719	37334	8216	1505	0.218	18
13	Urine 50 ppb Mes	708	9.33	9023	52651	13757	1902	0.369	109
14	Urine 100 ppb T	67	2.00	1391	11554	1157	440	0.075	40
15	Urine 100 ppb P	73	1.33	1965	542	894	348	0.078	9
16	Blank urine	56	1.67	1231	4652	971	199	0.054	153
17	Reagent blank	0	0	0	0	0	0	0	0
18	DMSO blank	23	2.00	1189	-8006	355	-153	0.030	-50
19	0	0	0	0	0	0	0	0	0
20	2 nM E2	58	-0.33	-518	-11125	368	-127	0.062	-56
21	6 nM E2	49	0.67	-445	-2017	486	-127	-0.005	40
22	20 nM E2	73	1.00	38	-4734	1569	-68	0.055	101
23	60 nM E2	404	4.00	3683	28305	5312	902	0.071	190
24	200 nM E2	817	10.00	10540	65143	16438	1947	0.408	1179
25	600 nM E2	881	9.00	11139	68913	18152	1451	0.452	1691
26	2000 nM E2	859	8.33	10861	50054	17661	1203	0.417	1725
Corresponding EC_{50} [nM]		**0.64**	**0.63**	**0.74**	**0.61**	**0.83**	**0.59**	**0.95**	**0.83**
EC_{50} own E2 stocks [nM]		0.64	0.37	0.28	1.09	0.77	0.35	1.10a	0.54

[1] Highest concentration of E2 not included in calculation and in grey the samples that not fulfil the criterion.

reagent blank, i.e. sample #17, and fluorescence signals of samples #19-#26 were corrected for the fluorescence signal obtained with DMSO only, i.e. sample #19. Table 1 shows the results of these corrected fluorescence signals measured at t=24h. The big differences between the laboratories of the absolute fluorescence values that were measured are probably due to differences between the specific types of 96-well plates used and the type and settings of the fluorometers.

Fig. 2a shows the dose-response curves obtained with the eight 17p-estradiol stock solutions, see Table 1 samples #19-#26, that were sent to the seven laboratories. Final concentrations were 1% of those shown in Table 1, representing the stock solutions. Fig. 2b shows the dose-response curves obtained with 17p-estradiol stock solutions that were prepared by the participants themselves. The data of both complete dose-response curves were fitted and Table 1 shows the corresponding EC50 values. EC50 values of the 17p-estradiol dose-response curves obtained using the delivered stock solutions ranged from 0.59 to 0.95 nM. Proving that the differences between the laboratories of the absolute fluorescence values
are indeed due to differences between the specific types of 96-well plates used and the type and settings of the fluorometers. The EC50 values of the 17p-estradiol dose-response curves obtained with each participant's own prepared stock solutions ranged from 0.28 to 1.10 nM. The broader range of the latter is probably due to small differences in weighing and dilution of 17p-estradiol during the preparation of the standards at the different laboratories or due to differences in the chemical standard, e.g. purity and age.

The eight urine samples that were screened at our Institute as compliant before in a national control program and were used as blanks in this study, were analysed with GC/MS/MS as described before [18] in order to prove that real estrogen blank urine samples were used to perform this inter-laboratory comparison. The results in Table 2 show that these urine samples all contain less than "lngmL^{-1} 17p-estradiol equivalents" as estrone and 17a-estradiol are respectively 5 and 10 less potent than 17p-estradiol [10]. In addition, the GC/MS/MS analysis demonstrated that the samples contained no 17a-ethyl-5p-estrane-3a,17p-diol, norethandrolone,

