Plant Constituents Interfering with Human Sex Hormone-Binding Globulin. Evaluation of a Test Method and Its Application to *Urtica dioica* Root Extracts

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Sex Hormone-Binding Globulin, *Urtica dioica* (Roots), Secoisolariciresinol, 9,10,13-Trihydroxy-11-octadecenoic Acid, 9,12,13-Trihydroxy-10-octadecenoic Acid

A test system is described, which allows the search for compounds interfering with human sex hormone-binding globulin (SHBG) even in complex plant extracts. The method has been evaluated and applied to *Urtica dioica* root extracts. The lignan secoisolariciresinol (**5**) as well as a mixture of isomeric (11*E*)-9,10,13-trihydroxy-11-octadecenoic and (10*E*)-9,12,13-trihydroxy-10-octadecenoic acids (**3** and **4,** resp.) were demonstrated to reduce binding activity of human SHBG. Methylation of the mixture of **3** and **4** increased its activity about 10-fold.

Introduction

Sex hormone-binding globulin (SHBG) is a serum protein, which binds androgens and (somewhat less effectively) estrogens in plasma reversibly and with high affinity (Petra, 1991; Rosner, 1991). In a series of experiments Rosner and coworkers provided evidence for SHBG to play a role in cell activity regulation of prostate cells (Hryb *et al.,* 1985; Hryb *et al.,* 1990; Nakhla *et al.,* 1990; Rosner *et al.,* 1991; Plymate *et al.,* 1991; Rosner *et al.,* 1992; Nakhla *et al.,* 1994).

Since *U. dioica* root extracts are successfully applied in the treatment of early stages of BPH (Dathe and Schmid, 1987), the positive effects of this drug in BPH could (at least in part) be based on the action of compounds interfering with SHBG. In this paper we describe the evaluation of a test system which allows the search for SHBG-active constituents in complex plant extracts. Then we applied this method to non-polar extracts of *U. dioica* roots.

Materials and Methods

Preparation of serum

Pooled third trimester human pregnancy serum was used as source for SHBG. Since human serum albumin (HSA) also binds sex steroids (Vermeulen *et al.,* 1971), the serum was pretreated in order to remove HSA (all the following operations were carried out at 4 °C and in plastic vessels): *ca.* 8 ml freshly delivered and cooled serum was centrifuged (10 min, 6000×*g*) and the supernatant applied to a Concanavalin A-Sepharose® 4 B column (Lea, 1979). For removal of albumin the column was washed with buffer A (25 mM Tris-(hydroxymethyl-)aminomethane-hydrochloride (Tris-HCl), 10 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 nM radioinert 5α-dihydrotestosterone (DHT), 0.05% (w/v) NaN_3 in H_2O/glycerol 9:1; pH = 7.4), until the albumin peak had been eluted completely (monitored by measurement of extinction of the effluent at 280 nm). Then Con A-bound globulins were eluted with buffer B (buffer A + 2% (w/v) methyl-α-D-glucoside) and precipitated with half-saturated $(NH_4)_2SO_4$ solution (Rosner, 1972). Precipitated globulins were dissolved in buffer C (25 nM Tris-HCl, 10 mM $CaCl_2$, 1.5 mM EDTA, 1% (w/v) bovine serum albumin (BSA) in H_2O/glycerol 9:1; pH = 7.4) to give a concentration of about 2.6 mg/ml human protein. This solution was stored at −20 °C until use. 1% (w/v) BSA was added to minimize non-specific adsorption of DHT to the walls of the assay tubes. It was demonstrated not to affect specific binding of DHT to SHBG.

