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Interactions between human plasma sex hormone-binding globulin and xenobiotic ligands

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Abstract

Human sex hormone-binding globulin (SHBG) binds sex steroids with high affinity. In plasma, the number of SHBG steroid-binding sites far exceeds the molar concentrations of sex steroids, and will accommodate other ligands such as phytoestrogens and fatty acids. We have therefore developed a screening assay to identify ligands for SHBG, which exist in our diet or environment. This assay allows the binding of potential ligands to SHBG to be assessed under physiological conditions, and is sensitive to the effects of plasma constituents. Several classes of endocrine active compounds were tested, including hydroxy-polychlorinated biphenyls (HO-PCBs), phthalate esters, monoesters, chlorinated pesticides, as well as synthetic estrogens and phytoestrogens. The relative binding affinities (RBAs) of various compounds to SHBG were determined in competitive displacement assays, by comparison with 17β-estradiol (RBA = 100). Synthetic estrogens bound SHBG with RBAs of 0.4 (ethinylestradiol)-0.2 (diethylstilbestrol), while some phytoestrogens bound with RBAs of 0.12 (coumestrol)-0.04 (naringenin). Many compounds did not bind to SHBG with sufficient affinity to allow RBA measurements, and these include: several phytoestrogens, such as genistein and kaempferol, polychlorinated biphenyls, phthalate esters and monoesters. Of nine HO-PCB congeners tested only 4-OH-2′, 3′, 4′, 5′-tetraCB and 4-OH-2, 2′, 3′, 4′, 5′-pentaCB bound SHBG in undiluted serum with RBAs of 0.05 and 0.11. Although all test compounds bound to SHBG with much lower affinity than endogenous sex steroids, these interactions may be physiologically relevant in situations where plasma SHBG levels are high and endogenous sex steroid levels are low, such as in pre-pubertal children and women taking oral contraceptives. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Xenobiotics that interfere with endocrine systems are thought to represent a health risk, while dietary phytoestrogens are considered to have beneficial effects in terms of reducing the relative risk of developing sex-steroid hormone-dependent cancers of the breast [1] and prostate [2]. Both classes of compounds act as hormone agonists and/or antagonists in vitro [3], but their activities under physiological conditions are poorly defined [4,5]. In this context, their accessibility to tissues in the body is an important question that needs to be addressed.

A plasma glycoprotein, known as sex hormone-binding globulin (SHBG), regulates the access of testosterone and 17β-estradiol (E_2) to their target tissues [6,7]. In humans, the serum concentrations of SHBG are sexually dimorphic [8]. They undergo remarkable changes in women during pregnancy [8], as well as in infants during postnatal development [9,10], and are influenced by pharmacological treatments with synthetic estrogens and thyroid hormone [8,10]. Serum levels of SHBG are relatively high in boys and girls until puberty [11,12] and SHBG steroid-binding sites are largely unoccupied during childhood because plasma sex steroid levels are negligible [13]. During puberty, serum SHBG levels in boys decrease by ap-

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proximately one-half, while the amounts of circulating testosterone increase substantially [13]. This results in a large reduction in the number of unoccupied SHBG steroid-binding sites in boys as they mature sexually. In girls, there is a much smaller decrease in circulating SHBG during puberty [11], and endogenous sex steroid levels are much lower in women than in men. As a result, ~80% of the SHBG steroid-binding sites are unoccupied in women versus only 44% in men [14]. In women, the use of certain oral contraceptives not only markedly reduces the gonadal production of sex steroids, but causes a three to five fold increase in serum SHBG levels [15], and the vast majority of SHBG steroid-binding sites will be unoccupied under these conditions. Thus, in children and women, and particularly in women taking oral contraceptives, large numbers of SHBG steroid-binding sites are available to bind non-steroidal ligands.

It has been reported that human SHBG binds phytoestrogens [16,17], fatty acids [18] and several pharmaceutical agents [19], but little is known about its interactions with synthetic non-steroidal compounds [16,20]. In previous studies, the ability of test compounds to interact with the SHBG steroid-binding site has been determined using purified SHBG, or SHBG in diluted serum samples, and the influence of other serum constituents, including other ligand-binding proteins, cannot be assessed under these conditions. We have therefore adapted a simple method of measuring SHBG levels [21] as a means of screening for compounds that bind to SHBG in undiluted serum, and which might therefore displace endogenous steroids from its binding site under certain circumstances.

2. Methods

2.1. Chemicals

[2,4,6,7-^{3}H]17β-Estradiol (S.A. 91 Ci/mmol), [1α-2α(n)-^{3}H]5α-dihydrotestosterone (S.A. 50 Ci/mmol) and Aqueous Counting Scintillant (ACS) were purchased from Amersham LIFE SCIENCES (Arlington Heights IL). Indenestrol A and Indenestrol B were from Dr K.S. Korach (National Institute of Environmental Health Sciences, NC). Ammonium sulfate, *cis*-stilbene, daidzein, 5,7-dihydroxy-4-methoxyisoflavone, diethylstilbestrol (DES), ethinylestradiol (EE_2), 17β-estradiol-17-acetate (estradiol acetate), 17β-estradiol-3-benzoate (estradiol benzoate), genistein, harmane, hexachlorocyclohexane (γ-isomer), kaempferol, methoxychlor, methoxypsoralen, naringenin, nordihydroguaiaretic acid (NDGA), norharmane, *trans*-stilbene, tropolone, and quercetin were purchased from Sigma (St. Louis, MO), and E_2 and 5α-dihydrotestosterone (DHT) were from Steraloids (Wilton, NH). Coumestrol was from Acrōs Organics (New Jersey), and o,p′ DDE and p,p′ DDE were from Supelco Canada (Mississauga, Canada). 4-Octylphenol, β-sitosterol, 4(*tert*-octyl)phenol and *trans*-chalcone were from Aldrich (Milwaukee WI). Phthalate esters and monoesters were provided by the Toxicology Research Task Group. Polychlorinated biphenyls and hydroxy-polycholorinated biphenyls (HO-PCBs) were generously provided by Dr S.H. Safe (Texas A&M University, College Station, TX).

