Bull Environ Contam Toxicol (2011) 86:461–464
DOI 10.1007/s00128-011-0251-x

A Comparison of Recombinant Receptor-Reporter Gene Bioassays and a Total Estrogen Enzyme Linked Immunosorbent Assay for the Rapid Screening of Estrogenic Activity in Natural and Waste Waters

Mayumi Allinson · Fujio Shiraishi · Graeme Allinson

Received: 15 September 2010 / Accepted: 16 March 2011 / Published online: 26 March 2011

Abstract In this study, we compared the performance as screening tools of two yeast-based recombinant receptor-reporter gene bioassays with a commercial ELISA kit for measurement of total estrogens. For WWTP effluents there was a very good correlation between the measured total estrogen concentrations (ELISA) and estrogenic activity by the hERα bioassay ($r^2 = 0.93$), but not for the medERα bioassay ($r^2 = 0.50$). For freshwater samples, the correlations between bioassay response and ELISA ES measurments were very good ($r^2 > 0.95$). There was no correlation between bioassay response and ELISA ES measurments for estuarine samples.

Keywords Estrogenic activity · Recombinant receptor-reporter gene bioassay · Total estrogens ELISA

The occurrence of endocrine disrupting chemicals (EDCs) in the aquatic environment and their impact on indigenous fauna has generated a significant amount of scientific and public interest in the last decade. The naturally-occurring, steroidal estrogenic sex hormones are the source of much of the hormonal activity in many waterways, but residues of a diverse range of estrogenic chemicals may also be present. Typically such compounds are found at levels lower than required to cause cellular expression of estrogenicity, but may act cumulatively to do so (Sonnenschein and Soto 1998). In short, the behaviour of estrogenic substances is considered to be additive, and this has become the basis for quantitatively assessing total estrogenic activity using in vitro assays (Kinnberg 2003). Several in vitro assays have been developed to screen the estrogenic activity of compounds in natural and waste waters, including ligand-binding assays, recombinant receptor-reporter gene bioassays, assays based on the measurement of cell proliferation, and enzyme-linked immunosorbent assays (ELISA; Streck 2009; Kinnberg 2003). Recombinant receptor-reporter gene bioassays, such as the yeast two-hybrid assay used in this study, measure the activation of an estrogen receptor, and so they can both provide a broad measure of the hormonal potency of samples, and an indication of the level of anti-estrogenic activity; ELISAs have potential utility as a method for the rapid mapping of contaminant levels across large areas, and to optimise the design of monitoring networks (Morozova et al. 2005).

Estrogenic steroid hormones in water samples can be reliably and definitively quantified using instrumental methods based on liquid chromatography – mass spectrometry (LC–MS). However, because of the large number of known and potential estrogenic compounds, and concomitant resource implications (including the need for expensive instrumentation and high level technical expertise in its operation), preliminary screening of samples using rapid assessment tools, such as in vitro assays, is an attractive prospect for waterways managers. One of the major advantages of using in vitro assays is that the total

M. Allinson · G. Allinson
CAPIM, Bio 21 Institute, University of Melbourne, Parkville, VIC 3052, Australia

M. Allinson · G. Allinson (✉)
Future Farming Systems Research Division, Department of Primary Industries, PO Box 114, Queenscliff, VIC 3225, Australia
e-mail: graeme.allinson@dpi.vic.gov.au

F. Shiraishi
Environmental Quality Measurement Section, Research Centre for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

estrogenic activity observed when the assays are challenged by sample extracts can be expressed as 17β-estradiol equivalents (EEQ), which allows for quantification of estrogenic activity without having to know the precise chemical make up of the sample. However, few laboratories are able to undertake such screening using recombinant receptor-reporter gene assays compared to the number potentially able to screen suitably prepared samples using commercial ELISA kits. As part of studies assessing the hormonal activity of Victorian waterways we were able to obtain samples from a range of sources, including municipal waste water treatment plant (WWTP) effluents, rivers and estuaries. Thereafter, in this study we compared the performance of two recombinant receptor-reporter gene bioassays with a commercial ELISA kit for measurement of total estrogens, in order to assess whether using a commercial ELISA kit for the measurement of total estrogens is a reasonable surrogate for bioassay screening.

