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Endocrine disruptor activity in bottled mineral and flavoured water

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ABSTRACT

A panel of reporter gene assays (RGAs) coupled with a single solid phase extraction (SPE) step was developed and used to screen bottled mineral water for the presence of four classes of endocrine disruptors (EDs), oestrogens, androgens, progestagens and glucocorticoids.

Fourteen brands of bottled mineral water in triplicate (42 samples) were analysed. Overall, hormonal activity was found in 78% of the samples. Oestrogenic, androgenic, progestagenic and glucocorticoid activity was found in 38%, 38%, 36% and 55% of the samples, respectively at an average concentration of 10 ng/l 17β-estradiol equivalent (EEQ), 26 ng/l testosterone equivalent (TEQ), 123 ng/l progesterone equivalent (PEQ) and 13.5 ng/l hydrocortisone equivalent (HEQ).

The level of oestrogenic, androgenic and progestagenic activity observed is not considered a matter of concern for the consumers' health. It is unknown whether the glucocorticoid levels observed are safe. The ED source, long term exposure and mixture effects remain to be investigated.

1. Introduction

An endocrine disruptor (ED) is "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (OECD, 2010). EDs can interact with the endocrine system by mimicking or blocking natural hormones or their pathways, EDs may also influence the production, secretion or metabolism of endogenous hormones or/and their nuclear receptors (Tabb & Blumberg, 2006). Consequently, exposure to EDs may lead to detrimental health effects including interference with both the male and female reproductive systems, causing a spectrum of disorders throughout life, including sexual precocity, hormone related cancers e.g. testicular and breast cancer, reproductive tract abnormalities and infertility (Diamanti-Kandarakis et al., 2009). Other impacts of EDs may include effects on thyroid function, obesity and metabolism (Diamanti-Kandarakis et al., 2009).

It is now widely accepted that one of the major routes of ED exposure for humans is through the diet (Connolly, 2009). Consequently, with the consumption of bottled mineral water increasing worldwide (Doria, 2006) the presence of oestrogenic EDs in particular has become more widely investigated (Pinto & Reali, 2009; Wagner & Oehlmann, 2009; Wagner & Oehlmann, 2010). Pinto and Reali (2009) used an *in vitro* yeast bioassay to screen PET bottled mineral water and reported that 10% of the surveyed samples showed oestrogenic activity up to 23.1 ng/l 17β-estradiol equivalent (EEQ). Wagner and Oehlmann (2009) also used an *in vitro* yeast bioassay to provide evidence of oestrogenicity in glass and PET bottled mineral water with 60% of samples surveyed showing oestrogenic activity up to a maximum of 75 ng/l EEQ. They also reported that this contamination partly originates from compounds leaching from the PET bottles into mineral water as indicated by a molluscan model, whereby mud snails (*Potamopyrgus antipodarum*) that are highly sensitive to oestrogens were raised in glass and PET bottles and reproductive output measured. The results of the *in vitro* yeast based assay confirmed the oestrogenic results of the *in vivo* molluscan model in all except one PET bottled sample which showed a minimal response in the yeast assay but induced a more significant response in the mud snail experiment. Therefore, this result may imply that the *in vivo* assay is more sensitive than the *in vitro* assay, or that bottled water contains a mixture of EDs, potentially of other classes. For example, if EDs such as anti-androgens are present, they will remain undetected by the oestrogen receptor specific *in vitro* assay, but may still have effects on reproduction (Tillmann, Schulte-Oehlmann, Duft, Markert, & Oehlmann, 2001). Further confirmation of the presence of oestrogenic compounds in bottled mineral water has again been reported by Wagner and Oehlmann (2010) using an improved extraction procedure and the E Screen bioassay.

Three potential sources of oestrogenic ED contamination have been outlined by Wagner and Oehlmann (2009); contaminated source water, contamination with plastic components or detergents used in the production process or substances migrating from packaging materials. EDs found in the environment and having the potential to be present in resources used for drinking water

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production include: natural hormones and their metabolites, synthetic hormones, phyto- and mycooestrogens, drugs with hormonal side effects, heavy metals, pesticides and their metabolites, and industrial and household chemicals and their by-products (Burkhardt-Holm, 2010). Most research so far has focused on the detection of compounds in bottled water that bind to the oestrogen receptor, however, ligands for other hormone receptors may also be present. Multiple ED classes have previously been shown to exist in wastewater effluents and surface waters using a panel of reporter gene cell lines (Van der Linden et al., 2008). This study showed that androgen, progestagen and glucocorticoid hormonal activities were present at similar or much higher levels than that of oestrogens. The identity of many of these substances have yet to be established. Cleaning reagents with ED activity may originate in the production process and have been observed in bottled water previously (Diana & Dimitra, 2011). The widespread use of compounds such as parabens and perfluoronited compounds (PFCs) has lead to their detection in drinking water resources, lower levels of PFCs have been observed in bottled water (Richardson and Ternes, 2005). Low levels of bisphenol A has recently been reported in bottled mineral water, with bottle closures, the water itself or recycled PET being suggested as possible sources of contamination (Bach, Dauchy, Chagnon, & Etienne, 2012). Phthalates also have the potential to leach from plastics (Casajuana & Lacorte, 2003) and individual phthalates such as DBP and butyl benzyl phthalate (BBP) have been reported as weakly oestrogenic (Jobling, Reynolds, White, Parker, & Sumpter, 1995), while others such as the plasticiser DEHP has been shown to be non-oestrogenic. However, all of these phthalates have been shown to have anti-androgenic effects (Parks et al., 2000; Sohoni & Sumpter, 1998; Takeuchi et al., 2005). Therefore not only oestrogenic EDs but a range of ED classes may be present in bottled mineral water, this remains to be investigated.