2.2. Screening assay of SHBG–ligand interactions

The binding of synthetic estrogens and non-steroidal compounds to human SHBG was evaluated using undiluted serum in a screening assay based on the ammonium sulfate precipitation method [21]. For these studies, female serum was used because plasma SHBG levels are higher in women than in men, and there is a greater proportion of unoccupied SHBG binding sites in female serum [7]. Serum samples taken from women under different physiological conditions were also tested. The concentrations of SHBG in these serum samples were determined using an established saturation ligand-binding capacity assay [22] and were as follows: untreated female serum (61 nM SHBG), human pregnancy serum (370 nM SHBG) and serum from women taking oral contraceptives (167 nM SHBG). To determine the effect of removing endogenous ligands from SHBG and other plasma proteins, serum was incubated (15 min, room temperature) with a pellet of dextran-coated charcoal (DCC). The charcoal was sedimented by centrifugation (13 000***g***, 10 min) and the DCC-stripped serum was removed for the assay. Non-specific binding was determined in each case by first heat denaturing (30 min, 65°C) the serum samples.

In the screening assay, human serum samples (100 μl) were first incubated (30 min, room temperature) with a standard concentration (200 μM) of test ligand in glass test tubes. The serum and test ligand mixtures were then added to 40nM dried [^{3}H]-E_2 in glass test tubes and incubated at 37°C for 1 h, followed by 30 min at 4°C. The SHBG-ligand complex was precipitated by drop-wise addition of 100 μl ice-cold saturated ammonium sulfate. Following a 30 min incubation on ice, the precipitated SHBG-ligand complex was sedimented by centrifugation (13 000***g***, 15 min, 4°C). The supernatant was removed and discarded, and the pellet was re-suspended in 200 μl distilled water, 100 μl of which was added to 4 ml ACS and measured for radioactivity in a scintillation counter.

A similar protocol was used to generate complete displacement curves for potential ligands identified in the screening assay. This was achieved by first incubating (30 min, room temperature) the serum with increasing concentrations (typically 10 μM–10 mM) of each

test compound in glass test tubes prior to incubation with 40 nM [^{3}H]-E_2. All subsequent steps in the assay were then performed as described above.

2.3. Competitive binding assay of SHBG–ligand interactions in diluted serum

The relative binding affinities of ligands for SHBG identified in the screening assay were determined using diluted (1:200) human pregnancy serum containing ~2 nM SHBG in a competitive ligand-binding assay [22]. In brief, the pregnancy serum was diluted in a DCC slurry in phosphate-buffered saline (PBS) and incubated (15 min, room temperature) to remove endogenous steroids. Following centrifugation (10 000***g***, 10 min) to sediment the DCC, 100 μl diluted serum was added to glass test tubes containing increasing amounts of dried non-radioactive DHT, E_2 or test compounds, as competitors, and 100 μl PBS containing 10 nM [^{3}H]-DHT was added. Following incubations (1 h at room temperature and 30 min at 0°C), unbound [^{3}H]-DHT was separated by incubation (10 min at 0°C) with 600 μl ice cold DCC slurry and centrifugation (2000***g*** at 4°C for 10 min). Supernatants were decanted into 4ml ACS for radioactivity measurements to determine the amount of SHBG-bound [^{3}H]-DHT. Relative binding affinities (RBAs) of test compounds were determined from IC_{50} values when compared to E_2.

3. Results

3.1. Synthetic estrogens

Four synthetic estrogens used as pharmaceutical agents were tested as potential ligands for SHBG in the screening assay using human pregnancy serum. Each compound was tested in duplicate in at least three independent assays (n = number of assays), and the relative amounts of [^{3}H]-E_2 displaced from the SHBG steroid-binding site (mean percentages ± standard deviation) in serum and DCC-treated serum, respectively, were as follows: 20 μM estradiol acetate resulted in 29 ± 10% ($n = 3$) and 35 ± 5% ($n = 3$) displacement; 2 μM estradiol benzoate resulted in 49 ± 5% ($n = 3$) and 47 ± 3% ($n = 3$) displacement; 20 μM ethinylestradiol (EE_2) resulted in 29 ± 2% ($n = 3$) and 31 ± 3% ($n = 3$) displacement; and 20 μM diethylstilbestrol (DES) resulted in 30 ± 13% ($n = 4$) and 43 ± 4% ($n = 3$) displacement. The data indicate that endogenous ligands for SHBG or other serum proteins have essentially no impact on the abilities of these test compounds to interact with the SHBG steroid-binding site in serum. They also suggest that estradiol benzoate has a much higher affinity for the SHBG steroid-binding site when compared to EE_2or DES.