Performance of the test

Operations were carried out at 4 °C. Immediately before use endogenous steroids were re-

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moved from serum preparations by action of excess charcoal (2 h, 4 °C; "stripping"). Incubations contained (in 1.5 ml polypropylene Eppendorf tubes) 9.66 nM [1,2-^{3}H(N)]-5α-dihydrotestosterone ([^{3}H]DHT; NEN, Dreieich, Germany, sp. act. 1616.9 GBq/mmol [43.7 Ci/mmol]), 46 μg/ml stripped human protein and test extracts or test compounds (concentrations as indicated) in a total volume of 95 μl buffer C and 5 μl dimethylsulphoxide (DMSO). After 3 h at 4 °C 250 μl of a cooled 1.0% dextran-coated charcoal (DCC) suspension (1.0% (w/v) charcoal and 0.1% (w/v) dextran in buffer C) was added. Exactly 5 min later a centrifugation (15,000×*g*, 3 min) followed and an aliquot of the supernatant was transferred to a scintillation vial. Finally, individual samples were quantified in a scintillation counter at an efficiency of 39%. Values were corrected for non-specific binding (nsb). The remaining activity of SHBG in test incubations (test) was determined in comparison to reference incubations (ref) due to the following equation (reference incubations underwent the same experimental procedures, but contained the pure solvent instead of a solution of test compounds):

$$\text{remaining activity (\%)} = 100 \times (\text{cpm}_{\text{test}} - \text{cpm}_{\text{nsb}})/(\text{cpm}_{\text{ref}} - \text{cpm}_{\text{nsb}}).$$

Analytical methods

Melting points (m.p.): uncorr.; ^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR spectra at 125 MHz. Mass spectra were measured at 70 eV. TLC was carried out on silica gel sheets (0.4 mm, SIL G/UV 254) and prep. TLC on silica gel sheets (0.75 mm, PF_{254}).

Plant material

Dried and ground *U. dioica* roots were provided by BOOTS PHARMA GmbH, Höchstädt, Germany.

Work-up and screening procedure: 600 g of *U. dioica* roots were extracted ×3 with 4 l MeOH each (24 h, room temperature). Extracts were combined and the solvent removed *in vacuo*. The residue was suspended in 1000 ml H_2O. This suspension was successively extracted with cyclohexane (CH), Et_2O, EtOAc and *n*-BuOH (500 ml× 4 each – *solvent partition*). Combined organic layers were washed with 200 ml H_2O and evaporated to dryness to give CH, Et_2O, EtOAc and *n*-BuOH fractions (3.6 g, 1.2 g, 1.2 g, 4.8 g, resp.). These fractions were subjected to appropriate column chromatography (CC) and prep. TLC separation. Each step was monitored by tests for interference with human SHBG (concentration of test fractions ranging from 0.05 to 0.5 mg/ml). Active fractions were separated and tested again, until the effect could be attributed to individual compounds. These were then purchased, synthesized or isolated in pure form in order to further characterize their effect on SHBG.

Origin of individual compounds

2 was a generous gift from Dr. R. Kasper, Fachbereich Pharmazie, Freie Universität Berlin, Germany.

Isolation of the mixture of isomeric 9,10,13-trihydroxy-11-octadecenoic acids (**3**) and 9,12,13-trihydroxy-10-octadecenoic acids (**4**): 400 g dried and cut *U. dioica* roots were extracted ×3 with 2.5 l MeOH for 48 h each time. The solvent was removed *in vacuo*, the residue (28 g) was applied to a chromatography column (600 g silica gel) and eluted with CH–EtOAc–EtOH–HOAc 60:40:5:1. Elution was monitored by analytical TLC (silica gel sheets, EtOAc–HOAc 99:1). Fractions containing **3** and **4** isomers (R_f = 0.12–0.18 and 0.20–0.24, 8 isomers each) were collected and subjected to three successive prep. TLC separations (EtOAc–HOAc 99:1). Total yield of pure **3** and **4** isomers: 41 mg. Spectroscopical data (IR, MS) corresponded with those in the literature (Esterbauer and Schauenstein, 1977).

Isolation of **5:** 400 g dried and cut *U. dioica* roots were extracted ×3 with 2.5 l MeOH for 48 h each time. The crude extract was suspended in 700 ml H_2O and extracted ×5 with 300 ml EtOAc each. After removal of the solvent the extract (2.7 g) was separated by CC (150 g silica gel, CH–EtOAc 1:2). Three subsequent prep. TLC separations (silica gel, EtOAc) yielded 3.1 mg of pure **5** (R_f = 0.23–0.25).

