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Analytical methodology for the profiling and characterization of androgen receptor active compounds in human placenta

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ABSTRACT

The exposure to endocrine disrupting chemicals during foetal development has been proposed to cause reproductive dysfunctions in the neonate or later life. In order to support such studies, an analytical method was developed to profile the receptor mediated (anti)androgenic activities present in extracts of placenta samples. Placenta samples from women giving birth to healthy male neonates were extracted and fractionated by HPLC. Fractions containing androgen receptor (AR) activity were detected using an *in vitro* yeast-based human androgen receptor transcription screen. GC–MS analyses of receptor active fractions resulted in detection of chemical contaminants including antimicrobial and cosmetic compounds which exhibited AR antagonist activity in the yeast screen, and endogenously derived steroids which contributed to both the agonist and antagonistic activity in the samples. The bioassay-directed fractionation methodology developed in this study revealed the potential to identify mixtures of chemical contaminants that should be investigated for potential effects on the reproductive system.

1. Introduction

The exposure to endocrine disrupting chemicals (EDCs) in foetal life has been suggested to be one of the causative agents in the occurrence of testicular dysgenesis syndrome (TDS) in neonate or adult life. TDS is characterized by an increased prevalence of hypospadias, cryptochidism and testicular cancer and decreased semen quality in some populations in the world [1–3]. Although a causal relationship between exposure to EDCs and the development of male reproductive disorders has not been established for humans, evidence from laboratory animal and wildlife studies has confirmed that *in utero* or perinatal exposure of male animals to estrogens or antiandrogens results in impairment of reproductive health [1,2]. In mammals, male sexual differentiation is driven by androgens produced by the foetal testes and it is entirely androgen-dependent. EDCs that interfere with androgen action could impact male sexual differentiation, and environmental chemicals that alter endogenous levels of androgens in the rat foetus can result in symptoms similar to testicular dysgenesis syndrome [2,4]. In addition, a number of epidemiological studies have also shown increased prevalence of cryptochidism and/or hypospadias in sons of mothers exposed to persistent pesticides, many of which possess antiandrogenic activity [5–7]. Antiandrogens may act by disrupting androgen synthesis, clearance, or androgen receptor (AR) expression, however many EDCs can bind to the AR and act as agonists or antagonists [4,8]. In addition a wide range of chemical structures can bind to the AR making it difficult to predict the likely compounds contributing to (anti)androgen activity in human tissues [9–11]. Moreover, many antiandrogenic chemicals are present as mixtures in low concentrations, and animal and *in vitro* studies have shown that combinations of EDCs can act additively to effect sexual differentiation, reproductive health or receptor binding [12,13]. Hence, it is important to assess the total mixture of chemicals present in tissues, despite the fact that many contaminants may be present at too low a concentration to exert biological effects individually.

The use of bioassay-directed analytical methods to profile and identify androgen receptor active compounds has been used previously to detect EDCs in bile of fish exposed to wastewaters [14,15]. Bioassay-aided fractionation of extracts of human tissues has also been used to separate estrogenic contaminants from endogenous estrogenic hormones in order to estimate the total xenoestrogen burden in human tissues [16,17]. However, to date no analytical methodology has been reported for screening androgen receptor active activity in human tissues. In this work, we developed a method to profile the total AR mediated activity of placenta samples using an *in vitro* yeast-based human AR transcription

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screen (YAS). The effect of solvent extraction and HPLC fractionation on the recoveries of the AR activity from placenta samples was investigated. HPLC fractions containing androgen receptor activity were analyzed by mass spectrometry (MS) to identify receptor active compounds and, where available, pure standards of identified chemicals were tested for activity *in vitro* in the YAS.

2. Experimental methods

2.1. Chemicals

Androsterone, dihydrotestosterone (DHT), testosterone, estrone, 17β-estradiol, p,p′-dichlorodiphenylethane (p-p′-DDE), diethylphthalate, methylparaben, bisphenol A, procymidone (PRO), paracetamol [3,4,5,6-D4] n-dioctylphthalate (DOP-D4, 98% D atom), bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), Discovery® DSC-Si 200 mg SPE cartridges, EPA CLP Organochlorine Pesticide Mix and all other chemicals, including all the components of minimal medium and growth medium used in recombinant yeast assay, were purchased from Sigma–Aldrich (Dorset, UK), except for L-leucine, adenine, L-arginine, L-methionine, L-tyrosine, L-isoleucine, L-lysine–HCl, L-phenylalanine, L-glutamic acid, L-valine and L-serine, which were supplied by ICN Biomedicals Inc. (Aurora, OH, USA). Chlorophenol red-b-D-galactopyranoside (CPRG) was purchased from Roche Diagnostics (West Sussex, UK).

[2,4,16,16-D4] estrone (E1-D4, >98% D atom) and [2,2,4,6,6,17α,21,21,21-D9] progesterone (PG-D9, 98% D atom) were purchased respectively from Cambridge Isotope Laboratories Inc. (Andover, MA, USA) and CDN isotopes (Quebec, Canada). All solvents were of HPLC-grade from Rathburn Chemicals (Walkerburn, Scotland, UK).

2.2. Placenta samples

Placenta samples from healthy individuals were obtained from San Cecilio University Hospital of Granada between 2000 and 2002. Placenta was collected at delivery and examined and weighed, and a triangular portion was excised including maternal and foetal sides as well as central and peripheral parts of the placenta, and the total sample was mechanically homogenized. The samples were immediately coded, frozen and stored at −80 °C until analyses.

