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Short communication

Determination of trace endocrine disruptors in ultrapure water for laboratory use by the yeast estrogen screen (YES) and chemical analysis (GC/MS)

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ABSTRACT

High purity water for endocrine disruptors (EDs) analysis in experimental tests is an indispensable requirement for the preparation of reagents and solutions employed in biological laboratories. Commercial ultrapure water may contain traces of organic compounds, which can interfere with *in vitro* bioassays carried out to detect the potential estrogen-like activity of pure compounds and complex mixtures. This paper shows that solid-phase extracts of different types of ultrapure water (UPW) purchased or produced *in situ* for laboratory analysis (mQ-UPW) may contain organic molecules able to antagonize the binding of E_2 to the human estrogen receptor α in the yeast estrogen screen (YES) assay. GC/MS analysis detected the presence of bis(2-ethylhexyl) phthalate (DEHP) (0.033 ppm ± 0.006) in mQ-UPW extracts. The dose–response curve of DEHP in the YES assay showed a relevant antagonist effect of this phthalate. Agreement between content of DEHP chemically detected in UPW extract and the magnitude of biological effects induced was pointed out. It would be appropriate that chemical analyses were complemented by biological tests to establish concentration limits for chemical contaminants in UPW that do not induce biological effects detectable *in vitro*. The yeast assay used in this study has previously proved to be a sensitive tool in assessing the presence of agonistic/antagonistic chemicals at the ng/l level in complex mixtures and may be successfully used to identify trace amounts of estrogenic/antiestrogenic chemicals, which can represent critical issues influencing the experimental results in environmental testing laboratories.

1. Introduction

Endocrine disruptors (EDs) are a heterogeneous group of substances characterized by their potential to interfere with the function of the endocrine system in wildlife and humans. Potential targets of EDs are all tissues and organs containing specific receptors for steroid and thyroid hormones. For this reason much attention has been focused on substances with a structural similarity to steroid hormones, particularly estrogens, which may have high affinity for their receptors. The mechanism of action of these chemicals does not seem, however, limited to agents directly binding the hormone receptors, although this appears to be one of the most important mechanisms.

Other pathways may involve the synthesis, transport or metabolism of hormones as well as the activation of hormone receptors by different precursors [1].

The growing availability of scientific data has clearly demonstrated the ubiquitous presence of these substances which may cause deleterious effects, in the medium and long term, especially during fetal development and childhood [2–12], resulting in a potential multiple exposure of the biota.

Several *in vivo* and *in vitro* tests have been performed to assay hormone-like activity of xenobiotics. Since more information exists for receptor binding activity than for other classes of actions, particularly for estrogenic chemicals, a series of *in vitro* assays have been developed to measure the ability of xenobiotics and environmental mixtures to bind the human estrogen receptors (hERs), and their performance is continuously improving. The use of receptors linked to reporter genes in transformed cellular systems has been proposed as a reliable method for the screening of large amounts of xenobiotics, including water contaminants.

UPW is commonly used for the preparation of analytical solution and growth media for microbiological purposes; in control and calibration solutions as well as in chemical analysis. As several organic and inorganic contaminants may be present either in water dissolved or in suspended or colloidal particles, all these impurities should be removed from laboratory water before use.

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Conventional water purification techniques remove the bulk of organic compounds from water but some trace organics may still remain in ultrapure water released by the equipment used for its filtration and purification. The study of environmental hormones or hormone-like chemicals requires very sensitive tests for analysis, therefore there is a need for endocrine-disruptors free ultrapure water.

During a funded research project aimed at assessing the possible leaching of complex mixtures of biologically active migrants from plastic medical devices by an *in vitro* assay using a yeast strain transfected with a human estrogen receptor (ER) (yeast estrogen screen, YES) we observed some critical points in experimental procedures due to the ultrapure water used.

On this basis, we decided to check the adequacy of several UPWs to be used for studying the endocrine deregulation induced by several complex matrices and/or individual compounds. This short paper shows that UPWs, commercially available or produced in research laboratories by a certified equipment, may contain traces of organic substances able to interfere with experimental results in a biological assay to assess hormone-like activity of chemical compounds.