M.p.: 106–109 °C; $[\alpha]_D^{20}$ = −13.44° (mixture of (+)- and (−)-enantiomers 3:7, as $[\alpha]_D^{20}$ is reported to be −30.8 °C for (−)-**5** (Erdtman and Tsuno, 1969) (structural formula shown in Fig. 3 is that of (−)-**5**); ^{1}H NMR (acetone-d_6) and MS data cor-

responded with those given in the literature (Andersson *et al.*, 1975; Powell and Plattner, 1976).

8 was prepared from oleic acid *via* epoxidation with *m*-chloroperbenzoic acid, subsequent opening of the oxirane ring with aqueous H_2SO_4 and final purification with prep. TLC (CH–EtOAc–HOAc 60:38:2; R_f = 0.42). Melting point and spectroscopical data corresponded with those in the literature (Capella and Zorzut, 1968; Schmidt, 1987).

9 was synthesized from linoleic acid by oxidation with O_2/soybean lipoxygenase, subsequent reduction with $NaBH_4$ and finally purified by prep. TLC (Gardner, 1975). Spectroscopical data (IR, MS, ^{1}H NMR) corresponded with those in the literature (Hamberg, 1975; Vick and Zimmerman, 1976).

11 was obtained as a by-product during oxidation of β-sitosterol with sodium dichromate (Fieser, 1953). R_f (CH–EtOAc 4:1) = 0.56; m.p. (EtOH): 154–156 °C; IR ($CHCl_3$: ν (cm^{-1}) = 2950, 2850, 1705, 1460, 1440, 1380, 1360, 1260, 1150, 950; MS *m/z* (rel. int.): 414 (M^+, 59), 399 (14), 271 (16), 255 (12), 232 (48), 231 (100), 217 (24), 215 (11); ^{1}H NMR ($CDCl_3$, methyl protons): δ (ppm) 0.64 (s), 0.77 (d, *J* = 6.8 Hz), 0.79 (d, *J* = 6.8 Hz), 0.80 (t, *J* = 7.4 Hz), 0.87 (d, *J* = 6.5 Hz), 0.97 (s); ^{13}C NMR ($CDCl_3$): δ (ppm) 211.9 (C-3), 56.2 (C-17)[a], 56.1 (C-14)[a], 53.8 (C-9), 46.6 (C-5), 45.8 (C-24), 44.6 (C-4), 42.5 (C-13), 39.8 (C-12), 38.5 (C-1), 38.1 (C-2), 36.1 (C-20), 35.6 (C-10)[b], 35.3 (C-8)[b], 33.8 (C-22), 31.7 (C-7), 29.1 (C-25), 28.9 (C-6), 28.2 (C-16), 26.0 (C-23), 24.2 (C-15), 23.0 (C-28), 21.4 (C-11), 19.8 (C-26), 19.0 (C-27), 18.7 (C-21), 12.0 (C-18), 11.9 (C-29), 11.4 (C-19).

Results and Discussion

The described test for compounds interfering with human SHBG (see Materials and Methods) is based on methods designed for the determination of the SHBG concentration in blood plasma. In contrast to these methods this assay procedure serves to determine influences of extracts, mixtures or individual compounds on SHBG. Therefore we modified test systems described by Dennis *et al.* (1977) and Hammond and Lähteenmäki (1983), resp. Briefly, methodology is as follows: a SHBG preparation from human pregnancy serum is incubated together with tritium-labelled 5α-dihydrotestosterone (DHT) **1** and test extracts or test compounds.

After an appropriate incubation period (see Materials and Methods) dextran-coated charcoal (DCC) is added, which adsorbs free DHT while SHBG-bound DHT under appropriate conditions is affected only to a negligible extent. SHBG-bound DHT is quantified in the supernatant after centrifugation. Interference of extracts or individual test compounds with SHBG is indicated by reduction of DHT-binding to SHBG compared to reference incubations.

Key modifications of the test method are 1. the use of organic solvents and 2. the separation of bound and non-bound DHT in the presence of a large excess of other compounds (*e.g.* plant constituents).