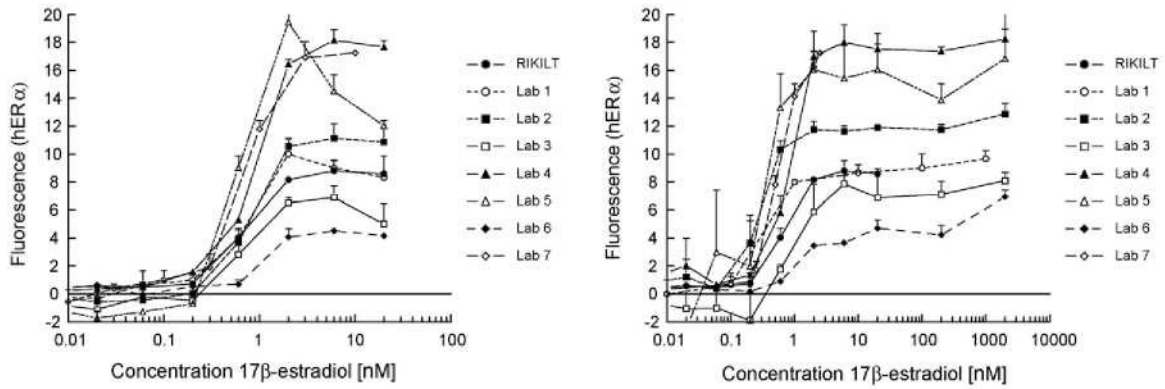

Fig. 2 - (A) Dose-response curves obtained at the seven laboratories with the eight 17p-estradiol stock solutions, see Table 1 samples #19-#26. Final concentrations were 1% of those shown in Table 1, representing the stock solutions. (B) Dose-response curves obtained with 17p-estradiol stock solutions that were prepared by the participants themselves.

norgestrel, 17a-nor testosterone, 17p-nortestosterone and 17a-methyltestosterone (data not shown). All samples contained low amounts of 17a-testosterone (range <1 to 88.4ngm.L~[1]) and sample #7, containing 88.4ngm.L~[1], also contained l.lngmL^{-1} 17p-testosterone while the other blank urines contained no 17p-testosterone. However, the latter two compounds are almost inactive in the yeast estrogen bioassay [10,16]. Moreover, these GC/MS/MS results are in agreement with earlier findings, showing that 17a-testosterone is the most abundant natural hormone residue in urine of both male and female calves and increased levels 17a-testosterone are associated with the occurrence of low 17p-testosterone levels [18].

The decision limit CCa for an initial validation is defined as the mean of at least 20 blank samples plus three times its corresponding standard deviation. Complete initial validations of the yeast estrogen bioassay for the detection of estrogenic activity in calf urine and animal feed are described in [16] and [19]. Here we calculated a decision limit from the corrected fluorescence signals of only seven blank urine samples, i.e. samples #l-#7, as this inter-laboratory comparison is only meant as a kind of transfer validation for the yeast estrogen bioassay. Samples giving a signal lower than this determined CCa are classified as compliant or negative. Samples giving a signal higher than the decision limit CCa
are classified as suspect. The results in Table 1 demonstrate that the blank urine samples #l-#7 and also the blank urine sample #16 fulfil the CCa criterion, i.e. give signals below the CCa and are thus classified as negative. Also the negative controls, performed with blank urine samples spiked with lOOngmL^{-1} 17p-testosterone or progesterone (#14 and #15), a reagent blank (#17) and a DMSO control (#18), give signals below the determined CCa and are classified as negative. The positive control samples #8-#13, containing extracts of blank urine samples that were spiked with 1 and 5ngmL^{-1} 17p-estradiol, 1 and 5ngmL^{-1} 17a-ethynylestradiol and 10 and 50ngmL^{-1} mestranol respectively, nearly all give signals that are higher than the determined CCa and are thus classified as suspect. Only laboratory #5 and #7 had false negative screening results versus their individual CCa values. The percentage of false negative screening results is 9.5% (4 out of 42). Laboratory #5 screened the urine samples spiked with lngmL^{-1} 17a-ethynylestradiol and IOngmL^{-1} mestranol as compliant. However, both samples gave clear responses compared to blank and the mean of the blank samples and the corrected signals were just below the determined decision limit. Laboratory #7 screened the urine samples spiked with 10 and 50ngmL^{-1} mestranol as compliant. However, laboratory #7 deviated from the protocol and diluted the DMSO samples 1:200 in the yeast suspension instead of 1:100 and samples

Table 2 - Levels (ng mL [a]) of natural and synthetic steroids and their metabolites determined in calf urine samples from a national control program using GC/MS/MS and that were screened negative in the yeast estrogen bioassay.

Blank urine sample #	Bioassay result	DE	Hex	EE2	cDES	tDES	E1	17αE2	17βE2	17αT	17βT
# 1	N	N	N	N	N	N	<1	<1	N	1.3	N
# 2	N	N	N	N	N	N	<1	<1	N	<1	N
# 3	N	N	N	N	N	N	<1	1.1	N	<1	N
# 4	N	N	N	N	N	N	<1	<1	N	<1	N
# 5	N	N	N	N	N	N	N	<1	N	3.1	N
# 6	N	N	N	N	N	N	<1	1.7	N	47.7	N
# 7	N	N	N	N	N	N	<1	1.3	N	88.4	1.1
# 16	N	N	N	N	N	N	<1	<1	N	17.4	N

N = negative, DE = dienestrol, Hex = hexestrol, EE2 = 17α-ethynylestradiol, cDES = cis-diethylstilbestrol, tDES = trans-diethylstilbestrol, E1 = estrone, 17αE2 = 17α-estradiol, 17βE2 = 17β-estradiol, 17αT = 17α-testosterone and 17βT = 17β-testosterone.

were distributed over four 96-multi well plates instead of two as shown in Fig. 1. The latter is of minor importance, but diluting the samples 1:200 instead of 1:100 is reason enough to exclude the data from this laboratory when calculating the % of false negative results. The percentage of false negative screening results is therefore 5.6% (2 out of 36), which is near the acceptable percentage of 5% as prescribed in EC Decision 2002/657 for a complete initial validation of a qualitative screening method [17].

