ANALYTICA CHIMICA ACTA 637 (2009) 235–240

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Biosensor-based detection of reduced sex hormone-binding globulin binding capacities in response to growth-promoter administrations

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ARTICLE INFO

Article history:
Received 20 June 2008
Received in revised form
19 August 2008
Accepted 20 August 2008
Published on line 28 August 2008

Keywords:
Growth-promoters
Sex hormone-binding globulin
Biomarker
Biosensor

ABSTRACT

Growth-promoting agents are illicitly used during animal rearing processes and the detection of their use is limited by new compounds and dosing practices that limit the efficiency of current testing which is based on residue analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography–mass spectrometry (GC–MS) methodology. An alternative approach is to use indirect biological evidence as a screening tool to identify growth-promoter treated animals thus improving the effectiveness of residue testing through the targeted sampling of these animals. Sex hormone-binding globulin (SHBG) is a glycoprotein which binds and controls the levels of sex-hormones within the circulation. Using a biosensor assay based on measurement of binding to an immobilised 1α-dihydrotestosterone (1α-DHT) derivative, reduced SHBG binding capacities were detected in growth-promoter treated animals. During the course of a veal treatment regime based on repeated oestradiol benzoate, nortestosterone decanoate and dexamethasone administrations, treated male and female calves were shown to have significantly lower SHBG capacities. To assess the effectiveness of using SHBG binding capacities as a biomarker of treatment and to investigate the role of individual growth-promoter components to the SHBG capacity lowering effects, adult heifer animals were subjected to repeated doses of nortestosterone decanoate. These animals also demonstrated a reduction in SHBG capacity levels at Day 39 of the study, in contrast to oestradiol benzoate treated adult steers who were found to have unaltered levels. These findings suggest that the measurement of SHBG binding capacities using a biosensor assay has potential in the identification of illegally treated animals, particularly those exposed to androgens.

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doi:10.1016/j.aca.2008.08.024

1. Introduction

The effectiveness of chemical growth-promoting agents as a tool to increase yields during bovine meat producing processes is well documented [1], but their use is banned within the European Union under Directive 88/146/EEC [2]. The detection of the illicit use of such agents is based on the direct detection of drug residues via highly sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography–mass spectrometry (GC–MS) analysis of various matrices, such as muscle tissue and urine, which are randomly sampled from the herd population [3]. Although there is and will be continued progress in this area in terms of instrumentation and data handling, MS-based residue analysis is currently unable to test comprehensively for the vast array of readily available and ever expanding range of growth-promoting compounds. The increasing use of both naturally occurring hormones and cocktail administrations consisting of low doses of multiple growth-promoting agents [4] further limits the viability of direct residue-based analysis. The expensive and laborious nature of the sampling and analysis procedures required also means that only a relatively small proportion of animals are ever subjected to any form of residue analysis. As a consequence, the positive identification of growth-promoter treated animals occurs infrequently which is at variance with evidence that there is continued sporadic use of illicit growth-promoters throughout Europe [5].

The use of biological evidence from a range of sources has been proposed as a potential additional tool to improve the effectiveness of procedures to detect illicit growth-promoter use [6–8]. An indirect approach based on detecting deviant or unusual perturbations in the circulating levels of plasma analytes has recently been shown to demonstrate potential [9]. Those plasma constituents which demonstrate an increase or decrease in their circulating concentrations in response to exposure to growth-promoters can be used as biomarkers to identify treated animals. Reduced plasma sex hormone-binding globulin (SHBG) binding capacities measured using a radioligand binding technique were identified as a potential biomarker of the treatment of calves with a known growth-promoter regime typically associated with use during veal production and consisting of oestradiol, nortestosterone and dexamethasone administrations [9]. SHBG binds and transports sex-hormones thus acting as a reservoir modulating variations in concentrations over time, and the control of SHBG levels is thought to involve the competing effects of androgens which lower, and oestrogens which elevate levels [10]. In addition to its role in regulating steroid hormone concentrations, the identification of binding sites for SHBG on tissue specific cell membranes has led to suggestions that SHBG may actively participate in steroid uptake by responsive tissues [11,12]. Since growth-promoter administration often occurs in groups rather than individual animals, successful application of a biomarker-based strategy to detect abuse will require examination of a greater proportion of the cattle population than that offered by existing residue analysis. Measurement of SHBG binding capacities currently requires use of radioligand-binding methodology [13,14] which is not suitable for adaptation to high-throughput screening applications. The aim of the present study was to investigate the potential of using a ligand-binding assay on a biosensor device to measure SHBG binding capacities to identify animals treated with growth-promoting agents.

2. Materials and methods

2.1. Reagents

The synthesis of 1-α-dihydrotestosterone (1α-DHT) derivative (1α-aminohexyl-17β-hydroxy-5α-androstan-3-on) has been reported previously [15]. Nortestosterone decanoate was sourced from Organon (OSS, the Netherlands) and 17β-oestradiol benzoate and dexamethasone were from Intervet (Boxmeer, the Netherlands). All water used in these experiments was purified using a Milli-Q Water Purification System (Millipore, Milford, MA, USA).