Increasing concentrations of these pharmaceutically active estrogens, or the major serum metabolites of DES (Indenestrol A and Indenestrol B), were then used to generate displacement curves in undiluted serum (Fig. 1(A–B)). This was done to assess more accurately their RBAs for the SHBG steroid-binding site, and the results were compared to those obtained using a conventional competitive ligand binding assay with SHBG in diluted (1:200) serum (Fig. 2(A–B)). These data confirm that all four synthetic estrogens, which were

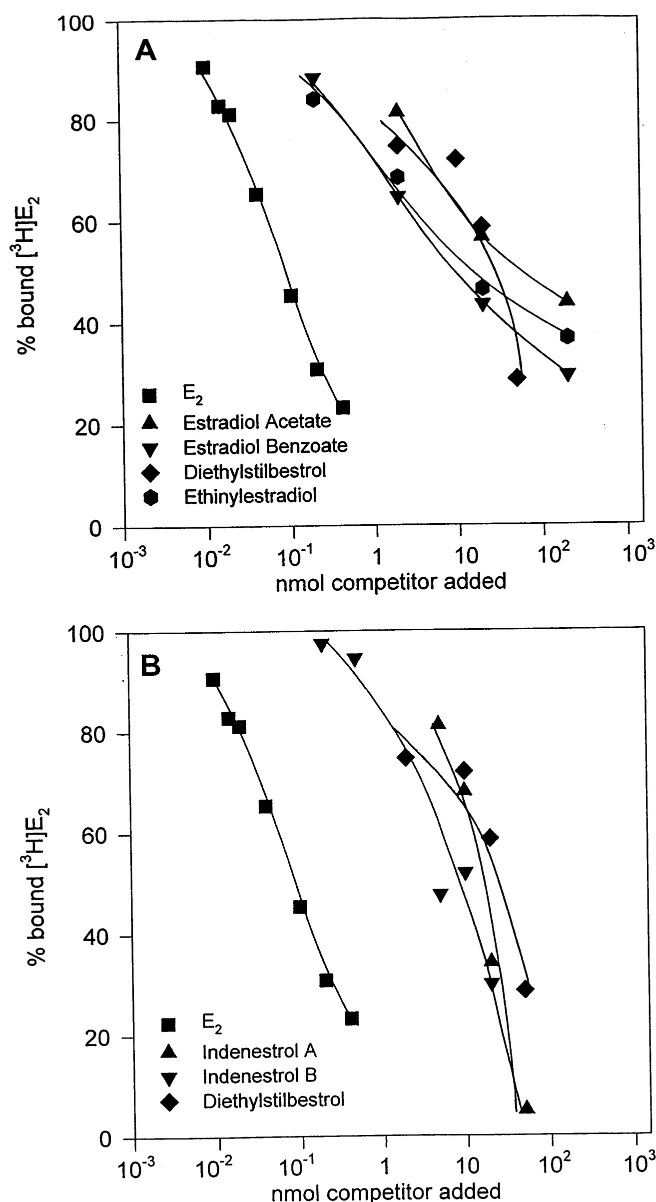

Fig. 1. Competitive displacement of [^{3}H]-E_2 from the SHBG steroid-binding site by: (A) pharmaceutical estrogens; and (B) the major serum metabolites of DES for human SHBG relative to unlabeled 17β-estradiol (E_2) Displacement curves were generated using undiluted human pregnancy serum in the presence of increasing amounts of unlabeled test compounds. Data points are the means of three assays.

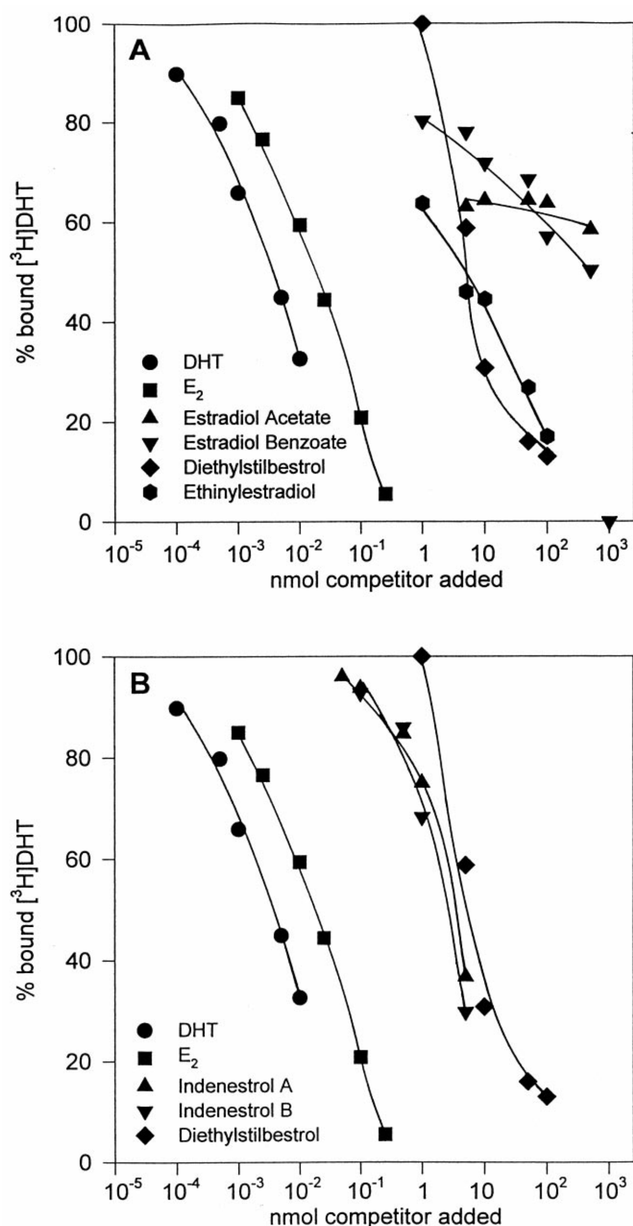

Fig. 2. Competitive displacement of [^{3}H]-DHT from the SHBG steroid-binding site by: (A) pharmaceutical estrogens; and (B) the major serum metabolites of DES for human SHBG relative to 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT). Displacement curves were generated using diluted (1:200) human pregnancy serum in the presence of increasing amounts of unlabeled test compounds. Data points are the means of three assays.