2. Experimental

2.1. Samples

The following water samples were analyzed:

Sample A (Brand 1): "endotoxin-free" UPW, sterile-filtered, cell culture tested, marketed in plastic containers. The following specifications are given: Total impurities ≤1.0 EU/ml endotoxin; refractive index n20/D 1,34 (lit.); pH = 6.0–8.0; boiling point = 100 °C at 760 mmHg (lit.); density = 1 g/cm^3 at 3.98 °C (lit.), TOC (not indicated).
Sample B (Brand 2): UPW for HPLC analysis. This water is marketed in glass containers. The following specifications are given: TOC ≤ 0.1 ppm; nitrate ≤ 0.1 ppm; Heavy metals (Pb) ≤ 0.1 ppm; filtered at 0.2 μm.
Sample C: UPW for dialysis produced by Certified Laboratory (UNI EN ISO 9001-2000, CERTIQUALITY).
Sample D: mQ (Millipore® Quality) water produced in a biological laboratory (Lab 1) by using Millipore Equipment (Milliq Academic Q GAR DOOR 1/2008).
Sample E: mQ-water produced in a NRC chemical laboratory (Lab 2) by using Millipore Equipment (Millipore Elix-Type II analytic grade water).
Sample F: bidistilled water produced in a biomedical laboratory (Lab 3) by using an old glass apparatus connected to a deionizing tank through a polyethylene (PE) pipe.
Sample G: Preliminary experiments indicated that local municipal drinking tap water (spring water treated with sodium hypochlorite) showed, in repeated experiments, neither estrogenic activity [13] nor inhibitory activity (maximum inhibitory activity, 2.25%, data not shown), so it was used as control water.

2.2. Chemical standards

17β-Estradiol (E_2), diethylstilbestrol (DES), 4-hydroxytamoxifen (OHT), bisphenol A (BPA), di(2-ethylhexyl) phthalate (DEHP), 4-octylphenol (OP), and 4-n-nonylphenol (4-n-NP), hexadecane (99%), were purchased from Sigma (Milan, Italy). Methanol (HPLC gradient grade) was purchased from Mallinckrodt Baker Italia (Milan, Italy) and n-hexane (95%) from Panreac Quimica (Barcelona, Spain). Dimethyl sulfoxide (DMSO 99.5%) was purchased from Riedel-de Haën (Seelze, Germany).

2.3. Solid-phase extraction (SPE)

Water samples (1000 and 200 ml) were extracted by solid-phase extraction (SPE) on C18 cartridges (1 g/6 ml) (Supelco, Milan, Italy), OASIS-HLB cartridges (60 mg/3 ml) (Waters Corp., USA) and XAD-2 resins. Water was filtered by entering it directly into the cartridges to avoid interference due to the leaching of organic substances from pipes used for water adduction, at a flow rate of 5 ml/min. C18 cartridges were previously conditioned with 15 ml methanol and 15 ml n-hexane, dried overnight at 30 °C and eluted with 10 ml methanol and 10 ml n-hexane [14,15]. The eluate was dried in rotavapor and the extract dissolved in DMSO and stored at −20 °C.

OASIS™ cartridges were conditioned with 15 ml methanol and 15 ml water, dried overnight at 30 °C, washed with a mixture 5% (v/v) of methanol–water and eluted with 12 ml methanol [16,17]. The solvent was evaporated in rotavapor and the extract dissolved in DMSO and stored at −20 °C.

The extraction on XAD-2 resins was performed to test 1 l of water [18–20]. The glass columns were filled with the resin (10 cm). Water was filtered at a flow rate <10 ml/min. Columns were dried under a gentle flow of nitrogen, eluted with 15 ml methanol and 15 ml n-hexane. The solvent was evaporated in rotavapor, the extracts were dissolved in DMSO and stored at −20 °C.The extraction procedures were previously developed to assess the pollution of surface marine water from natural and synthetic hormones (research project supported by the Italian Ministry of the Environment, Directive UE/60/2000) and DES was used as a tracer to test the recovery efficiency of the hormones and hormone-like substances from water samples. The recovery efficiency of DES from water by SPE on XAD-2 and C18/OASIS cartridges was evaluated using a commercial UPW (Brand 1, lot 1) spiked with 10 nM DES and it was assessed both using the YES assay than chemical analysis (GC/MS) [20].

