Food and Chemical Toxicology 69 (2014) 260–266

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Validation and application of a reporter gene assay for the determination of estrogenic endocrine disruptor activity in milk

E. Wielogorska [a,b], C.T. Elliott [a], M. Danaher [b], L. Connolly [a,*]

[a] Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Northern Ireland, United Kingdom
[b] Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland

ARTICLE INFO

Article history:
Received 14 January 2014
Accepted 15 April 2014
Available online 24 April 2014

Keywords:
Milk
Endocrine disrupting compounds
Reporter gene assay

ABSTRACT

Endocrine disruptors (EDs) are compounds known to interfere with the endocrine system by disturbing the action or pathways of natural hormones which may lead to infertility or cancer.

Our diet is considered to be one of the main exposure routes to EDs. Since milk and dairy products are major components of our diet they should be monitored for ED contamination. Most assays developed to date utilise targeted, chromatography based methods which lack information on the biological activity and mixture effects of the monitored compounds.

A biological reporter gene assay (RGA) was developed to assess the total estrogen hormonal load in milk. It has been validated according to EU decision 2002/657/EC. Analytes were extracted by liquid–liquid extraction with acetonitrile followed by clean up on a HLB column which yielded good recovery and small matrix effects. The method has been shown to be estrogen specific, repeatable and reproducible, with covariance values below 20%. In conclusion, this method enables the detection of low levels of estrogen hormonal activity in milk with a detection capability of 36 pg g^{-1} EEQ and has been successfully applied in testing a range of milk samples.

1. Introduction

Food is a major route of exposure to EDs for the human population (Thomson, 2009). Naturally occurring EDs such as phytoestrogens in plants and endogenous steroid hormones in food of animal origin can contribute to this exposure. Additionally, man-made chemicals have been reported to enter the food chain through agricultural or industrial practices resulting in environmental and food chain contamination (Connolly, 2009). Consequently, we are exposed to a cocktail of compounds on an every day basis. As EDs have been linked to various health disorders such as infertility, urogenital tract abnormalities (cryptorchidism, hypospadias) and cancers (Diamanti-Kandarakis et al., 2009), monitoring EDs in food is of great importance for consumer health.

Milk and dairy products are one of the main components of our diet (IUNA, 2011). Thus there is a great interest in monitoring this matrix for ED contamination. Various analytical methods have been developed to monitor the presence of diverse groups of EDs in milk. These include liquid chromatography – tandem mass spectrometry (LC–MS/MS) methods for detection of more polar compounds such as phytoestrogens (Flachowsky et al., 2011; Krajcova et al., 2010), mycotoxins (Sorensen and Elbaek, 2005), perfluoroalkylated compounds (Noorlander et al., 2011) and nonylphenols (Shao et al., 2007). Also gas chromatography (GC) – MS has been employed in the assessment of less polar EDs such as dioxins (Abad et al., 2002), phthalates (Casajuana and Lacorte, 2004) polybrominated diphenyl ethers, polychlorinated biphenyls and other persistent organic pollutants (Petro et al., 2010) as well as natural hormones (Courant et al., 2008). MS based analytical methods provide excellent tools for ED detection with very precise quantitation. Nevertheless, due to the chemical diversity of the analytes there is a need for the development of a separate method devoted to each group. Additionally, the development of such methods requires expensive equipment, experienced staff and is time consuming.

Abbreviations: ADI, acceptable daily intake; ANOVA, analysis of variance; $CC\alpha$, decision limit; $CC\beta$, detection capability; CV, covariance; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; EC_{50}, concentration yielding 50% increase in maximal response; ED, endocrine disruptor; EEQ, 17β-estradiol equivalents; EU, European Union; GC, gas chromatography; LC, liquid chromatography; MeOH, methanol; MS, mass spectrometry; MTT, thiazolyl blue tetrazolium bromide; PBS, phosphate buffered saline; P, Progesterone; RGA, reporter gene assay; RLU, relative light unit; SD, standard deviation; TBME, tert-butyl methyl ether; Te, testosterone.