identified as potential ligands of SHBG in the screening assay, compete with E_2 for the SHBG steroid-binding site in undiluted serum (Fig. 1(A)), and that the metabolites of DES have a 2.0–3.6-fold higher affinity for SHBG than DES itself (Fig. 1(B)). However, when the results of the two assays are compared, the RBAs of estradiol acetate and estradiol benzoate for SHBG appear to be much higher when measured in undiluted serum (Fig. 1(A)) than in diluted serum (Fig. 2(A)). This indicates that a serum component increases the availability of these compounds as potential ligands for SHBG in some way, and demonstrates the value of comparing the results obtained by both assays. By contrast, the RBAs of EE_2, DES and the metabolites of DES were similar when measured in undiluted or diluted serum. Thus, the results of two independent assays indicate that these compounds are capable of displacing E_2 from the SHBG steroid-binding site, and that the metabolites of DES have a higher affinity for SHBG than DES itself. In terms of their RBAs, EE_2 and the DES metabolites bind SHBG with ~250-fold less affinity than E_2.

3.2. Phytoestrogens

A wide variety of plant-derived compounds with estrogenic properties were tested in the serum screening assay (Table 1). These data indicate that several phytoestrogens (genistein, naringenin, coumestrol and β-sitosterol) are potential ligands for SHBG, as defined by a > 20% displacement of [^{3}H]-E_2 from SHBG when

Table 1
Displacement of [^{3}H]-Estradiol by test ligands in the screening assay using undiluted human pregnancy serum (HPS) and human pregnancy serum from which endogenous steroid hormones have been removed by DCC adsorption (DCC-treated HPS)[a]

Test compound (200 μM)	% [^{3}H]-Estradiol displaced	
	DCC-treated HPS	HPS
Bisphenol A	18.8 ± 16.7 (3)	16.0 ± 26.9 (3)
Black liquor	20.0 ± 3.0 (3)	21.6 ± 12.4 (8)
Trans-chalcone	26.6 ± 17.7 (5)	6.0 ± 7.7 (4)
Coumestrol	55.8 ± 18.8 (6)	68.8 ± 22.0 (4)
Daidzein	3.7 ± 3.5 (3)	12.0 ± 1.7 (3)
o,p′ DDE	20.5 ± 10.1 (8)	13.5 ± 1.3 (6)
p,p′ DDE	6.3 ± 4.2 (3)	10.3 ± 6.5 (3)
DDT	13.4 ± 8.5 (8)	12.6 ± 13.1 (6)
5,7-Dihydroxy-4-methoxyiso-flavone	25.2 ± 21.4 (5)	10.6 ± 9.6 (5)
Genistein	40.4 ± 7.8 (5)	57.8 ± 24.5 (9)
Harmane	40.8 ± 21.2 (4)	24.3 ± 10.1 (5)
Hexachlorocyclohexane γ	32.8 ± 16.8 (4)	13.0 ± 13.2 (5)
Kaempferol	16.0 ± 5.3 (5)	18.4 ± 14.8 (7)
Methoxychlor	19.3 ± 21.5 (3)	8.3 ± 14.4 (3)
Methoxypsoralen	35.5 ± 11.1 (4)	22.7 ± 25.1 (5)
Naringenin	34.3 ± 13.5 (4)	49.2 ± 24.6 (12)
Nordihydroguaiaretic acid	19.0 ± 16.5 (5)	14.8 ± 13.3 (8)
Norharmane	28.0 ± 18.4 (12)	30.5 ± 19.3 (11)
4-Octyl phenol	15.5 ± 9.0 (4)	11.7 ± 13.2 (3)
4(Tert)octylphenol	44.6 ± 23.2 (5)	20.5 ± 12.3 (12)
Quercetin	10.5 ± 11.6 (4)	1.5 ± 1.9 (4)
β Sitosterol	39.2 ± 17.1 (6)	26.5 ± 11.2 (4)
cis-Stilbene	24.0 ± 20.6 (5)	15.2 ± 17.2 (5)
trans-Stilbene	15.8 ± 15.7 (4)	4.8 ± 1.9 (4)
Tropolone	39.3 ± 20.6 (4)	13.6 ± 14.6 (4)

[a] Data are presented as mean ± SD (n = number of assays).