Materials and Methods

Water samples were collected as 'grab' or spot samples from 39 WWTPS, or about 20% of the state's WWTPs, at the point of discharge, i.e. the point at which effluent enters the environment, either as recycled water for agricultural or other purposes, or direct discharge to the receiving water, in the 4 weeks from February 12–March 7, 2007. A single reconnaissance survey was conducted on 6 estuaries and 16 freshwaters (rural, peri-urban and urban streams, wetlands and lakes) in the weeks between February 18 and March 12, 2009, and February 9 and April 1, 2009, respectively. Samples were directly collected in glass bottles, stored on ice, and then at 4°C until processed (generally within 36 h of collection). For each WWTP site, an aliquot of the effluent (1L) was extracted for the measurement of estrogenic activity using a yeast two-hybrid bioassay, and a second aliquot (500 mL) extracted for measurement of total estrogens (ES) by ELISA. The sample preparation methods for these tests are described elsewhere (Shiraishi et al. 2000; Allinson et al. 2007, 2008, 2010) but involved filtration and adding buffer solution to the sample to ensure an acid pH (according to JEA 1998), filtration through GF/C filters to remove particulate matter, and then solid phase extraction (SPE; Octadecyl C18FF disk (Empore; 47 mm; 3 M, MN, USA)) and elution of analytes with methanol. After evaporation, the sample was re-suspended in a mixture of 3:1 hexane: dichloromethane (1 mL), and loaded onto a florisil column (Varian Bond Elut-FL, 500 mg, 3 mL; CA, USA). Thereafter, elution protocols separated the extract into three fractions, first a 3:1 hexane:dichloromethane fraction (H/D), second a 1:9 acetone:dicholoromethane fraction (A/D), and finally a methanol fraction (MeOH).

Measurement of hormonal activity was undertaken with a yeast two-hybrid recombinant receptor-reporter gene bioassay system using yeast cells (*Saccharomyces cerevisiae* Y190) in accordance with the method of Shiraishi et al. 2000 although, in this case, yeast into which the human estrogen receptor ERα or the estrogen receptor from Japanese medaka (*Oryzias latipes*) had been inserted (hERα and medERα, respectively; Nishikawa et al. 1999). In short, samples and standards were dissolved in dimethyl sulphoxide. Yeast cells were cultured in a modified SD (Sabouraud Dextrose) medium (lacking tryptophan and leucine). Modified SD solution (60 µL) was added to each well of the first row of a 96-well culture plate. Thereafter, 2% DMSO/modified SD solution (60 µL) was automatically added to each well of the 2nd–8th rows of the plate. Six samples were run on each plate, with aliquots of each sample (60 µL), added to two, neighbouring wells of the 1st row of the plate. An aliquot was removed from each well of row 1, and added to row 2 to dilute twofold. This process was then repeated from rows 2–7. No sample solution from row 7 was added to the 8th row. Thereafter, yeast solution (60 µL) was added to all wells, the plate shaken (30 s) and then incubated (30°C, 4 h). After incubation, a mixed solution (80 µL) for inducing chemiluminescence and for enzymatic digestion was then added to each well, and the plate incubated (37°C, 1 h). Thereafter, a light emission accelerator solution (50 µL) was added to each well, and the chemiluminescence produced by released β-galactosidase measured with a 96-well plate luminometer. Agonist activity was recorded as the EC $\times$ 10 (defined as the concentration of test solution producing a chemiluminescent signal 10 times that of the blank (negative) control). The method limits of reporting (LOR) for the hERα and medERα bioassay were 0.1 and 0.4 ng/L 17β-estradiol equivalents (EEQ), respectively. In order to verify the reproducibility of the bioassay measurements, $\sim$10% of the effluent water samples were processed in duplicate. The average difference between these repeat measurements was 47% and 26% in the hERα and medERα bioassay, respectively.

Measurement of total estrogens (ES) was undertaken using commercial ELISA kits in accordance with the manufacturer's instructions (Ecologiena® Estrogenes (E1/E2/E3) ELISA Kit (Tokiwa Chemical Industries, Japan)). In order to verify calibration accuracy, check standards (i.e. standards from the kit run as samples) were run in duplicate on each ELISA plate during each ELISA test. The ratio of nominal concentrations and result values were 105% for total estrogen. Consequently, to assess the relative response of the ELISA kit, the kits were challenged with estrone (E1), estriol (E3), and both 17α-and 17β-estradiol (E2). The average ratio of nominal concentrations and result values were: E1, 70%; E3, 50%; 17α-E2, 10%; 17β-E2, 100%. The ELISA method LOR was 0.1 ng/L EEQ.