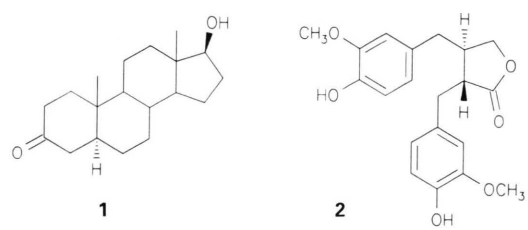
1. Organic solvents

Since we investigated non-polar extracts of *U. dioica* roots, solubility of the extracts and individual compounds in the aqueous test medium was limited. These solubilities could be increased by addition of organic solvents to the incubation mixtures. We tested the binding activity of SHBG towards DHT in the presence of the following solvents (Table I).

With the exception of dimethylsulphoxide (DMSO) each of the tested solvents decreased binding activity of SHBG towards DHT, probably due to conformational changes in the protein structure. For that reason we used DMSO as solvent, whenever possible.

2. Separation of bound and non-bound DHT

A series of techniques can be applied for the separation of SHBG-bound and non-bound DHT:



1 2

Fig. 1. Structural formulas of 5α-dihydrotestosterone (**1**) and (–)-matairesinol (**2**).

[a,b] Assignments may be interchangeable.

Table I. Binding activity of sex hormone-binding globulin (SHBG) towards 5α-dihydrotestosterone (DHT) in the presence of organic solvents. *c* (DHT) varied from 5.7 to 17.3 nmol/l; 46 μg/ml human protein each. Each value represents 4 to 6 individual tests.

Solvent (percentage [v/v])	Remaining binding activity [%]
EtOH (3%)	88.7 ± 7.0
EtOH (6%)	84.7 ± 7.4
EtOH (3%)/Et_2O (2%)	74.7 ± 7.2
EtOH (3%)/acetone (2%)	74.5 ± 7.4
DMSO (5%)	98.0 ± 4.1

e.g. equilibrium dialysis (Rosner and Smith, 1975), precipitation of the SHBG-DHT complex with $(NH_4)_2SO_4$ (Blank *et al.*, 1978), gel filtration (Cowan *et al.*, 1976). In spite of certain disadvantages (measurement under non-steady-state conditions; requirement of precise handling to ensure reproducibility and correctness) we chose the DCC method, because it is inexpensive and can be performed rapidly. Thus it allows the investigation of high numbers of samples. For determination of SHBG concentrations in blood plasma, it has been evaluated by Hammond and Lähteenmäki (1983).

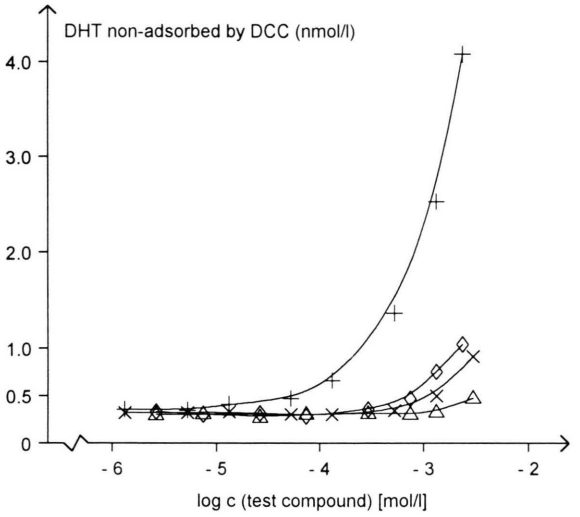


Fig. 2. Adsorption of 5α-dihydrotestosterone (DHT) to dextran-coated charcoal (DCC) in the presence of varying concentrations of test compounds. Addition of 250 μl DCC suspension to 100 μl of a solution of DHT (9.66 nmol/l) and test compounds (concentrations as indicated). **2** with 0.3% (+) and 1.0% (×) DCC suspension; mixture of **3** and **4** isomers with 0.3 (◇) and 1.0% (△) DCC suspension. Each value represents the mean of duplicate determinations.

We additionally had to check the possibility that our test compounds could occupy DCC's surface and thereby would cause insufficient adsorption of non-bound DHT, thus leading to incorrect results. In an experiment we mixed DHT and test compounds ((−)-matairesinol (**2**) on the one hand and the mixture of **3** and **4** isomers on the other hand) without addition of SHBG and determined DCC's ability to adsorb DHT (Fig. 2).