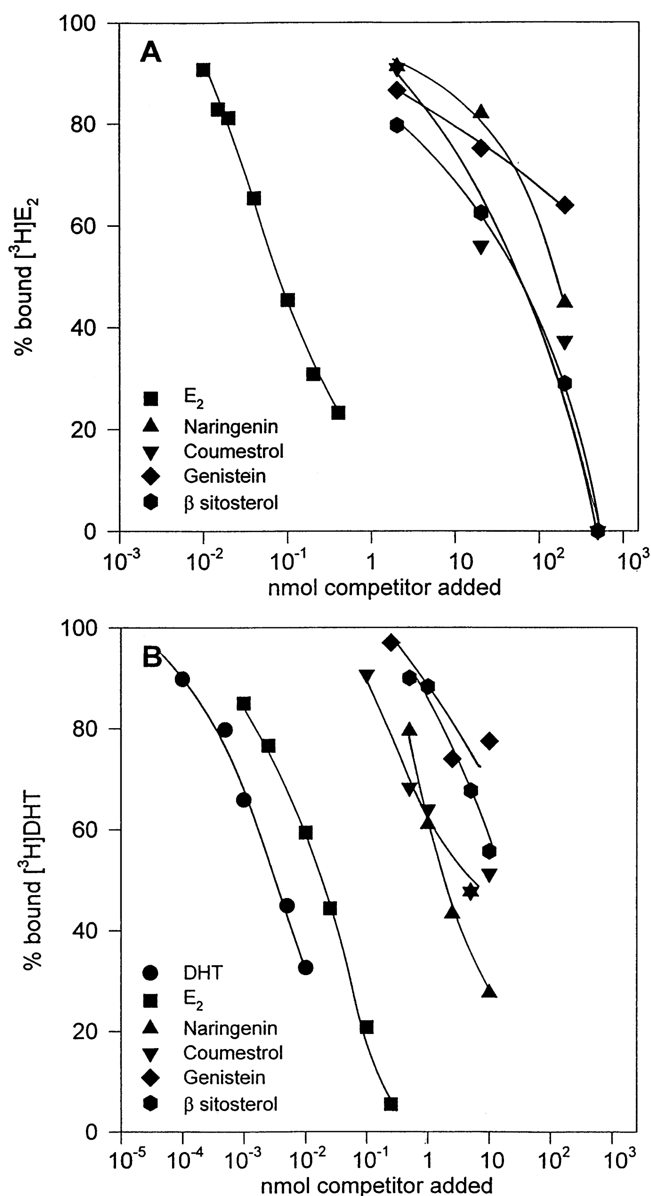

Fig. 3. Competitive displacement of: (A) [^{3}H]-E$_2$; or (B) [^{3}H]-DHT from the SHBG steroid-binding site by plant estrogens. Displacement curves were generated using undiluted human pregnancy serum (A) or diluted (1:200) human pregnancy serum (B) in the presence of increasing amounts of unlabeled test compounds. Data points are the means of three assays.

tested at a standard concentration of 200 μM in the screening assay using either untreated or DCC-treated serum. These compounds were therefore tested further to assess their RBAs for SHBG in undiluted (Fig. 3(A)) or diluted (Fig. 3(B)) serum. We were unable to generate full displacement curves for genistein in these assays due to precipitation at high concentrations, and its affinity for the SHBG steroid-binding site is much lower than the other three compounds tested (Fig. 3(B)). The RBAs of coumestrol, naringenin and β-sitosterol for SHBG measured in undiluted serum are within the same order of magnitude, and are 900–2500-fold lower than that of E$_2$. By contrast, the RBAs of coumestrol and naringenin are only 100–500-fold lower than E$_2$ when measured in diluted serum. Thus, naringenin and coumestrol bind with relatively high affinity to the SHBG steroid binding site, but some plasma constituent appears to reduce the amounts of these phytoestrogens available to interact with SHBG in undiluted serum.

3.3. Industrial compounds

A variety of industrial compounds were examined as potential ligands for SHBG in the screening assay, and the results are shown in Table 1. When tested at a standard 200 μM concentration, most of these compounds failed to displace significant amounts (i.e. $>20\%$) of [^{3}H]E$_2$ from the SHBG steroid-binding site. Two of these compounds (o,p′ DDE, p,p′ DDE) were further tested to assess their RBAs for SHBG in competition assays using undiluted (Fig. 4(A)) or diluted (Fig. 4(B)) serum. As expected from the screening assay, both isomers of DDE are poor competitors for the SHBG steroid-binding site.

A panel of polychlorinated biphenyls (PCBs) and hydroxy-polychlorinated biphenyls (HO-PCBs) was examined in the screening assay, and the results are summarized in Table 2. Although the PCBs have little or no ability to interact with SHBG, at least three HO-PCBs (HO-PCB2, HO-PCB3 and HO-PCB4) were identified as potential ligands for SHBG. These compounds were tested further to determine their RBAs for SHBG, and HO-PCB1 (4-OH-2, 3, 5, 3′, 4′, 5′-hexaCB) was used as a negative control in these assays. In undiluted serum, HO-PCB1 (Fig. 4(A)) and HO-PCB2 (4-OH-2, 3, 5, 3′, 4′-pentaCB, not shown) failed to displace [^{3}H]-E$_2$ from the SHBG binding site, while full displacement curves were generated for HO-PCB3 (4-OH-2′,3′,4′,5′-tetraCB, Fig. 4(A)) and HO-PCB4 (4-OH-2, 2′, 3′ 4′, 5′-pentaCB, not shown). By contrast, all four of these HO-PCBs clearly interact with SHBG in diluted serum (Fig. 4(B) and data not shown). This indicates that some HO-PCBs, as exemplified by HO-PCB1 and HO-PCB2, interact with a plasma constituent that decreases their ability to interact with SHBG in undiluted serum.

All the PCBs and HO-PCBs included in the screening assay were therefore re-tested in the competition assays using either undiluted or diluted serum, and the results are shown for those compounds for which an IC$_{50}$ could be obtained (Table 3). None of the PCBs interacted with the SHBG steroid-binding site in either assay, and the same was true for HO-PCB5 (4-OH-3, 5, 2′, 3′, 4′-pentaCB), HO-PCB6 (4-OH-2, 3, 5, 2′, 3′, 4′-hexaCB) and HO-PCB8 (4-OH-2, 3, 5, 2′, 3′, 4′, 5′-heptaCB) (data not shown). However, these experi-

ments demonstrated conclusively that only HO-PCB3 and HO-PCB4 can displace 50% of [^{3}H]-DHT and [^{3}H]-E_2 from the SHBG ligand-binding site in diluted and undiluted serum, respectively. By contrast, HO-PCB1, HO-PCB2, HO-PCB3, HO-PCB4, HO-PCB7 (3-OH-2, 4, 5, 2′, 3′, 4′, 5′-heptaCB) and HO-PCB9 (4-OH-2, 3, 5, 6, 2′, 4′, 5′-heptaCB) all effectively compete for the SHBG steroid-binding site only when tested using diluted serum (Fig. 4(B); Table 3). These data provide further evidence that plasma constituents other than SHBG, and most probably other binding proteins, interact with these latter HO-PCBs and restrict their access to SHBG under physiological conditions, i.e. in undiluted serum.