Results and Discussion

In 2009, as part of larger programs investigating the hormonal activity of Victorian waters we were able to compare the performance of two recombinant receptor-reporter gene bioassays with a commercial ELISA kit for measurement of total estrogens to assess the applicability of using the latter for rapid screening of water samples. In that context, the total estrogen ELISA system worked well for WWTP samples. For instance, almost all of the WWTP effluents effluent samples collected in 2007 (Allinson et al. 2010) showed estrogenic activity in both the hERα and medERα bioassays (hERα, <0.1–16 ng/L EEQ; medERα, <0.4–18 ng/L EEQ). In this study we confirmed the presence of steroidal estrogens using the ELISA kit (ES, 0.2–41.1 ng/L EEQ), and found a very good correlation between the measured ES concentrations (ELISA) and estrogenic activity of the WWTP samples determined by the hERα bioassay ($r^2 = 0.93$; Table 1), but not for the medERα bioassay ($r^2 = 0.50$; Table 2). The stronger correlations observed for the hERα bioassay with the WWTP samples may be due to the greater sensitivity of this bioassay towards steroid hormones compared to the medERα bioassay, and, in turn, the weaker correlation observed for the medERα bioassay due to that bioassay's greater sensitivity to a wider range of estrogenic compounds than the hERα bioassay, including its sensitivity to many of the industrial compounds often found in WWTP effluent. There was also good correlation between ES concentrations in the WWTP samples measured in this study and the sum of specific estrogen concentrations reported by Allinson et al. (2010) calculated using relative response of the total estrogen ELISA system of E2 1.0, and E1 0.7 ($r^2 = 0.85$).

A measurable response was observed using the ELISA kit in each freshwater sample, although on the whole the level of estrogen observed was low (<2 ng/L; one outlier, a WWTP impacted site, with an ES of 12.4 ng/L EEQ). Despite this, there was still a very good correlation between estrogenic activity and the measured total estrogen concentrations for freshwater samples for both the hERα bioassay ($r^2 = 0.98$; Table 1), and the medERα bioassay ($r^2 = 0.97$; Table 2). That the correlations were poor for the medERα bioassay when evaluating WWTP effluents but good when assessing the freshwater samples may have been due to there only being steroid estrogens as the source of steroid estrogenic activity in the freshwaters examples, regardless of its source (rural stream or urban river).

There was no correlation for estuarine samples between observed ES concentrations using the ELISA test kit and estrogenic activity as measured by either bioassay ($r^2 < 0.1$). The reason for the difference between the results of ELISA and the bioassay for estuarine samples is not yet fully understood, but is consistent with Hashimoto et al. (2007), who reported that chemical analysis of estuarine samples from Suruga Bay, Japan (EC: ~12–33 mS cm^{-1}) showed higher calculated estrogenic activities relative to estrogenic activities obtained by the same bioassays (and bioassay laboratory) used in this study.

In 2010, the United States Environmental Protection Agency (US EPA) contracted AOAC INTERNATIONAL (AOAC) to develop standard method performance requirements (SMPRs) for test kits for EDCs, specifically for a quick, cheap and easy way for state and local authorities to

Table 1 Comparison and correlation of estrogenic activity (hERα) with measured ES concentration (ELISA)

Sample type	n	Estrogenic activity (hERα)		Total estrogens (ELISA)		Correlation (r^2)
		Mean	(Minimum–maximum)	Mean	(Minimum–maximum)	
		(ng/L EEQ)				
WWTP	39	1.4	(<0.1–16.0)	3.8	(0.2–41.1)	0.93
Fresh	16	1.1	(<0.1–12.0)	1.5	(0.1–12.4)	0.98
Estuary	12[a]	0.1	(<0.1–0.4)	3.3	(0.6–8.4)	0.02

n, number of samples; [a] Each estuary sampled twice

Table 2 Comparison and correlation of estrogenic activity (medERα) with measured ES concentration (ELISA)

Sample type	n	Estrogenic activity (medERα)		Total estrogens (ELISA)		Correlation (r^2)
		Mean	(Minimum–maximum)	Mean	(Minimum–maximum)	
		(ng/L EEQ)				
WWTP	39	3.1	(<0.4–18.0)	3.8	(0.2–41.1)	0.50
Fresh	16	0.9	(<0.4–11.0)	1.5	(0.1–12.4)	0.97
Estuary	12[a]	<0.5	(<0.4)	3.3	(0.6–8.4)	–

n, number of samples; [a] Each estuary sampled twice

monitor steroid estrogen hormones in surface waters potentially contaminated by livestock operations, e.g. intensive and extensive dairy operations, swine and poultry farms (AOAC 2010). In this context there are a number of advantages to immunochemical methods when screening for trace organic contamination of natural and waste waters, such as (Morozova et al. 2005) simplicity of operation, rapidity of determinations, and the relatively low cost of determinations once the samples have been prepared (in this case, about Aus $35–45 per sample depending on exchange rate fluctuations, compared to several hundred dollars for each of the yeast two-hybrid bioassays, and higher for LC–MS techniques). Moreover, because of the large number of known and potential estrogenic compounds, and concomitant financial implications associated with definitive tests (such as LC–MS/MS techniques) preliminary screening of samples using rapid assessment tools is an attractive prospect for waterways managers. However, the success or failure of any chemical analytical technique is primarily due to the ability of analysts to remove as many extraneous compounds as possible from the sample prior to measurement. The samples investigated in this study were prepared using fairly straightforward process involving solid-phase extraction, a process eminently suitable to many laboratories.