2.3. Solvent extraction of placenta samples

In initial work, three solvents, ranging from non polar to polar (hexane, acetonitrile and a 1:1 (v/v) mixture of acetonitrile:methanol) were tested to compare the efficiency of extraction of AR activity from placenta samples. For this purpose, replicate composite placenta samples were prepared by combining 4 different individual placentas. Composite samples (1.0 g) were extracted twice with 4 mL of solvent using a Microson XL2000 Ultrasonic probe (Misonix Farmingdale) at 10 W for 45 s. Each extract was vortexed for 45 s, centrifuged (3000 rpm, 5 min) and the two supernatants combined, concentrated to dryness under vacuum, and re-dissolved in 500 μL of acetonitrile. To remove the proteins from the solvent extract, it was sequentially filtered using 0.2 μm and 10 kDa pore size centrifuge filters (Whatman VectaSpin Micro with Anopore filter membrane and Millipore, Amicon Ultra with Ultracel-10 membrane) at 13,000 rpm for 7 and 15 min respectively. The solvent was removed under vacuum, and the residue was resolubilised in 100 μL of either acetonitrile:water (3:1) or hexane:isopropanol (3:1) for fractionation on reversed or normal phase HPLC (RP- or NP-HPLC) respectively. Blank (solvent only) samples were prepared to monitor for contamination during the workup procedures.

2.4. HPLC fractionation of placenta extracts

Aliquots of extracts (equivalent to 0.5 g equivalents of wet placenta) were fractionated using either RP- or NP-HPLC. For RP-HPLC an analytical C18 column (Waters, SunFire 3.5 μm particle size; 4.6 mm × 100 mm) with a guard column (Waters, SunFire 3.5 μm particle size; 4.6 mm × 10 mm) was used with a mobile phase of water (0.2% acetic acid, 5% acetonitrile) as solvent A, and acetonitrile (0.2% acetic acid) as solvent B at a starting ratio 100:0. The separation was performed at room temperature (flow rate 1.0 mL/min) with a linear gradient program of 0.0–3.0 min (100:0, A:B), 3.0–10.0 min (52:48, A:B), 10.0–21.5 min (29:71, A:B), 21.5–26.0 min (0:100, A:B) for 19 min.

For NP-HPLC fractionation an analytical silica column (Waters Spherisorb, 3 μm particle size, 4.6 mm × 100 mm) was used with a guard column (5 μm, 4.6 mm × 10 mm). Three mobile phases were used; solvent A, hexane (0.2% (v/v) of acetic acid); solvent B, hexane/methanol/isopropanol, 50/25/25 (0.2% (v/v) of acetic acid) and solvent C, isopropanol (0.2% (v/v) of acetic acid). The separation was performed at 32 °C with a flow rate at 1.5 mL/min, using a binary gradient of 0.0–4.0 min (98:2, A:B); 4.0–5.0 min (94:6, A:B); 5.0–17.0 min (94:6, A:B); 17.0–17.5 min (50:50, B:C) for 17 min.

In further work, semi-preparative NP-HPLC was used to fractionate 25 g equivalent amounts of placenta extracts in order to obtain enough material to identify structures of chemicals in fractions containing AR activity. A Waters SunFire column (5 μm particle size 10 mm × 150 mm) equipped with a guard column (5 μm particle size; 10 mm × 10 mm) was used. The solvents and temperature were the same as used for the NP-HPLC analytical separation but with a flow of 7 mL/min and gradient of 0.0–9.0 min (98:2, A:B); 9.0–9.5 (93:7, A:B); 9.5–23.0 (93:7, A:B); 23.0–23.5 min (50:50, B:C) for 12 min. Some fractions from semi-preparative NP-HPLC were repurified on a longer NP-HPLC analytical column (Phenomenex Luna Silica 3 μm particle size; 250 mm × 4.6 mm) using a gradient of hexane (0.2% (v/v) of acetic acid, solvent A) and hexane/isopropanol/methanol (75/12.5/12.5 with 0.2% (v/v) of acetic acid, solvent B). The separation was performed at 32 °C with a flow rate at 1.5 mL/min, using a binary gradient of 0.0–20.0 min (98:2, A:B); 20.0–21.0 min (97:3, A:B); 21.0–75.0 min (97:3, A:B); 75.0–110.0 min (84:16, A:B); 110.0–130.0 min (75:25, A:B).

All HPLC separation programs in reversed and normal phase modes included a re-equilibration time between runs of 30 min at starting conditions.

HPLC fractions were collected every minute, the solvent removed and the extract re-dissolved in ethanol (100 μL). Aliquots were used for the determination of androgenic and antiandrogenic activities in YAS and injected onto the GC–MS or LC–MS for compound identification. Blank workup samples were fractionated to check for contamination.

2.5. Yeast-based androgen receptor bioassay

The androgenic activity and antiandrogenic activity of whole placenta extracts and HPLC fractions were quantified using an *in vitro* recombinant yeast screen (YAS) expressing the human androgen receptor (hAR). The YAS was supplied by Prof. J. Sumpter, Brunel University, and has been validated for a range of androgen receptor agonists and antagonists [18,19]. Briefly, the hAR gene has been stably integrated into the yeast genome together with an expression plasmid containing the androgen-response element (ARE), which controls expression of the reporter gene Lac-Z (encoding the enzyme β-galactosidase). Activation of the receptor hAR, by binding of a ligand, causes binding to the ARE and consequently the