Although the complete initial validation and this inter-laboratory exercise of the yeast estrogen bioassay were performed as a qualitative screening method for the determination of estrogenic activity in calf urine, signals can be converted to concentrations using a 17p-estradiol standard dose-response curve. Using this semi-quantitative approach, the decision limit CCa for calf urine that was determined earlier at our laboratory correlated with "0.22ngmL^{-1} 17p-estradiol equivalents" [16]. Also the results shown in Tables 1 and 2 show that there is a clear correlation between the presence of little amounts of 17a-estradiol in the blank urine samples (Table 2) and the obtained signals in the bioassay (Table 1). Samples #3, #6, and #7 contained low levels of 17a-estradiol, respectively 1.1, 1.7, and 1.3ngmL^{-1}, and gave higher signals than the blank samples containing amounts lower than lngmL^{-1}. However, 17a-estradiol is about ten times less potent than 17p-estradiol and thus sample #6, containing lJngmL^{-1} 17a-estradiol, contains "0.17ngmL^{-1} 17p-estradiol equivalents", which is below the concentration of "0.22ngmL^{-1} 17p-estradiol equivalents" that correlated with the determined CCa at our laboratory and is thus classified as negative.

Moreover, the IOngmL^{-1} mestranol spiked sample showed the lowest response of all positive controls at all laboratories. It gave signals just above the determined CCa at the six laboratories that screened this sample as suspect. The 50ngmL^{-1} mestranol spiked sample showed the second lowest response of all positive controls at six of the eight laboratories. Thus diluting the samples twice as much as prescribed in the protocol possibly explains the false negative screening results obtained with the 10 and 50ngmL^{-1} mestranol spiked samples at laboratory #7. In addition, also the 50ngmL^{-1} mestranol spiked sample at laboratory #7 showed a clear elevated signal compared to the blank and the mean of the blank urine samples.

In general the higher concentration-spiked samples gave higher reporter responses, but lab 1 and lab 3 obtained a higher response with the lngmL^{-1} EE2 than with the 5ngmL^{-1} EE2 spiked sample and lab 5 obtained a higher response with the lngmL^{-1} E2 than with the 5ngmL^{-1} E2 spiked sample. However, this is due to the fact that these lower concentration-spiked samples already gave a response that was at least equal to the maximal response obtained at these laboratories with the dose-response curve obtained from the 17p-estradiol stocks (see Table 1).

4. Conclusions

Although all except one laboratory used the same protocol, there are of course differences between the laboratories due to differences in temperature, preparation and storage of reagents and media, and between chemicals and equipment used. Despite these differences, the results of this inter-laboratory comparison prove that the yeast estrogen bioassay is a robust test for the qualitative screening of estrogenic activity in biological samples. All blank samples and negative controls at each of the seven laboratories gave responses below the determined decision limits (CCa) and thus were screened as compliant. Positive controls, i.e. the urine samples spiked with two concentrations of *Uβ*-estradiol, 17a-ethynylestradiol and mestranol, were almost all screened as suspect, i.e. gave signals above the determined CCa. Only two out of seven laboratories had false negative screening results, one of which used its own protocol and diluted the extracts 1:200 instead of 1:100 as prescribed in the protocol and the other laboratory obtained signals with two positive controls that were just below the determined decision limit. The percentage of false negative screening results of 5.6% is near the acceptable percentage of 5% as prescribed in EC Decision 2002/657 for a full initial validation [17]. Determined EC50 values calculated from the 17p-estradiol dose-response curve obtained by the seven laboratories ranged from 0.59 to 0.95 nM and from 0.28 to 1.10 nM if the participants used their own prepared stock solutions. Therefore, these results demonstrate the reproducibility and robustness of the yeast estrogen bioassay for expressingyEGFP upon exposure to estrogens.

This inter-laboratory comparison is a kind of transfer validation. If the other laboratories want to use this as a validated method for screening calf urine samples they have to determine at least a CCa based on own prepared extracts of 20 blank samples and test whether the percentage of false negatives is below 5%.

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