2.4. The yeast estrogen screen (YES assay)

For this study an *in vitro* assay using the *Saccharomyces cerevisiae* yeast strain RMY326 [21] containing a plasmid expressing the human estrogen receptor α (hERα) and a reporter plasmid carrying an estrogen responsive element (ERE) bound to the lac*Z* reporter gene encoding for the enzyme β-galactosidase (β-gal) was used. The yeast strain was kindly supplied by Prof. D. Picard, University of Geneva. The assay was previously described [22,23]. Briefly, the activation of the receptor due to formation of a receptor-ligand complex causes the expression of the lac*Z* reporter gene. The production of the enzyme (OD_{420} nm) is normalized to the number of cells assayed (OD_{600} nm). The extracts were added to the yeast culture (final concentration 2×) so that the solvent concentration did not exceed 1% (v/v). 17β-Estradiol (E_2) was used as a positive control, solvent as a negative control. Agonist activity was expressed as Relative Inductive Efficiency (RIE) calculated as the ratio between the maximal β-gal activity obtained with each water extract and that elicited by 10 nM E_2 × 100.

To test for antagonist activity samples were co-treated with 1 nM E_2. Samples able to inhibit the activity of the natural ligand E_2 led to a dose-dependent decrease in β-gal production in the medium. Antagonist activity is expressed as percent inhibition of the enzymatic activity induced by 1 nM E_2.

The drug 4-OHT, widely regarded as an antagonist of the estrogen action in yeast [21,24,25], was used as a control to measure the inhibitory activity.

Bisphenol A (BPA), di(2-ethylhexyl) phthalate (DEHP), 4-octylphenol (OP), and 4-n-nonylphenol (4-n-NP), were used to assess the YES sensitivity to most common water contaminants [26].

2.5. Statistical analysis of biological results

In order to define whether or not a compound is to be considered estrogenic/antiestrogenic we adopted the following criteria: according to our observations depending on dose–response repeated experiments, we can consider positive a compound if the variability due to the treatment (doses) is statistically significant greater than that due to the inter-experiment variability (experiments), and this effect was assessed by multifactor analysis of variance (MANOVA). The other criterion relies on the assessment of the presence of a significant dose–response relationship within a range of doses, at least two effective ones, and it was assessed by the multiple range test (MPR) and linear regression analysis (LRA). Statistical analysis was performed using Statgraphic *Plus* version 5.1 software. (Statistical Graphics Corporation, 2001, Rockville, USA). A p value less than 0.05 was used as the level of significance. All data are presented as mean and standard error of at least three independent experiments.

2.6. Chemical analysis

Two μl of the extracts were injected into a 6890N GC System Gas Chromatograph (Agilent Technologies), coupled with a 5975 Mass Selective Detector (Agilent Technologies) single quadrupole mass spectrometer equipped with PTV injector. The mass spectrometer was operated in the EI positive mode (70 eV). The MS transfer line temperature was 280 °C; the MS ion source temperature was kept at 230 °C and the MS quadrupole temperature at 150 °C. For the gas chromatographic separation an HP-5MS fused silica capillary column (5% diphenyl–95% dimethyl-polysiloxane, 30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Agilent Technologies, Palo Alto, CA, USA) coupled with a deactivated silica pre-column (2 m × 0.32 mm i.d., J&W Scientific Agilent Technologies, Palo Alto, CA, USA) using a quartz press fit, was used. The carrier gas (He, purity 99.995%) was used in the constant flow mode at 1.2 ml/min. For the analysis of phthalates, the PTV injector was used in splitless mode at 300 °C and the chromatographic oven was programmed as follows: 80 °C, isothermal for 2 min, 6 °C/min up to 200 °C, 200 °C, isothermal for 4 min, 10 °C/min up to 280 °C, 280 °C, isothermal for 30 min.

Mass spectra assignment was based on the direct match with the spectra of WILEY 275 library and comparisons with mass spectra of pure compounds were made.

The method has been optimized for the determination of bis(2-diethylexyl) phthalate, whose retention time is 23.63 min. Hexadecane (99% Sigma) has been used as internal standard. Regarding quantitation, selected ion monitoring (SIM) acquisition was used by selecting the fragments at 149, 167 and 279 m/z in the time interval 23–24.50 min. Blanks of the analytical procedure exhibit absence of the chosen analyte. Calibration curves are linear ($R^2 > 0.9881$) in the range 0.5–7 ppm. The method shows a limit of detection (LOD) of 0.01 ppm and a limit of quantification (LOQ) of 0.10 ppm.