Adsorption of DHT to DCC is dependent on the nature and the concentration of added test compounds. With the exception of very high concentrations (10^{-3} mol/l), for addition of the mixture of isomeric fatty acids **3** and **4** both DCC suspensions yielded the same results. However, the lignan **2,** an aromatic compound, is adsorbed very strongly because of π−π interactions. In the presence of concentrations of **2** higher than 10^{-4} mol/l a 0.3% DCC suspension is no longer able to adsorb all of the DHT, while a 1.0% DCC suspension does up to lignan concentrations of 10^{-3} mol/l.

Subsequently we demonstrated the 1.0% DCC suspension not to influence binding of DHT to SHBG when applied for only 5 min.

For test compound concentrations $<10^{-3}$ mol/l a 1.0% DCC suspension under these conditions guarantees complete adsorption of non-bound DHT while not affecting SHBG-bound DHT.

Finally we checked the serum preparation procedure and the modified test system by determination of association constants for SHBG and DHT at 4 °C, according to Scatchard (1949). For three preparations from two different human pregnancy sera we obtained in independent incubations association constants of 0.88×10^9 M^{-1}, 0.76×10^9 M^{-1} and 0.76×10^9 M^{-1}, thus demonstrating reproducibility. These values are in reasonable agreement with that given in the literature (2.4×10^9 M^{-1} (Rosner and Smith, 1975)), which is somewhat higher because of measurement under steady-state conditions.

Screening of U. dioica roots

U. dioica roots were extracted and divided into groups of different polarity (*solvent partition* – see Materials and Methods). Since natural ligands of SHBG (androgens, estrogens) are rather non-polar compounds, we focussed on the investigation of the non-polar fractions (CH, Et_2O, EtOAc).

Tests of these crude fractions did not indicate a distinct effect on SHBG. However, further fractionation (CC, prep. TLC) in combination with tests after each step finally suggested a mixture of isomeric trihydroxyoctadecenoic acids **3** and **4** to interfere with human SHBG. So the mixture of these isomers was isolated from *U. dioica* roots (see Materials and Methods) and tested in pure form.

To check, what structural features might be responsible for this effect we also tested *threo*-9,10-dihydroxyoctadecanoic acid (**8**), (9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (**9**) and the methyl esters of **3** and **4, 6** and **7,** resp. Palmitic acid (**10**) was tested in order to exclude a detergent action of the fatty acids on SHBG in the concentrations employed.

Only trihydroxyoctadecenoic acids and their methyl esters showed significant, although rather weak effects on the binding activity of SHBG towards DHT (Fig. 4). As the other tested hydroxy acids also are C-18 fatty acids, obviously the structural feature –CHOH–CH=CH–CHOH–CHOH–, having a *trans*-configurated double bond, seems to be responsible for these effects. Since **6** and **7** display an about 10-fold activity compared to **3** and **4,** the acidic function is not a necessary feature for activity.

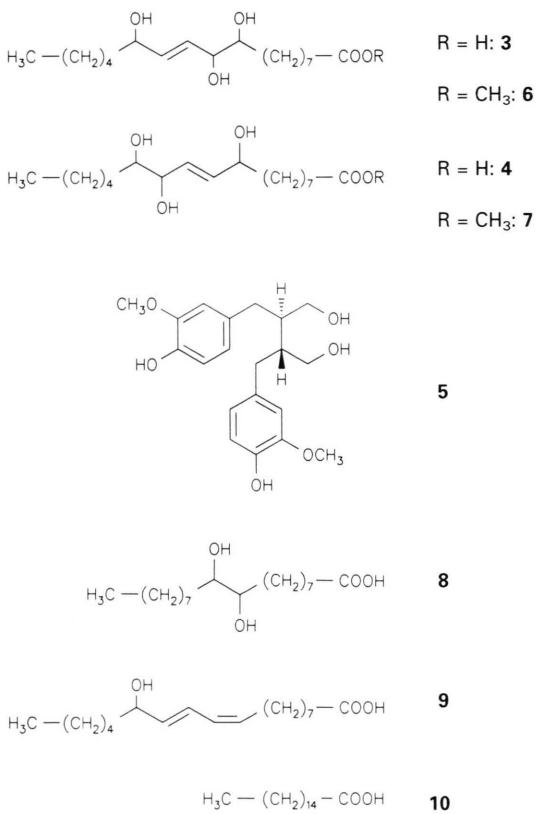


Fig. 3. Compounds tested for interference with human sex hormone-binding globulin (SHBG).