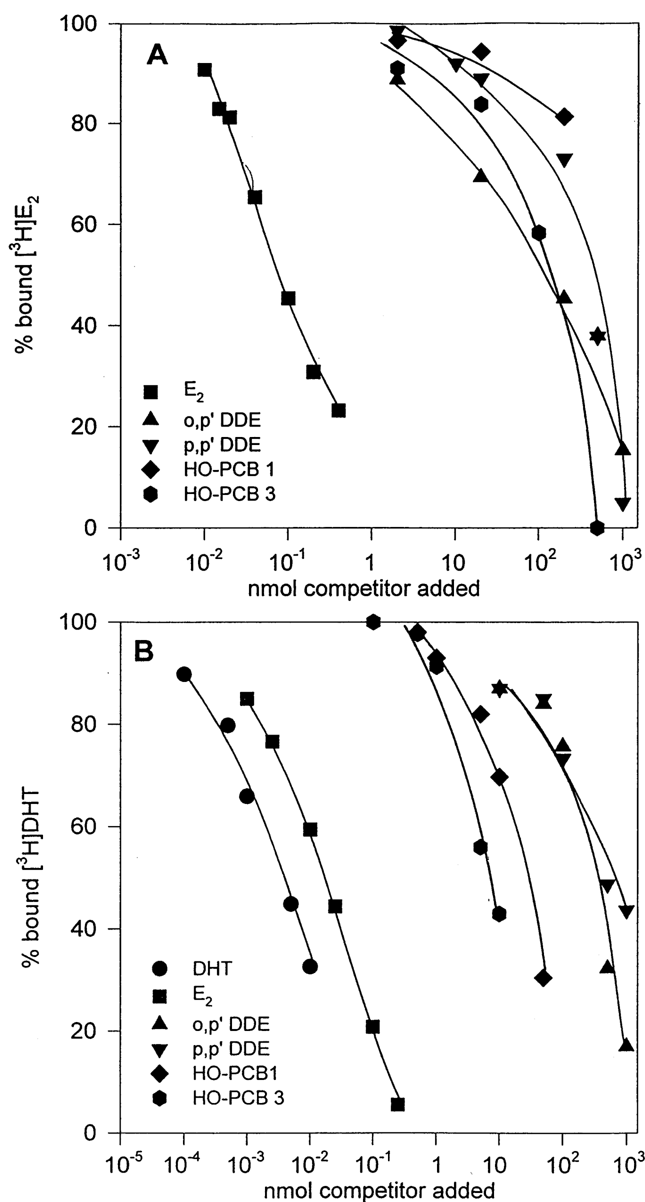

Fig. 4. Competitive displacement of: (A) [^{3}H]-E_2; or (B) [^{3}H]-DHT from the SHBG steroid-binding site by industrial chemicals. Displacement curves were generated using undiluted human pregnancy serum (A) or diluted (1:200) human pregnancy serum (B) in the presence of increasing amounts of unlabeled test compounds. Data points are the means of three assays.

Table 2
Displacement of [^{3}H]-Estradiol by polychlorinated biphenyls (PCB), and hydroxy-PCBs (HO-PCB) in the screening assay using undiluted human pregnancy serum (HPS) and human pregnancy serum from which endogenous steroid hormones had been removed by DCC adsorption (DCC-treated HPS)[a]

Test compound (200 μM)	% [^{3}H]-Estradiol displaced	
	DCC-treated HPS	HPS
PCB-28	11.3 ± 4.7 (3)	2.3 ± 4.0 (3)
PCB-52	8.0 ± 8.0 (3)	25.7 ± 12.5 (3)
PCB-105	4.7 ± 5.0 (3)	21.3 ± 37.0 (3)
PCB-118	0.3 ± 0.5 (3)	22.0 ± 37.2 (3)
PCB-153	11.7 ± 11.7 (3)	33.3 ± 38.2 (3)
PCB-156	10.0 ± 2.7 (3)	37.0 ± 33.9 (3)
PCB-170	10.0 ± 16.5 (3)	15.0 ± 26.0 (3)
PCB-180	5.7 ± 5.1 (3)	30.3 ± 29.0 (3)
HO-PCB 1	1.3 ± 2.5 (4)	12.0 ± 16.1 (7)
HO-PCB 2	20.3 ± 12.6 (3)	11.3 ± 10.1 (7)
HO-PCB 3	18.4 ± 13.4 (5)	23.9 ± 11.9 (10)
HO-PCB 4	47.7 ± 13.5 (3)	27.0 ± 26.7 (4)
HO-PCB 5	6.3 ± 11.0 (3)	0 ± 0 (3)
HO-PCB 6	4.3 ± 5.2 (3)	0 ± 0 (3)
HO-PCB 7	11.2 ± 9.6 (5)	10.0 ± 8.7 (9)
HO-PCB 8	12.0 ± 15.2 (6)	4.8 ± 5.5 (4)
HO-PCB 9	1.8 ± 3.5 (4)	15.3 ± 20.3 (5)

[a] Data are presented are mean ± SD (n = number of assays).

Phthalate esters have been used as plasticizers as well as in inks, adhesives vinyl flooring and emulsion paints, and monoesters are the first step metabolites of these compounds in the gut [23]. Some phthalate esters exhibit estrogenic properties in E-screen assays [24,25], and monoesters have been implicated in reproductive

Table 3
Relative binding affinity of hydroxy-polychlorinated biphenyls (HO-PCBs) for SHBG as compared to 17β-estradiol in undiluted versus diluted human serum based on displacement of 50% of labeled ligand in displacement curves

HO-PCB	Relative binding affinity[a] for SHBG	
	Diluted serum (versus [^{3}H]-DHT)	Undiluted serum (versus [^{3}H]-E_2)
HO-PCB 1	0.137	[b]
HO-PCB 2	0.250	[b]
HO-PCB 3	0.125	0.059
HO-PCB 4	0.098	0.067
HO-PCB 7	0.077	[b]
HO-PCB 9	0.137	[b]

[a] Calculated at 50% displacement of labeled ligand using displacement curves similar to those in Figs. 1 and 2.