3. Results and discussion

The three SPE procedures showed a recovery efficiency of DES, which was higher than 95%. Filtration on OASIS cartridges showed the best recovery (99.2%) and the results of the chemical determinations (GC/MS) of DES showed a strong level of consistency with the biological activity of extracts detected in the YES assay. Therefore, SPE on OASIS was used for the experiments.

All UPW extracts showed a very low estrogenic activity (RIE < 10% E_2), but were able to antagonize the binding of E_2 at different extents, depending on the type of water tested (Table 1).

Table 1
Antagonistic activity of different water samples. The activity is expressed as inhibition (%) of the enzymatic activity induced by 1 nM E_2. Values represent mean ± standard error.

Samples	Antagonistic activity (% inhibition of 1 nM E_2)
A. UPW endotoxin-free (Brand 1)	45.8 ± 5.5
B. UPW for HPLC (Brand 2)	46.4 ± 5.3
C. UPW for dialysis (CL)	26.4 ± 5.6
D. mQ W, biological lab (Lab 1)	23.7 ± 3.2
E. mQ W, chemistry lab (Lab 2)	45.4 ± 8.4
F. bidistilled W, biomedical lab (Lab 3)	38.9 ± 4.0
G. treated municipal tap water	3.4 ± 0.3

Marketed UPWs showed the highest inhibitory activity (samples A and B), even if for one brand (sample A) a different percentage of inhibition was observed as a function of the production lot (Fig. 1). Tap water extract was neither estrogenic nor antiestrogenic. Sample D (mQ-UPW from Lab 1) showed the lowest inhibition value (23.7% inhibition of E_2) in the YES assay and it underwent chemical analysis.

Fig. 2 reports the GC/MS chromatogram of sample D together with the blank: the peak at 23.63 min is unequivocally identified as DEHP by the mass spectrum shown in the inset. The quantitative analysis performed on four replicates of mQ-water extracts from Lab 1 (sample D) showed that DEHP has a concentration of 0.033 ppm ± 0.006.

The sensitivity of the yeast assay to detect analytes was: <1 ng/l for DES, 1 μg/l for BPA, 1 μg/l for 4-n-NP, 1 μg/l for 4-OP, and <0.39 μg/l for DEHP. BPA did not exhibit antagonistic activity in the YES assay, while 4-n-NP, 4-OP and DEHP showed high values of antiestrogenicity (23% and 62% inhibition, respectively). The dose–response curve of DEHP in the YES assay showed that 0.39 ppb induced a 23.0% inhibition. Taking into account the dilution factor of the UPW extract in the *in vitro* assay we confirm that there is a strong agreement between concentration of DEHP detected by chemical analysis and the extent of biological results assessed for antagonistic activity. Biological results were confirmed by chemical analyses as DEHP was identified as the contaminant responsible for the antagonistic activity.

This paper inequivocally shows that UPWs used in laboratory analyses, commercial or otherwise produced by certified equipment, may contain organic chemicals in trace which can mimic an antiestrogenic response in *in vitro* biological assay and interfere with experimental results. It should be emphasized that an *in vitro* bioassay has revealed a technical problem caused by UPW related to traces of unknown bio-active contaminants able to induce misinterpretation of biological results.

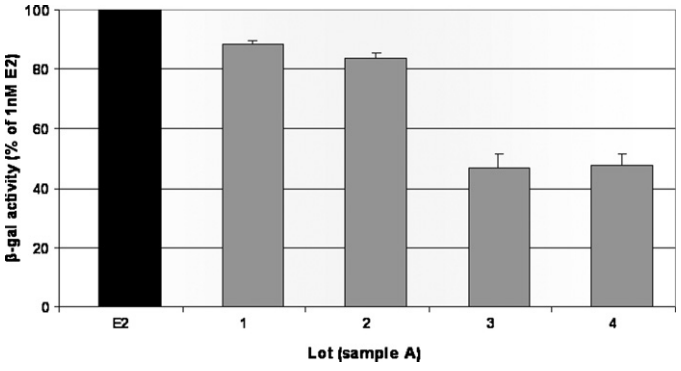

Fig. 1. Antagonistic activity of different lots of UPW (Brand A). The *Y*-axis indicates relative β-galactosidase activity induced by 1 nM E_2 in co-treated samples, in comparison with a control (100%) (E_2 alone). The bar at each point is the standard error of three independent experiments.

