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Journal of Chromatography A, 1066 (2005) 33–40
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analytical methodology for the identification of estrogenic contaminants in fish bile

R. Gibson[a], C.R. Tyler[b], E.M. Hill[a,*]

[a] *Centre for Environmental Research, School of Life Sciences, Chichester Building, University of Sussex, Brighton, East Sussex BN1 9QJ, UK*
[b] *Environmental and Molecular Fish Biology Group, School of Biological and Chemical Sciences, Hatherly Laboratories, University of Exeter, Devon EX4 4PS, UK*

Received 24 September 2004; received in revised form 9 January 2005; accepted 20 January 2005
Available online 1 February 2005

Abstract

Effluents from wastewater treatment works (WwTWs) contain estrogenic contaminants that can cause feminised responses in fish. In order to assess the identity of estrogenic contaminants taken up by fish exposed to effluents, an analytical method was developed to detect estrogenic substances in fish bile, where many xenobiotics are excreted and concentrated. Estrogenic metabolites in bile were deconjugated using enzymatic hydrolysis and the estrogenic activity was determined using a yeast estrogen receptor transcription screen (YES). Hydrolysed samples were concentrated by solid-phase extraction (SPE) prior to fractionation by reversed-phase high-performance liquid chromatography (HPLC). Active HPLC fractions were detected by YES assay and analysed by gas chromatography–mass spectrometry (GC–MS) after trimethylsilylation. The method was validated using bile samples from immature female rainbow trout, which had been exposed to either tap water or an undiluted estrogenic effluent for 10 days. Hydrolysis of bile from effluent-exposed fish was complete within 16 h and most of the estrogenic activity in the bile was released by β-glucuronidase rather than sulfatase or β-glucosidase treatment. The estrogenic activity of hydrolysed bile from effluent-exposed fish ranged between 530 and 1440 ng E2eq/mL and was 17–48-fold greater than the activity of bile from reference fish exposed to tap water. The estrogenic activity of bile samples decreased with time in storage (at −70 °C by 7% per month). The recovery of estrogenic activity from SPE was 96 ± 7% (mean ± SD), from HPLC fractionation 87 ± 7% and for the whole method 81 ± 7% ($n = 7$). 17β-Estradiol, estrone, 17α-ethinylestradiol, nonylphenol and short-chain nonylphenol polyethoxylates were all identified from GC–MS analysis of active HPLC fractions of bile from effluent-exposed trout, whereas only 17β-estradiol was detected in bile from fish exposed to tap water. There were also several other minor estrogenic components, at present unidentified, in bile of effluent-exposed fish. The work shows that fractionation of fish bile is a useful approach to identifying mixtures of estrogenic contaminants taken up by fish from WwTW effluents and has the potential for application in the detection of other endocrine disrupting chemicals in fish tissues.

Keywords: Bile; HPLC; GC–MS; Estrogen; Effluent; Nonylphenol

1. Introduction

Effluents from UK wastewater treatment works (WwTWs) were first reported to be estrogenic to fish in 1994 [1], and since then estrogenic contaminants have shown to be widespread in effluents discharging into rivers in England and Wales as well as in Europe and the USA [2–5]. The major estrogenic components in WwTWs effluents have been identified as the natural estrogens 17β-estradiol (E2) and estrone (E1) and the synthetic estrogen 17α-ethinylestradiol (EE2) [6]. In addition, estrogen mimics such as alkylphenols, short-chain alkylphenol polyethoxylates and bisphenol A have also been detected in WwTWs receiving industrial inputs [7,8]. Estrogenic effluents are suspected to be responsible for the high incidence of intersex fish documented in some native fish populations and there is concern about the reproductive capabilities of affected fish [9–11]. However, no conclusive association has been proven between environmentally relevant concentrations of any of the estrogenic contam-

* Corresponding author. Tel.: +44 1273 678382; fax: +44 1273 677196.
E-mail address: e.m.hill@sussex.ac.uk (E.M. Hill).

0021-9673/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.01.045

inants in effluents and the induction of intersexuality in wild fish. For vitellogenin induction, a biomarker response for estrogen exposure, steroid estrogens and steroid estrogens in combination with xenoestrogens have been shown to induce additive effects [12,13]. It is possible that mixtures of estrogenic contaminants accumulate in fish tissues that can act additively to cause intersex. Although there has been much work analysing concentrations of estrogenic compounds in effluents we know very little about the nature of mixtures of estrogenic contaminants in fish tissues [6,14–16]. A wide variety of classes of compounds have been shown to be weakly estrogenic [17], and as typical effluents are complex mixtures which contain many thousands of chemicals from numerous domestic and industrial sources, it is important to identify the most relevant mixture of environmental estrogens that may accumulate to a high degree in fish.