The present study aimed to investigate the presence and activity profile of a broader range of EDs in bottled mineral water samples by combining a suitable sample pre-treatment step prior to analysis in oestrogen, androgen, progestagen and glucocorticoid responsive RGAs.

2. Material and methods

2.1. Chemicals and reagents

All cell culture reagents were supplied by Invitrogen Ltd. (Paisley, UK). Reference standards including 17β-estradiol, testosterone, progesterone and hydrocortisone, HPLC grade water and tert-Butyl-methyl-ether were obtained from Sigma (Poole, Dorset, UK). Falcon tissue culture flasks were supplied by BD Biosciences (Oxford, UK). Specialised RGA plates were supplied by Greiner Bio-One (Stonehouse, UK). A luciferase kit (Promega, E1501) was obtained from Promega (Southhampton, UK). Oasis HLB glass cartridges (5 cc/200 mg) were supplied by Waters Chromatography Ireland (Dublin, Ireland). Methanol and acetone were obtained from BDH (Poole, Dorset, UK).

2.2. Reporter gene assay analysis

Oestrogen (MMV-Luc), androgen (TARM-Luc), progestagen (TM-Luc) and glucocorticoid (TGRM-Luc) responsive mammalian reporter cell lines were previously produced by stable transfection with the MAR-Vit-Luc vector into the MCF-7 cell line in the case of oestrogen, and with the MMTV-Luc vector into the T47D cell line for each of the other three hormone specific cell lines (Willemsen, Scippo, Maghuin-Rogister, Martial, & Muller, 2002; Willemsen et al., 2004). Additionally for the androgen cell line the pSV-AR_0 expression vector was inserted, as the T47D cell line does not contain the androgen receptor gene. The RGA procedure was followed as described in a previous study by Plotan et al. (2011). The progestagen and glucocorticoid responsive RGAs were performed exactly as for the androgen assay using their corresponding standards. 17β-Estradiol, testosterone, progesterone and hydrocortisone were applied as the standards for the oestrogen, androgen, progestagen and glucocorticoid RGAs, respectively.

2.3. Cytotoxicity testing

All extracts produced for validation of the assay and the samples used in the screening study were tested for cytotoxicity on all four cell lines, MMV-Luc, TARM-Luc, TM-Luc and TGRM-Luc. The thiazolyl blue tetrazolium bromide (MTT) based cytotoxicity assay was used to check the toxicity of the applied samples, the protocol used was as described previously by Frizzell et al. (2011).

2.4. Oasis HLB cartridge SPE

Solid phase extraction (SPE) was used for the extraction of EDs from the bottled mineral water samples. Waters Oasis HLB glass columns (5cc/200 mg) were used. The extraction procedure included conditioning with 3 ml of tert-Butyl methyl ether, methanol and HPLC grade water; loading of the sample (10, 20, 50 and 250 ml); washing with 3 ml of 5% methanol in water and elution with 6 ml of 10% methanol in tert-Butyl methyl ether. Eluent was evaporated under a nitrogen stream at 40 °C and resuspended in 50 μl methanol.

2.5. Extraction recoveries

Pure HPLC grade water (250 ml) was spiked with 17β-estradiol, testosterone, progesterone and hydrocortisone at 0.5, 2.9, 5 and 100 ng/ml, respectively, and extracted using the SPE procedure described in Section 2.4. The recoveries were determined based on the RGA results for these extracts which were compared with the spiked concentrations results for each of the standards. The extraction recoveries for each RGA was determined from seven independent experiments. All concentrations (ng/ml) presented in the manuscript describe final well concentrations.

2.6. Influence of sample volume on the HLB extraction

In order to investigate the influence of the sample volume on the HLB extraction and subsequent RGA results, a series of volumes of HPLC grade water was extracted and applied to the androgen responsive cell line. Two samples of each of four volumes of water were measured: 10, 20, 50 and 250 ml. One was left unspiked and the other was spiked with 29 ng testosterone (to give a 2.9 ng/ml final well concentration in each case), both were then extracted and applied to the androgen RGA. The influence of the sample volume was measured based on the extraction recoveries determined (Section 2.5) and this was investigated in duplicate.

2.7. Repeatability study

Repeatability of the oestrogen, androgen, progestagen and glucocorticoid RGAs was investigated by triplicate analysis of (un)-spiked HPLC grade water, this was performed in a single experiment. Three water samples (250 ml) were left unspiked and the other three water samples of the same 250 ml volume were spiked with one of the four hormone standards at the same concentrations: 17β-estradiol (0.5 ng/ml), testosterone (2.9 ng/ml), progesterone (5 ng/ml) and hydrocortisone (100 ng/ml). These