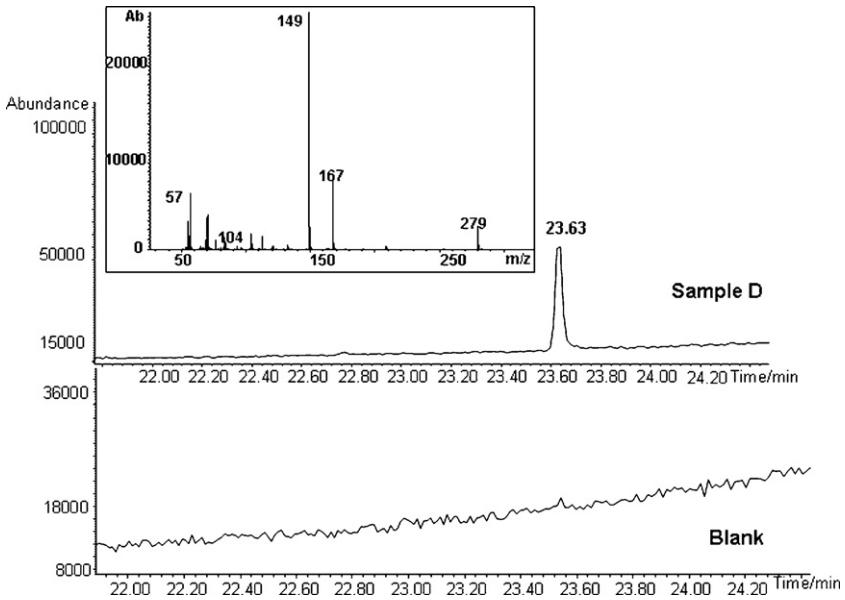

Fig. 2. Chromatogram of a blank and sample D; the inset shows the mass spectrum of the peak with retention time of 23.63 min.

4. Conclusions

The biological results of this study indicate the presence in UPW of trace organics able to interfere with the interpretation of the results of bioassays used for the detection of estrogenic/antiestrogenic chemicals. Some EDs are ubiquitous in the environment and may contaminate glassware and solvents [27,28]. Water contamination may depend on the materials making up the equipment for water purification, as many plastic materials used for membranes, resin housings and piping may leach out EDCs.

In previous studies carried out in our laboratory, the release under dynamic flow conditions (15 ml/min) of estrogen-like compounds from plastic pipes (polyethylene, polyvinyl chloride, silicon, Tygon®, and Teflon®) to water was assessed in the YES assay. Several migrating compounds from plastic materials were identified using chromatography–mass spectroscopy (GC/MS) and solid-phase microextractions (SPME) techniques. Migrating compounds (range 1–10 μg/l) from plastic tubes included dibutyl phthalate, diisooctyl phthalate, ethyl hexanol, hexamethylcyclotrisiloxane, 2,4-dichlorobenzoic acid, isobenzofuranone, methyl cyclo-pentanone, dimethylbenzene methanol, benzoisothiazolin, methylundecene, di-butyl-methyl-phenol, tetramethyl-phenyl-bicyclo-hept-2-ene. Teflon® leached traces of benzoic acid-butylester (unpublished data). A strong correlation between the chemical release and the estrogenic activity measured *in vitro* with the yeast assay was observed. Teflon® did not induce any estrogenic activity.

These results demonstrate that many additives used in the plastic industry, to produce supplies for laboratory also, may interfere with bioassay, especially enzymatic assays [29,30]. UPW production systems commercially available ensure only a partial removal of organic contaminants and the TOC parameter still accepted today fails to distinguish the contaminant species [26]. The YES bioassay showed a high sensitivity in detecting biological activity due to endocrine disruptor chemicals at level <1 ppb for many chemicals [22].

This paper shows that conventional water purification techniques do not completely remove contaminants which induce biological effects in *in vitro* tests. As UPWs can be a critical factor for life scientists, this problem must be taken into account by analysts during the evaluations of the results. It is therefore necessary that strategies are developed leading to real high purity of water for laboratory use, including a careful selection of the materials used in the purification systems. Moreover, there is the need to establish concentration limits for chemical contaminants in UPW that do not induce biological effects *in vitro*, in order to certify high quality UPW. Therefore, it would be appropriate that chemical analyses were complemented by a bioassay, such as the method proposed in this report, which as shown itself to be particularly effective in detecting estrogenic/antiestrogenic-disrupting contaminants.

Conflict of interest

There are no competing interests.

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