[b] Indicates incomplete displacement curves which did not reach 50% displacement and therefore no relative affinity could be calculated.

Table 4
Displacement of [^{3}H]-Estradiol by phthalate esters and monoesters in the screening assay using undiluted human pregnancy serum (HPS) and human pregnancy serum from which endogenous steroid hormones had been removed by DCC adsorption (DCC-treated HPS)[a]

Test compound (200 μM)	% [^{3}H]-Estradiol displaced	
	DCC-treated HPS	HPS
Benzyl butyl phthalate	9.7 ± 12.4 (6)	7.4 ± 15.8 (7)
di-Butyl phthalate	5.9 ± 6.4 (7)	1.0 ± 1.5 (6)
diiso-Decyl phthalate	0 ± 0 (4)	1.8 ± 3.5 (4)
di-2-Ethyl hexyl phthalate	6.8 ± 5.6 (4)	0.3 ± 0.7 (3)
di-Hexyl phthalate	34.0 ± 42.7 (3)	17.7 ± 25.3 (5)
diiso-Heptyl phthalate	7.0 ± 4.7 (3)	9.0 ± 10.8 (3)
diiso-Nonyl phthalate	5.4 ± 4.5 (5)	3.8 ± 7.5 (4)
di-n-Octyl phthalate	8.3 ± 4.9 (4)	8.6 ± 6.9 (5)
Benzyl monoester	31.7 ± 24.0 (3)	34.0 ± 24.8 (3)
Decyl monoester	24.3 ± 23.6 (3)	15.7 ± 10.7 (3)
Ethyl monoester	33.0 ± 17.1 (3)	19.7 ± 5.0 (3)
Heptyl monoester	14.0 ± 7.9 (3)	16.0 ± 7.9 (3)
Hexyl monoester	26.3 ± 13.3 (3)	14.0 ± 8.7 (3)
Nonyl monoester	26.0 ± 30.8 (3)	21.3 ± 14.4 (3)
Octyl monoester	26.7 ± 22.3 (3)	18.7 ± 2.1 (3)

[a] Data are presented as mean ± SD (n = number of assays).

toxicity [26,27]. A comprehensive series of phthalate esters was therefore tested in the screening assay (Table 4) but none of them displace appreciable amounts of E_2 from SHBG when tested at a 200 μM concentration. Although some of the monoesters appear to be potential ligands for SHBG in this assay (Table 4), further testing of multiple concentrations of the phthalate esters and monoesters showed that none of them displace sex steroids from the SHBG ligand binding site (data not shown).

3.4. Effect of physiological status on SHBG ligand interactions

Most of the compounds shown to interact with SHBG were also tested in the screening assay with serum samples in which the SHBG concentrations are quite different, i.e. from untreated women, women taking oral contraceptives, and from pregnant women. This was done to determine whether these physiological states might influence the binding of test ligands to SHBG, but their ability to interact with SHBG was relatively consistent in all serum samples tested (Table 5).

4. Discussion

It is difficult to assess the potential health risks posed by endocrine active compounds in our diet and environment because their effects in organisms are complex. Several assays using cell cultures or laboratory animals have been devised to address this issue [28], but in most cases these neglect the influence of species-specific serum binding proteins, such as SHBG [29,30]. Furthermore, existing assays for identifying SHBG ligands do not take into account how their binding to SHBG may be influenced by other plasma constituents in a physiological context, and we have therefore developed a simple assay to identify compounds that interact with SHBG in undiluted serum.

Our results indicate that a wide variety of pharmaceutical estrogens, phytoestrogens and industrial chemicals bind SHBG with varying affinities. Several of these compounds have been examined as potential SHBG ligands by others [17,19,20] and the RBA data reported previously are for the most part similar to our own.

Table 5
Displacement of [^{3}H]-Estradiol from the SHBG ligand-binding site in control female serum, human pregnancy serum and serum from women taking oral contraceptives by test compounds shown to bind SHBG in human serum[a]

Test compound (μM)	% [^{3}H]-Estradiol displaced		
	Female serum	Pregnancy serum	Oral contraceptives
Coumestrol (200)	55.2 ± 14.5 (5)	65.5 ± 13.9 (4)	54.3 ± 30.9 (3)
o,p′ DDE	28.0 ± 16.5 (8)	53.7 ± 13.2 (4)	54.7 ± 23.0 (3)
p,p′ DDE	52.3 ± 8.1 (3)	45.3 ± 18.6 (5)	43.3 ± 17.2 (3)
Daidzein (200)	58.7 ± 32.9 (3)	77.5 ± 9.5 (4)	77.0 ± 19.9 (3)
Diethylstilbestrol (20)	47.8 ± 10.2 (4)	32.8 ± 7.2 (4)	47.3 ± 12.6 (3)
Estradiol acetate (20)	52.2 ± 8.2 (5)	67.0 ± 12.3 (3)	32.7 ± 1.5 (3)
Estradiol benzoate (2)	65.4 ± 10.6 (5)	52.0 ± 9.1 (4)	64.3 ± 6.5 (3)
Ethinylestradiol (20)	69.3 ± 2.5 (4)	76.7 ± 16.4 (3)	65.3 ± 4.6 (3)
Genistein (200)	53.6 ± 19.3 (5)	82.3 ± 15.2 (4)	76.0 ± 27.4 (4)
Naringenin (150)	67.5 ± 7.6 (4)	80.5 ± 16.4 (4)	70.0 ± 17.4 (4)
Nordihydroguaiaretic acid (200)	41.3 ± 8.1 (4)	30.0 ± 14.1 (3)	20.3 ± 2.3 (3)
Norharmane (200)	11.0 ± 11.2 (4)	37.5 ± 12.7 (4)	38.0 ± 14.1 (2)

[a] Data are presented as mean ± SD (n = number of assays).

