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Detection of estrogenic activity in municipal wastewater effluent using primary cell cultures from three-spined stickleback and chemical analysis

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ABSTRACT

Environmental estrogens are substances that imitate the effects of endogenous estrogens. Effluents from municipal wastewater treatment plants are known to contain substances with estrogenic activity including steroidal estrogens and xenoestrogens. In the current study, a combination of biological and chemical analysis was applied to determine the estrogenic activity in municipal wastewater effluents in Finland. The male three-spined stickleback (*Gasterosteus aculeatus*) hepatocyte assay with vitellogenin induction as an endpoint was used for the detection of estrogenic activity in solid phase extracts of wastewater effluents, and 17β-estradiol (E2) as a positive control. The wastewater extracts and E2 were found to induce vitellogenin production. The extracts were also subjected to chromatographic fractionation and the collected fractions were assayed. The only active fraction was the one in which E2, estrone and ethynylestradiol were eluted. Its activity corresponded to the activity of the original wastewater extract. The LC–MS/MS analyses of the wastewater extracts showed that the concentration of estrone was about 65 ng L^{-1}, the concentration of E2 was less than 1 ng L^{-1}, while estriol and 17α-ethynylestradiol could not be detected. These findings showed that the activity of the wastewater extracts and the chromatographic fraction was much higher than the activity which could have been expected on the base of the chemical analysis. This strongly indicates that other compounds, possibly acting by additivity or synergism, are playing a major role in the induced vitellogenin production by the hepatocytes.

1. Introduction

Endocrine disrupting compounds (EDCs) are chemicals which interfere with the endocrine system causing adverse reproductive effects in exposed organisms. Adverse effects with regard to human health, namely the increasing number of endocrine responsive cancers and the decreasing reproductive fitness of men, are thought to be attributed to estrogenic EDCs (Daston et al., 1997; Juberg, 2000). A number of natural and synthetic compounds have been identified as belonging to this group of chemicals, including natural and synthetic steroid hormones, phytoestrogens, alkylphenols, pesticides, surfactants and polychlorinated biphenyls (Jobling et al., 1995; Soto et al., 1995; Routledge and Sumpter, 1997).

Many EDCs are potentially released into the environment through discharges from wastewater treatment plants (WWTPs), surface non-point runoff, and atmospheric deposition of particulates and aerosols (Campbell et al., 2006). Natural steroid hormones and the synthetic ethynylestradiol, alkylphenols and bisphenol A are EDCs that have been identified in sewage effluents (Desbrow et al., 1998; Lye et al., 1999; Ternes et al., 1999; Körner et al., 2000; Kuch and Ballschmiter 2001; Spengler et al., 2001).

The chemical structures of compounds with estrogenic activity show a high diversity, which makes it difficult to predict estrogenic potency solely on the basis of compound structure. The ability of the compounds to interact with the human estrogen receptor has been used in a variety of different screening tests. Recombinant yeast assays based on genetically modified yeast cells in which the expression of a reporter gene is driven by the activation state of the human estrogen receptor have been proposed as useful screening methods (Roda et al., 2006). However, studies on the endocrine activity of wastewater samples that have been carried out to date have yielded controversial results.

In particular, several aquatic ecosystems have been studied for the effect of effluents from WWTPs (Jobling et al., 1998; Routledge et al., 1998; Tilton et al., 2002), with fish being the primary targets for waterborne endocrine disrupters. The most widely accepted endpoint for the assessment of estrogenic activity of EDCs has been the production of female yolk protein vitellogenin (VTG). VTG is naturally produced in the liver of oviparous organisms after stimulation with endogenous estrogens, but exogenous estrogenic chemicals can do exactly the same. Detection of VTG using

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enzyme-linked immunosorbent assay (ELISA) has been used in many male fish species to screen estrogenic and anti-estrogenic activity of chemicals and wastewater effluent *in vivo* (Iguchi et al., 2006). However, *in vivo* experiments for investigating estrogenic effects are generally time consuming and expensive, therefore sophisticated analytical techniques for the measurement and assessment of EDCs are highly valued. Since VTG response can also be measured from isolated fish hepatocytes, the use of such a bioassay has been suggested as an *in vitro* screening for identifying estrogen-active substances (Pelissero et al., 1993; Smeets et al., 1999; Segner and Braunbeck, 2003). The hepatocyte VTG assay has the potential to detect effects of estrogenic metabolites and anti-estrogenic effects (Navas and Segner, 2006).

Recently, the three-spined stickleback (*Gasterosteus aculeatus* L.) was introduced as a model species for evaluation of effects caused by endocrine disrupters (Katsiadaki et al., 2002, 2007; Hahlbeck et al., 2004). The three-spined stickleback is naturally present in most fresh, brackish and marine environments (Curry-Lindahl, 1985) over the whole of the North Hemisphere, which makes this fish species an excellent sentinel and surrogate species. Primary liver cell and tissue slice cultures isolated from male three-spined stickleback has recently been characterized and tested for detection of estrogenic activity (Björkblom et al., 2007).