* Corresponding author. Address: The Institute for Global Food Security, Queen's University Belfast, David Keir Building, Stranmillis Road, Belfast BT9 5AG, Northern Ireland, United Kingdom. Tel.: +44 028 90976668; fax: +44 028 90976513.
E-mail address: l.connolly@qub.ac.uk (L. Connolly).

http://dx.doi.org/10.1016/j.fct.2014.04.028
0278-6915/

Also, such chemical methods lack information regarding possible biological effects that can be exerted by chemicals both on their own and as a mixture. This information can be obtained using *in vitro* bioassays, which are cheaper and more efficient as a screening tool. Various bioassays such as the ER-CALUX, E-Screen and YES assay (Bovee and Hoogenboom, 2009) have been developed for the screening of estrogenic activity in food. Nevertheless, there is little information regarding the total estrogenic activity of milk. Only two bioassays have been developed and validated in milk for screening of dioxins and dibenzofurans (Chou et al., 2008) or polychlorinated biphenyls (Bovee et al., 1998). An additional two studies, focusing on phytoestrogen fractions, cite estrogenicity in bovine milk extracts as 6.25 ng EEQ L^{-1} and 0.75 ng EEQ L^{-1} (Dip et al., 2008; Nielsen et al., 2009). Concerns have also been raised regarding exposure to milk produced by pregnant cows in recent years. Significant changes in the hormonal profiles of both men and children after consumption of milk have been reported (Maruyama et al., 2010), suggesting that the ordinary intake of cow's milk can affect sexual maturation of prepubertal children. In light of the ADI levels for 17β-estradiol (0–50 ng kg bw^{-1}), established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1999), the results of Maruyama et al. (2010) are of concern as the reported levels of natural hormones in milk are well below these established limits, nevertheless milk consumption was shown to affect consumers. A number of studies report the presence of other EDs in milk, such as organochlorine compounds, perfluoroalkylated compounds, phenols or phthalates (Casajuana and Lacorte, 2004; Noorlander et al., 2011; Petro et al., 2010; Shao et al., 2007), all of which may add to the total hormonal load. Milk evaluation is also of a great importance as a variety of dairy products including cheese and butter may possibly aggregate the hormonal load present in the raw product (Behr et al., 2011). Thus, the purpose of this study is to develop and validate a novel estrogenic bioassay which allows the assessment of the true hormonal load of milk (i.e. the sum of free natural hormones and exogenous ED contaminants).

2. Materials and methods

2.1. Chemicals

Ultra-pure water (18.2 MΩ cm^{-1}) was generated in-house using a Millipore (Merk Millipore, Billerica, MA, USA) water purification system. 17β-Estradiol (E2), progesterone (P), testosterone (Te), methanol (MeOH), acetonitrile, tert-butyl methyl ether (TBME), dimethyl sulfoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cell culture reagents i.e. Dulbecco's Modified Eagle Medium (DMEM), general foetal bovine serum, hormone depleted serum, penicillin streptomycin, L-glutamate were supplied by Invitrogen Ltd. (Paisley, UK). Phosphate buffered saline (PBS) was supplied by SAFC Biosciences (Lenexa, Kansas, USA).

E2 standard solution was prepared in MeOH at 4 mg mL^{-1}. An intermediate standard solution was prepared at 40 μg mL^{-1}. Both primary and intermediate stock standard solutions were found to be stable for at least 12 months when stored at −20 °C. A working E2 standard solution was prepared from the intermediate mixed standard solution at the following concentrations of 3240 (std 5), 324 (std 4), 32.4 (std 3), 6.4 (std 2), 1.6 (std 1) ng mL^{-1}. Extracted milk matrix calibrants were prepared by spiking negative milk samples (10 g) with 50 μL of the working standard solutions prior to extraction. This gave a five point calibration curve in the range 0.008–16.2 ng g^{-1}. Recovery controls were prepared by spiking blank sample post-extraction with std 3 (50 μL) to monitor for loss of analytes during extraction.

2.2. Milk samples

Milk samples used in this study included a wide range of matrices i.e. raw milk collected from farms' bulk tanks, commercially available bovine milk of different fat content (from 0% fat to 3%), butter milk, goat's milk, infant formulae, organic and fortified milks. The raw milk samples were collected in glass bottles previously washed in detergent and rinsed thoroughly with methanol and acetonitrile. Commercial milk samples included those packaged in plastic, carton and glass containers. All samples were either analysed immediately after collection or stored in their original packaging at −20 °C for no more than two weeks, before being thawed for analysis purposes.