Although there is one report of genistein displacing [^{3}H]-labelled sex steroids [16] from purified SHBG, the same authors found that < 20% of testosterone or E_2 was displaced from the SHBG binding site when tested in human serum. Furthermore, in another report using semi-purified SHBG only partial displacement of SHBG ligands by genistein was observed [17].

Once identified in the serum screening assay, displacement curves for potential SHBG ligands were therefore generated using diluted as well as undiluted serum. The RBA values obtained using these two assays differed considerably for some compounds, and this was particularly evident for some phytoestrogens and HO-PCBs.

Although coumestrol and naringenin bound relatively well to SHBG in diluted serum, their RBAs for SHBG were much lower in undiluted serum. We interpret this as an indication that these phytoestrogens interact with one or more plasma constituents that decrease their abilities to bind to SHBG under physiological conditions. The urinary excretion of both compounds have been examined [31,32], but blood levels have not been measured, and it remains to be determined whether they interact with serum proteins other than SHBG, as our data suggest. Although all the phytoestrogens that have been shown to bind SHBG have relatively low affinities for its steroid-biding site, this could still be physiologically relevant because their blood concentrations can reach 100–1000 times that of E_2 [33,34]. At such high levels, some of these naturally occurring compounds in our diet might displace endogenous sex steroids from SHBG, especially during their first pass through the liver, and could thereby exert an indirect effect on androgen or estrogen regulated genes in hepatocytes.

The fact that six of the HO-PCBs tested bound to SHBG in diluted serum, while only two of them also bound SHBG in undiluted serum, can be attributed to an interaction between HO-PCBs and transthyretin in human serum [35]. Furthermore, both HO-PCBs that interact with human SHBG under physiological conditions lack the essential requirement for high affinity binding to transthyretin, i.e. one or more chlorine atoms substituted adjacent to the hydroxy group on the PCB molecule [35]. In fact, the two HO-PCBs that interacted with SHBG in undiluted serum have the least chlorine substitutions on the phenol ring of the congeners tested. A similar congener effect has been identified with respect to interactions with estrogen receptor (ER)α and β, in which chlorine substitutions on the phenol ring decreased affinity for the ER receptors by ten fold or more [36]. Although these data have led to the suggestion that less substituted HO-PCBs may be of greater concern in terms of health risks, due to their increased activity at the receptor level, our data imply that access of these particular HO-PCBs to target cells may be restricted due to their binding to SHBG.

The synthetic estrogen EE_2 was identified as a ligand for SHBG in the screening assay, and our measurements of its RBA for SHBG essentially confirm a previous report [19]. Although it is generally assumed that the relatively low affinity of EE_2 for SHBG is not biologically important, several studies have found that its pharmacokinetics are significantly altered during treatment with oral contraceptive formulations which cause a marked increase in plasma SHBG [37,38]. It has been suggested that the progestin content of these formulations may reduce the hepatic metabolism of EE_2 [37], but our data suggest that binding to SHBG may contribute to this.

Although DES has not previously been considered as ligand for SHBG [29] our data show that its affinity for SHBG is approximately equal to that of several phytoestrogens that have been reported as SHBG ligands [17]. Furthermore, the presence of other serum constituents does not affect the affinity of DES or its major metabolites (Indenestrol A and Indenestrol B) for SHBG. Although DES is no longer used as a pharmaceutical agent during pregnancy, an estimated 4 million pregnant women [39] ingested up to 150 mg DES/day [39]. The effects of fetal exposure to DES are well documented, but the potential for further effects as this exposed generation ages are not known [39]. Rodent experiments are commonly used to model the effects of DES in vivo, but the lack of SHBG in rats and mice may mean that the bioavailability of DES to target tissues will not be effectively modeled.

Screening test compounds using serum in the presence or absence of endogenous ligands gave similar results, and the results were not influenced by the physiological condition under which the serum was collected. It is therefore clear that exogenous compounds can interact with unoccupied SHBG steroid-binding sites, and the increased number of unoccupied SHBG binding sites during pregnancy, or oral contraceptive use, could result in greater amounts of xenobiotics being bound to SHBG under these circumstances. Although we have not measured this directly, because most of the test compounds are not available in radiolabelled form, we predict that the amount of an SHBG-bound xenobiotic ligand is directly proportional to the number of binding sites available based on what is known about natural steroid ligands for SHBG [7]. The physiological significance of this remains to be determined, but there are some obvious situations where SHBG-bound xenobiotics may be of concern. For instance, the plasma levels of sex steroids increase markedly during puberty, especially in boys, and will displace low affinity ligands bound to SHBG. In this context, it is also important to note that SHBG is located within the extra-vascular compartments of hormone sensitive tissues, including the interstitial compartment of the testis [40], and may influence the local

access of endocrine active compounds to specific cell types, such as the Leydig cells. This could be relevant because increased production of testosterone by Leydig cells during puberty will very effectively displace any xenobiotics from SHBG in the interstitial compartment of the developing testis.

In conclusion, the serum screening assay described here allows SHBG-ligand interactions to be assessed in a more physiological context, when compared to previous methods. In addition, when used in conjunction with other assays, it may indicate whether other plasma constituents influence the binding of xenobiotics to SHBG under various physiological conditions.

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