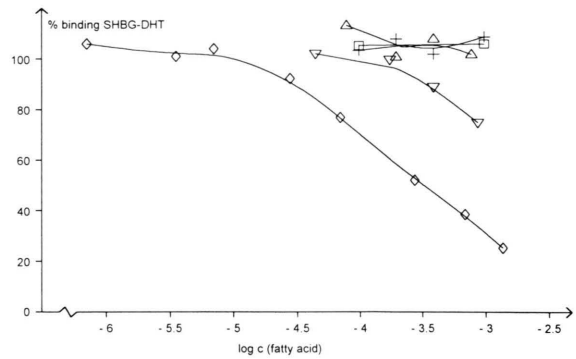
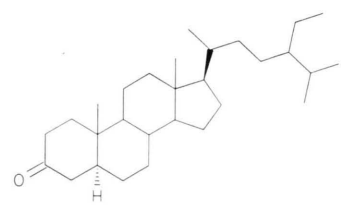


Fig. 4. Binding activity of sex hormone-binding globulin (SHBG) towards 5α-dihydrotestosterone (DHT) in the presence of various fatty acids. ◇: Mixture of **6** and **7** isomers (methyl esters of mixture of **3** and **4** isomers); ▽: mixture of **3** and **4** isomers (free acids); △: **8;** □: **9;** +: **10.** *c* (DHT) = 9.66 nmol/l; 46 μg/ml human protein. Each value represents the mean of 2 to 4 individual tests.

Secoisolariciresinol (**5**), the second-most abundant lignan in *U. dioica* roots, was also found to interfere with SHBG.

Although during our screening we did not get any indications for plant steroids to exert influences on SHBG, we additionally tested 5α-stigmastane-3-one (**11**), since it shows a high structural similarity to SHBG's preferential ligand DHT.

11 did not display any activity towards SHBG (Fig. 6). Obviously the bulky side chain on C-17 prevents plant steroids from interfering with the protein.



11

Fig. 5. 5α-Stigmastane-3-one.

In contrast **5** decreased binding activity of SHBG towards DHT in a concentration-dependent manner (Fig. 6), probably by competition with DHT for the binding site. Nevertheless its activity has to be considered as weak.

Recent research in this field carried out in our group revealed other lignans to develop considerably higher activity. The results of this investigation will be published in the near future.

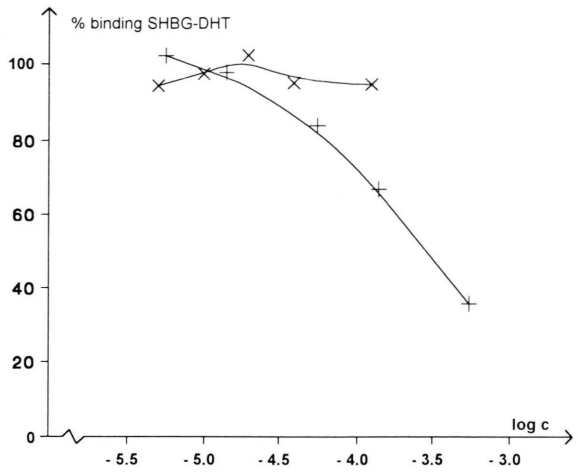


Fig. 6. Binding activity of sex hormone-binding globulin (SHBG) towards 5α-dihydrotestosterone (DHT) in the presence of **5** (+) and **11** (×). *c* (DHT) = 9.66 nmol/l; 46 μg/ml human protein. Each value represents 3 to 6 individual tests. Due to its limited solubility **11** could not be tested in higher concentrations.

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