Few laboratories are able to undertake screening for estrogenic activity using recombinant receptor-reporter gene assays compared to the number potentially able to screen suitably prepared samples using commercial ELISA kits. Consequently, this pilot study has shown that in those situations where there is a high probability that estrogenic activity is the result of steroid hormones, e.g. effluent from WWTPs receiving only domestic wastes, or animal feedlot operations, or where information on only the levels of steroidal estrogens is required, the use of commercial ELISA kits measuring total estrogens may be a cost-effective way to rapidly screen samples prior to more definitive (and more expensive) testing. Where there is no such a priori understanding of the source of potential estrogenic activity, or where information on the prevalence of anti-estrogenic materials in samples is required, the use of other in vitro assays is recommended as a screening technique.

Acknowledgments The research was primarily supported by the Department of Primary Industries (Project #06889, 08162), with additional assistance from Melbourne Water and the Victorian Water Trust (Project #33 V-4000). The authors confirm that they received no funding from, nor have any financial ties with the manufacturer of the ELISA kit, nor does its use in this study imply any endorsement of the kit by the authors or their respective organisations. The project team gives its thanks to Kathryn Hassell, Kavitha Chinathamby, Trevor Theodoropoulos and Adam Wightwick for assistance with collection of samples, and all the Victorian water authorities, who, in one way or another, contributed to the success of this project.

References

Allinson G, Allinson M, Salzman S, Shiraishi F, Myers J, Theodoropoulos T, Hermon K, Wightwick A (2007) Hormones in treated sewage effluent. Final report. A report prepared for the Victorian Water Trust. Department of primary industries, Queenscliff, Australia. Available on-line (last accessed: 4 January 2010): www.ourwater.vic.gov.au/programs/victorian-water-trust/publications

Allinson G, Allinson M, Shiraishi F, Salzman SA, Myers JH, Hermon KM, Theodoropoulos T (2008) Androgenic activity of effluent from forty-five municipal waste water treatment plants in Victoria, Australia. Trans Ecol Environ 110:293–304

Allinson M, Shiraishi F, Salzman SA, Allinson G (2010) In vitro and immunological assessment of the estrogenic activity and concentrations of 17β-estradiol, estrone and ethinyl estradiol in treated effluent from forty-five waste water treatment plants in Victoria, Australia. Arch Environ Contam Toxicol 58:576–586

AOAC (2010) AOAC signs new contract with EPA to develop SMPRs for endocrine disrupting compounds in animal feedlot runoff. Inside Lab Manag 14(3):13–14

Hashimoto S, Ueda Y, Kurihara R, Shiraishi F (2007) Comparison of the estrogenic activities of seawater extracts from Suruga Bay, Japan, based on chemical analysis or bioassay. Environ Toxicol Chem 26:279–286

JEA (1998) Draft manual for surveying exogenous endocrine disrupting chemicals (water, sediment, aquatic organisms). Japan Environment Agency, Water Quality Bureau, Water Quality Management Division, Tokyo, Japan (in Japanese)

Kinnberg K (2003) Evaluation of in vitro assays for determination of estrogenic activity in the environment (No. 43). Danish Environmental Protection Agency, Danish Ministry of the Environment. http://www2.mst.dk/udgiv/publications/2003/87-7972-922-3/pdf/87-7972-923-1.pdf

Morozova VS, Levashova AI, Eremin SA (2005) Determination of pesticides by enzyme immunoassay. J Anal Chem 60:202–217

Nishikawa J-I, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T (1999) New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicol App Pharmacol 154:76–83

Shiraishi F, Shiraishi H, Nishikawa J, Nishihara T, Morita M (2000) Development of a simple operational estrogenicity assay system using the yeast two-hybrid system. Kankyo Kagaku 10:57–64 (in Japanese)

Sonnenschein C, Soto AM (1998) An updated review of environmental estrogen and androgen mimics and antagonists. J Steroid Biochem Molec Biol 65:143–150

Streck G (2009) Chemical and biological analysis of estrogenic, progestagenic and androgenic steroids in the environment. Trends Anal Chem 28:635–652