Toxicology in Vitro 28 (2014) 70–75

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Evaluation of estrogenic activity in animal diets using in vitro assay

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ARTICLE INFO

Article history:
Received 23 October 2012
Accepted 7 October 2013
Available online 12 October 2013

Keywords:
Feed
Estrogenic activity
In vitro assay
Yeast culture

ABSTRACT

A yeast estrogen bioassay (RIKILT REA) was in-house validated for feed on the 5 μg 17β-estradiol-equivalents per kg level according to EC Decision 2002/657/EC. All the performance characteristics met the criteria as defined in the Decision and the REA is able to detect 17β-estradiol in animal feed at a low level of 1.15–2 μg kg^{-1}. Subsequently, the developed and validated procedure was applied to determine the estrogenic activity in 24 feed samples intended for food producing animals, pets and laboratory animals. Two batches of rodent diet Murigran and one dog feed have been presented as a suspect, i.e. gave responses above the determined decision limit (CCα) and detection capability (CCβ). In assessing the performance of the estrogenic activity in these diets evaluated by comparison with the 17β-estradiol calibration curve, 17β-estradiol-equivalence levels of 7.07 μg EEQ kg^{-1} and 9.54 μg EEQ kg^{-1} in two batches of rodent diet and 5.3 μg EEQ kg^{-1} in dog feed have been established. The activities observed in the rodent feed could be explained by chemical analysis, revealing high amounts of genistein, daidzein and trace amounts of zearalenone. In addition, the estrogenic activity in one of rodent feed was above the established CCα, but below the CCβ values established and all other samples showed no estrogenic activity with responses below the CCα value, which corresponds to levels below 2 μg EEQ kg^{-1}.

1. Introduction

In Europe, the prohibition of natural or synthetic steroid hormones (Council Directive 96/22) including estrogens, gestagens, and androgens as growth promoters triggered the need for efficient detection systems. Many analytical methods have been developed for the determination of these compounds in various matrices. Immunoassay methods allow examining of one or a few analytes simultaneously and are too specific to detect unknown compounds. Chromatographic methods such as the gas and liquid chromatography–mass spectrometry, more often being used for the screening studies are laborious and expensive and include a limited number of compounds. In view of the increasing number of growth promoters in that both natural and synthetic steroids, which can be used in the growth of animals for slaughter the use of multi-residue methods which include different classes of compounds is becoming more important. An alternative is to use the methods based on biosensors for screening purposes (Bovee and Pikkemaat, 2009; McGrath et al., 2012). The bioassay methods are of particular importance in the study of food (Behr et al., 2011; Nielsen et al., 2009), supplements (Plotan et al., 2011; Toorians et al., 2010) and feed (Rijk et al., 2011). Animal diets are produced from natural raw materials including grasses, corn, wheat, soybean and fishes. Some of the feed ingredients may contain various estrogens – like compounds derived from plants such as phytoestrogens and mycotoxins (Piersen, 2003; Mantovani et al., 2009) or xenoestrogens (insecticides, diethylstilbestrol, bisphenol A and polychlorinated biphenyls) for which pesticides, pharmaceuticals, industrial chemicals are the source of (McLachlan and Arnold, 1996). These compounds cannot be detected by the instrumental methods at low concentrations levels. The in vitro bioassays have many advantages: the detection of the effects caused by the compounds currently unidentified and integration the effects of complex chemical mixtures. Furthermore, quantification of estrogenic activity as estradiol equivalents (EEQ) facilitates the estimation of the total dietary intake of estrogenicity (Behr et al., 2011). Some papers indicate that the commercial laboratory feeds have estrogenic activity and may affect the results of estrogen assays by influence a number of different endpoints such as gene expression profile of the reproductive system in the immature female rats (Naciff et al., 2004), sexual development of rats (Odum et al., 2001), accessory sex organs of rat males (Stroheker et al., 2003), uterine weight of immature CD-1 mice (Thigpen et al., 2002), time of vaginal opening in immature CD-1 mice (Thigpen et al., 2003) and other (Unfer et al., 2004; Yamasaki et al., 2002). A critical value of dietary estrogenic intake depends on the species and endpoints measured. Therefore, according to OECD recommendations, the level of total daidzein and genistein in rodent diet should be less than 325 μg g^{-1} (Owens et al., 2003). In our laboratory the influence of diet on the immature hamsters' uterine weight was also observed. Therefore, in order to examine the estrogenic activity

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http://dx.doi.org/10.1016/j.tiv.2013.10.005

in animal feed, yeast estrogen bioassay described by Bovee et al. (2006) was introduced. In the present study, in house validation of the mentioned method and its application to test the estrogenic activity in different kind of feeds is presented.

2. Experimental

2.1. Reagents and chemicals

Standards of 17β-estradiol (⩾98%), 17α-estradiol (⩾98%), Estriol (⩾97%), Estrone (⩾99%), Diethylstilbestrol (⩾98%), Zeranol (⩾97%), Genistein (⩾98%), Coumestrol (⩾95%), Apigenin (⩾95%), Daidzein (⩾98%) were obtained from Sigma–Aldrich (Steinheim, Germany). Standards were stored according to the recommendations of the certificates. Primary standard stock solutions were prepared in DMSO at a concentration of 1 mg mL^{-1} and were stored below −18 °C. Working solutions were obtained by tenfold dilution of primary standard solutions to the concentration of 0.1 μg mL^{-1} in DMSO. All working standard solutions were stored at 2–8 °C for not longer than 6 months.