The low environmental concentrations of the causative agents, the complicated sample matrices, and the diversity of target compounds have generated a need for different robust *in vitro* bioassays. To identify individual estrogenic compounds in environmental samples, the results from biological assays must be combined with chemical analysis (gas or liquid chromatography coupled to mass spectrometry, GC–MS and LC–MS). In the present study, a combination of chemical analysis and biological analysis was applied to assess the estrogenic activity of effluents from a WWTP in Finland. A solid phase extraction and a chromatographic fractionation of the wastewater effluent was performed prior to assessment of estrogenic activity using VTG induction by primary hepatocyte cultures prepared from male three-spined stickleback. These results in combination with LC–MS/MS analysis were used for the determination of the compounds accounting for the estrogenic activity in wastewater effluent.

2. Materials and methods

2.1. Reagents and instrumentation for chemical analysis

Estrone (E1, purity 99%), 17β-estradiol (E2, 98%), 17α-ethynylestadiol (EE2, 98%) and estriol (E3, 99%) were purchased from Sigma–Aldrich. Acetonitrile (ACN), *n*-hexane, acetone, methanol, methyl-*tert*-butyl ether (MTBE) were all of analytical grade. Distilled water purified with Millipore Simplicity 185-system (Millipore S.A., Billerica, MA, USA) was used in all chemical sample treatment, as blank samples and for all chromatography. The deuterized internal standard of estrone, d_2-E1, was prepared as described by Block and Djerassi (1973). The purity and reaction recovery were checked by nuclear magnetic resonance (NMR, Bruker Avance 600 NMR spectrometer) and through direct infusion on an ion trap mass spectrometer (Agilent MSD SL Trap instrument). Standard solutions of $1\,g\,L^{-1}$ of the estrogens, except for the internal standard for which the concentration was $10\,mg\,L^{-1}$, were prepared in ACN and stored at −18 °C. From these, working solutions were prepared. All the glassware were washed with hot water, distilled water and acetone and dried in the oven at 250 °C for 8 h.

2.2. Sample collection

Twenty-four hours composite effluent samples from the municipal WWTP of the city of Turku, Finland, were collected in October 2006 in glass bottles. The municipal WWTP in Turku processes domestic and industrial wastewaters from a population of 160000 people and utilizes chemical and biological treatment processes. The influent flow is approximately $60000\,m^3\,d^{-1}$ and the Baltic Sea is the recipient of the plant final effluent. The samples were divided into smaller portions for the different preparations and the processing of the samples at the laboratory started immediately after collection to minimize possible degradation of the samples.

2.3. Solid phase extraction

Solid phase extraction (SPE) was used for isolation and concentration of the organic constituents of the wastewater effluent. Samples of 100 mL of effluent water were passed over the cartridge beds using the procedure previously described by Salste et al. (2007). In short, the filtrated samples were acidified and extracted on a pre-washed Oasis HBL 6 cc (200 mg, Waters, Helsinki, Finland) cartridge. After sample passages, the cartridges were washed and subsequently the elution was performed with 10% methanol/MTBE. The extract solvent was removed by evaporation under a gentle nitrogen stream. For the hepatocyte assay, the extracts were dissolved in 10 mL serum-free cell culture media, for the LC–MS/MS analyses they were dissolved in 100 μL 10% ACN in 10 mM NH_4OH, and for the chromatographic fractionation the extracts were dissolved in 1 mL methanol. The samples were stored at −20 °C until analysis. The internal standard (d_2-E1) was added ($100\,ng\,L^{-1}$) to the LC–MS/MS samples before extraction.

2.4. LC–MS/MS analysis

The quantitative analysis was performed on an Agilent 1100 HPLC-system (Agilent Technologies, Espoo, Finland) connected to a Quattro Micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). The concentration of the four estrogens (E1, E2, E3 and EE2, Scheme 1) in the wastewater effluent was determined by LC–MS/MS using deuterated E_1 (d_2-E1) as the internal standard. The relative recoveries (relative to the internal standard) were 105% for E1, 80% for E2, 43% for EE2 and 80% for E3. A detailed description of the LC–MS/MS method for the analysis of estrogens in wastewater has been reported previously (Salste et al., 2007).

2.5. Chromatographic fractionation

The chromatographic fractionation was performed on an Agilent 1100 Series liquid chromatographic system (Agilent Technologies, Espoo, Finland) as described by Salste et al. (2007). Shortly, 0.5 mL of the solid phase extracted effluent water was injected on a semi preparative reversed phase C-18 column (BDS Hypersil C18, 250 × 10 mm, 5 μm, Thermo Hypersil-Keystone, Krotek Oy, Tampere, Finland) and the column effluent was collected in fractions according to the retention time. The column was eluted with a gradient from 10% ACN in water to 80% ACN in 18 min and finally raised to 90% ACN. In all, six fractions were collected and each consisted of the eluent collected for a time period of 4.5 min. The fractionation was repeated twice so that each fraction corresponded to 100 mL of original wastewater effluent. To ensure that the HPLC system was free from contamination, it was washed extensively with 100% ACN and several gradients were run before injection of the actual samples. The fractions were concentrated with a rotary evaporator to dryness and reconstituted in 10 mL serum-free cell culture media for the hepatocyte assay and the samples were stored at −20 °C until analysis. Collected fractions were also recombined subsequent to fractionation, evaporated and reconstituted in 10 mL serum-free cell culture media.