The aim of this study was to develop an analytical method to isolate and identify the estrogenic components present in fish bile. Recent studies have shown that many substances, including estrogens and alkylphenols, concentrate as glucuronide and sulfate conjugates in the fish bile prior to excretion [18–21]. Analysis of bile fluid may be a good indicator of recent exposure to trace amounts of environmental estrogens and to investigate this, fish were exposed to a mixture of estrogenic substances contained within an effluent and their uptake investigated primarily by isolation of contaminants from bile. Bile samples were deconjugated, and estrogenic substances extracted on an OASIS HLB cartridge. The bile extracts were fractionated by reversed-phase liquid chromatography and the individual fractions analysed for estrogenic activity using a yeast estrogen screen (YES) assay. GC–MS was used to identify components present in estrogenically active fractions.

2. Experimental

2.1. Chemicals

E1, E2, EE2, technical 4-nonylphenol (NP), bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), β-glucuronidase (type VII-A extracted from *Escherichia coli*), β-glucosidase (type H-1 extracted from almonds), sulfatase (type VI from *Aerobacter aerogenes*) and all other chemicals were obtained from Sigma-Aldrich (Poole, UK). [2,4,16,16-$^4H_2$]E1 (E1-d_4), [2,4,16,16-$^4H_2$]E2 (E2-d_4) and [2,4,16,16-$^4H_2$]EE2 (EE2-d_4) (isotope purity 96%, chemical purity >98%) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). All solvents were of HPLC-grade purchased from Rathburn Chemicals (Walkerburn, UK).

2.2. Exposure of fish to WwTW effluent

Immature (1–2-year-old) female rainbow trout (*Oncorhynchus mykiss*), 250–320 g body weight, were obtained from Houghton Springs Farm, Dorset. Trout were exposed for 10 days in continuous flow through tanks containing either tap water or effluent from a WwTW. The WwTW site chosen for the study received an influent load of 138,000 population equivalents, and was primarily domestic, with only 6% of the load from industrial sources. The influent had been subjected to primary treatment, activated sludge and trickling filter secondary treatments. The final WwTW effluent has been shown to be estrogenic, and the estrogenic contaminants in the effluent have been well characterised from previous studies [14]. The volume of tanks containing the fish was approximately 1 m^3 and flow rate approximately 3–5 L/min. Fish were fed once daily with commercial trout pellets until 48 h prior to sampling when food was withheld to increase the bile volume. At the end of exposure the fish were sacrificed, and the gall bladder removed and stored immediately in dry ice then at −70 °C on return to the laboratory. All glassware used for sample storage and subsequent analysis was washed with detergent, thoroughly rinsed with water, washed with acetone, then baked at 500 °C for 2 h before use.

2.3. Enzymatic hydrolysis of estrogenic contaminants in the bile samples

Lyophilised enzyme material was dissolved in HPLC grade water to the following activities; β-glucuronidase 1000 units/mL, sulfatase 2 units/mL, and β-glucosidase 20 units/mL. The enzymes (200 μL of each solution) were added to 0.1 M phosphate buffer at pH 6.0 (1500 μL) and water (800 μL) containing bile (100 μL). A pH of 6.0 was found to give sufficient activity for all three enzymes. The solution was incubated for up to 16 h at 37 °C, then 300 μL of glacial acetic acid added. An aliquot of each hydrolysed sample was kept aside for direct determination of the total estrogenic activity and the remainder concentrated by solid-phase extraction. The activity and specificity of the individual enzymes was monitored separately by incubation with standard substrates (10 μg in 100 μL water) of nitrophenol glucuronide, nitrophenol sulfate and salicin. Deconjugation of the standards was determined by following the formation of 4-nitrophenol and 2-hydroxymethylphenol by HPLC with ultraviolet detection at 280 nm [22].

2.4. Solid-phase extraction of hydrolysed estrogenic components from bile

The hydrolysed bile was diluted with water (2 mL) and passed through an OASIS HLB cartridge (200 mg; Waters), which had been conditioned with methanol (5 mL) followed by water acidified with 1% acetic acid (5 mL). After sample loading, the cartridge was washed with water (2 mL), dried under vacuum, and the bile extract eluted with methanol (5 mL), ethyl acetate (3 mL) and hexane (3 mL). The ethyl acetate and hexane fractions were combined, evaporated to dryness, reconstituted in 500 μL of methanol then combined with the initial methanol fraction. The combined