Concentrated acetic acid (99.5% purity) and sodium acetate anhydrous (99.0% purity) of analytical grade were obtained from POCH (Gliwice, Poland). Sodium carbonate (99.8% purity) and L-leucine were provided from Sigma–Aldrich (Steinheim, Germany). Methanol (99.8% purity) and acetonitrile (99.8% purity) all resi grades were obtained from Mall Baker (Deventer, The Netherlands). Ammonium sulphate and dimethyl sulfoxide were obtained from Merck (Darmstadt, Germany). Purified water was achieved with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Solid-phase extraction (SPE) columns (Bakerbond® C_{18} 500 mg/3 mL and NH_2 500 mg/3 mL) were supplied by Mall Baker (Deventer, The Netherlands). Acetate buffer (0.05 M), pH 4.8 was prepared by mixing of 40 mL of 0.05 M acetic acid solution (1.42 mL in 500 mL of water) with 60 mL of 0.05 M sodium acetate solution (4.1 g in 1000 mL of water) and adjusting the pH value to 4.8.

Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were obtained from Difco (Detroit, MI, US).

The minimal medium with L-leucine (MM/L) consisted of: yeast nitrogen base without amino acids and without ammonium sulphate (1.7 g per L), dextrose (20 g per L), and ammonium sulphate (5 g per L) and was supplemented with L-leucine (60 mg per L).

All glassware used in yeast culture preparation were rinsed with ultrapure water and acetone and heated to 200 °C in order to eliminate estrogenic contamination.

2.2. Samples

The samples of feed for validation purposes were tested for the presence of estrogenic compounds. Prior to validation the feed for laboratory animals by liquid chromatography for the presence of phytoestrogens (Stochmal and Oleszek, 2007) and mycotoxins (Wisniewska-Dmytrow et al., 2012) was tested, while rest feeds by GC–MS method for the presence of stilbenes and zeranol, that might be used illegally in livestock production (Wozniak et al., 2010) were investigated. This method is used for study of hormones in feed in the frame of control programme.

Twenty four feed samples were assayed using yeast estrogen bioassay: commercial feeds for laboratory animals i.e. Murigran, LSM, LSK (AGROPOL, Poland) and Altromin 7010 (ALTROMIN, Germany); pet diets for cats (Royal Canin, Bozita, Friskies Whiskas, OKE, Kitekat, Purina Cat Chow); dog diets (N&D, Arion); livestock diets (feeds for sow, poultry and cattle).

2.3. Sample preparation

Extraction was performed using a modified protocol based on the procedure from Bovee et al. (2006). In brief, to 1 g of grinded and mixed feed sample placed in centrifuged tube, 4 mL of methanol and 4 mL of acetate buffer pH 4.8 was added. Estrogenic compounds were isolated from the sample matrix by ultrasonic extraction at 30 °C for 10 min. Then, the content of the tube was shaken followed by centrifugation (10 min at 4000 rpm). Supernatant was collected in glass tubes and pH with acetic acid to 4.8 was adjusted. The extract was applied onto C_{18} SPE column previously conditioned with 3 mL of methanol and 3 mL of acetate buffer (pH 4.8). The column was washed with 1.5 mL of 10% (w/v) sodium carbonate solution, 3 mL water, 1.5 mL sodium acetate, 3 mL water and finally with 2 mL of methanol/water mixture (50:50, v/v) and stored under vacuum. The compounds were eluted with 4 mL of acetonitrile and the eluate was directly loaded on SPE NH_2 column previously conditioned with 3 mL of acetonitrile. To improve the purity of the extract, NH_2 columns (500 mg, 3 mL) were used, instead of (100 mg, 3 mL) proposed by Bovee. The eluate was collected in glass tube and evaporated to dryness under the gentle stream of nitrogen at 40 °C (±2 °C). The residue was reconstituted in 200 μL of acetonitrile, transferred to conical tube and evaporated. Then, the residues were dissolved in 20 μL of DMSO and 2 μL aliquots of the solution were transferred to 96-well plate in triplicate. Application of 2 μL of DMSO extract instead 200 μL of acetonitrile extract proposed by Bovee, improved repeatability of the method about 14%, the recovery about 20% and reduced the time of analysis. Afterwards, the yeast estrogen bioassay was performed.

2.4. Yeast estrogen bioassay

The yeast estrogen bioassay was applied exactly according to procedure from Bovee et al. (2006). To each well of the microplate already containing the extracts of the feed samples, aliquots of 250 μL of the diluted yeast culture were pipetted. In each experiment 17β-estradiol dose-response curve, control samples and solvent control (DMSO) were included. To generate the standard curve a series of nine 1:3 dilutions of 17β-estradiol stock solution in DMSO (ranged from 0.62×10^{-9} to 4.05×10^{-6} M) were used. Fluorescence at time $t = 0$ and 24 h was measured in Synergy 2 micro plate reader (BioTek, US) using excitation at 485 nm and emission measurement at 530 nm. The absorbance was determined using micro plate reader RC (Labsystems) at 620 nm. The absorbance changes in the treated cells compared to controls gave an indication of toxicity. The results were acceptable if the cytotoxicity was less than 20%.

2.5. In house validation

The method was submitted to a validation process in accordance with Commission Decision, 2002/657/EC requirements for qualitative methods. To determine the decision limit (CCα) and detection capability (CCβ) of the method, extracts of 20 blank feed samples and 20 spiked feed samples were prepared and analyzed in the bioassay in three separate exposures. Each sample extract was assayed in triplicate. According to CD 2002/657/EC the decision limit CCα ($\alpha = 1\%$) is the result of mean signal of blank samples plus 2.33 times of their standard deviation. The samples with a signal below decision limit are classified as compliant whereas samples with a signal above CCα are classified as suspected. The detection capability (CCβ) value is the result of summing of the decision limits and 1.64 times the standard deviation of the signal of spiked feed samples. The criterion for the detection capability is that at least 19 out of the 20 spiked samples have to give a signal