2.3. Sample preparation

Milk sample (10 g) was transferred to a 50 mL tube and spiked with 50 μL of standard. The sample was allowed to stand for 10 min before adding 12 mL of 1% (v/v) acetic acid in acetonitrile, followed by addition of 2.5 g of $MgSO_4$ and 1.0 g of NaCl. Next samples were shaken by hand for 1 min prior to centrifugation at 4500 rpm for 10 min. The supernatant was transferred into a 15 mL tube and the solvent evaporated under a gentle stream of nitrogen at 60 °C for 60 min. Samples were reconstituted in 5 mL of H_2O and passed through glass HLB columns previously conditioned with 3 mL TBME, MeOH, and H_2O. The columns were washed with 3 mL of H_2O and analytes eluted with 6 mL of 1% acetic acid (v/v) in 10% MeOH in TBME into a 15 mL tube with 100 μL of DMSO as a keeper. The solvent was evaporated under nitrogen at 60 °C for 40 min and diluted with 200 μL MeOH/H_20 (1:3).

2.4. Cell culture

The estrogen specific RGA cell line (MMV-Luc) was developed as previously described (Willemsen et al., 2004). Briefly, this RGA cell line was generated from a human mammary gland cell line by stable transfection with the luciferase gene under the control of a steroid hormone inducible promoter. The cells were cultured in 75 cm^2 tissue culture flasks (BD Biosciences, Bedford, MA, USA) at 37 °C with 5% CO_2 and 95% humidity and routinely cultured in cell culture medium containing: Dulbecco's Modified Eagle Medium (DMEM) without phenol red which is a weak estrogen, 10% (v/v) foetal bovine serum and 1% (v/v) penicillin–streptomycin. Cells were transferred two passages prior to RGA analysis into assay media, containing hormone depleted serum.

2.5. Reporter gene assay

Cells were seeded at a concentration of 4×10^5 cells mL^{-1}, 100 μL $well^{-1}$, into white walled 96 well plates with clear flat bottoms (Greiner Bio-One, Frickenhausen, Germany) and incubated for 24 h. The following day, standard curves and samples extracts were prepared 1% (v/v) in assay media giving the final methanol concentration of 0.5% (v/v). For the E2 calibration curve following spiking concentration in range 0.008–16.2 ng g^{-1} was used. The cells were incubated for 24 h. The supernatant was discarded and the cells washed twice with PBS (pH 7.1) prior to lysis with 25 μL cell culture lysis buffer (Promega, Southampton, UK). Finally, 100 μL luciferase substrate (Promega, Southampton, UK) was injected into each well and luciferase activity measured using the Mithras Multimode Reader (Berthold, Other, Germany). The responses of the cells were measured and compared with the negative control (blank milk sample extract).

2.6. Cell viability assay

Viability was determined using the thiazolyl blue tetrazolium bromide (MTT) assay. Briefly, cells were seeded as for the RGA and the plates incubated for 24 h. The supernatant was discarded and the cells washed once with PBS. Fifty microliters of MTT solution (5 mg mL^{-1} stock in PBS, diluted 1:2.5 (v/v) in assay media) was added to each well and the cells incubated for 3 h. Viable cells convert the soluble yellow MTT to insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. The supernatant was once again removed and 200 μL of DMSO (Sigma–Aldrich, St. Louis, MO, USA) was added to dissolve the formazan crystals and the plate was incubated, with agitation, at 37 °C for 10 min. Optical density was measured in a Tecan Safire2 microplate reader (Tecan group Ltd., Männedorf, Switzerland) at 570 nm with a reference filter at 630 nm. Samples were assessed in triplicate and in three independent exposures. Viability was calculated as the % absorbance of the sample when compared with the absorbance of the control (reconstitution solvent).

2.7. Estrogen RGA validation

All the calculations and required tests were carried out in accordance with the European Union (EU) commission decision implementing Council Directive 2002/657/EC concerning the performance of analytical methods and interpretation of results (EU, 2002).

2.7.1. Blanks

Pre-screening for blank samples was performed in order to identify samples giving the lowest responses. As milk possesses free endogenous estrogens at a maximum level of 33 pg mL^{-1} (Courant et al., 2008) true blanks were not available for the majority of milk matrices. Samples selected were the lowest responders i.e. up to 3% maximal response (standardised to a curve prepared in solvent and solvent methanol blank being a reference).

2.7.2. Specificity and interference

The specificity of the estrogen RGA was investigated by spiking a blank milk sample with high levels of endogenous hormones (Te and P) which may potentially cross-talk in the estrogen RGA. The maximal level of Te and P reported in milk is 20 pg mL^{-1} (Courant et al., 2008) and 11 ng mL^{-1} (Hartmann et al., 1998) respec-