2.2. Animal studies

Procedures used in the growth-promoter treatment of young calves have been reported in detail previously [16]. Briefly, 24 crossbred (Holstein Friesian x Fries-Hollands) calves at an average age of 10 weeks were randomly divided into 2 control (male and female, $n=6$) and 2 treatment (male and female, $n=6$) groups. Calves within treatment groups received repeated i.m. (intramuscular) doses of 17β-oestradiol benzoate (25 mg) and nortestosterone decanoate (150 mg) at 14-day intervals (Days 0, 14 and 28) followed by a single subcutaneous dose of dexamethasone (4 mg) on Day 35 of a 42-day study period. Animals within control groups received matching volumes of peanut oil on treatment days.

In a second growth-promoter treatment study using adult animals, 20 continental crossbred heifers and steers at 16–18 months of age and with an average bodyweight of 350 kg were randomly placed into 2 control (male and female, $n=5$) and 2 treatment (male and female, $n=5$) groups. After an acclimatisation period and at 14-day intervals (Days 0, 14 and 28), female treatment cohort animals received i.m. administrations of nortestosterone decanoate (0.85 mg kg^{-1} bodyweight). Male treatment group animals received i.m. oestradiol benzoate (0.15 mg kg^{-1} bodyweight) at the same study timepoints.

Blood samples from control and treated animals in both calve and adult cattle studies were taken from the anterior jugular vein using lithium heparin Vacutainer tubes (Becton Dickinson, Oxford, UK). After sampling, plasma was acquired by centrifugation at 4 °C (1250 × g, 20 min) and stored at −20 °C prior to analysis. All animal studies were carried out in accordance with procedures as outlined in the UK Animals (Scientific Procedures) Act 1986.

2.3. Biosensor SHBG ligand-binding assay

SHBG binding capacity assay measurements were performed using a surface-plasmon resonance (SPR) Biacore Q system (Biacore AB, Uppsala, Sweden) based on binding of SHBG within plasma samples to an immobilised 1α-DHT derivative using a modification of the procedure as described previously

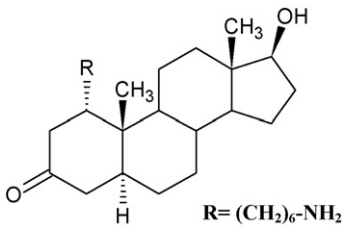

Fig. 1 – Chemical structure of 1α-DHT derivative (1α-aminohexyl-17β-hydroxy-5α-androstan-3-on) immobilised to CM5 chip surface and used to measure plasma SHBG binding capacity of plasma samples.

and interpretation of resulting SPR-sensograms [17]. The 1α-DHT derivative (Fig. 1) was immobilised onto CM5 chips (Biacore AB, Uppsala, Sweden) via amine coupling with the carboxyl groups of the chip dextran surface activated with an injection of a 1:1 solution of 0.2 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and 0.05 M NHS (*N*-hydroxysuccinimide) for 7 min at a flowrate of 5 μL min^{-1}. 35 μL of 100 μM 1α-DHT in 100 mM borate buffer (pH 8.5) was applied at a flowrate of 5 μL min^{-1} and this step was repeated three times. Unreacted ester groups were blocked by a 7-min injection of a 1.0 M ethanolamine hydrochloride solution (35 μL). A second flowcell was subjected to an identical immobilisation procedure in the absence of 1α-DHT derivative to allow for subsequent correction of sample non-specific binding (NSB) events. For the ligand-binding measurements, plasma samples were diluted 1:100 in HBS–EP+buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.05% (v/v) P20, pH 7.4). Biosensor ligand-binding assay conditions consisted of a cycle length of 3 min with a flowrate of 40 μL min^{-1} (cycle injection volume 120 μL). At the end of each biosensor assay cycle, the chip surface was regenerated using 20% (v/v) acetonitrile in 200 mM NaOH (30 μL, 45 s) followed by a 2-min stabilisation period. Plasma SHBG binding capacities were derived from resulting SPR-sensograms [17] and expressed in terms of observed binding to immobilised DHT derivative as measured by arbitrary response units (RU) following subtraction of corresponding sample NSB values.

2.4. *Statistical analysis*

All data shown are the mean ± S.E.M. of samples assayed randomly in triplicate. The differences in measured variables between control and treated groups were analyzed using unpaired Student's *t*-test and one-way ANOVA for repeated measurements followed by Tukey's post hoc test.

3. Results

3.1. *Animal studies*

Effects of the growth-promoter regime utilised on veal calves over the course of the experimental study [9] and the elimination profiles of administered substances [16] have been reported previously. In response to the growth-promoting regime used both male and female animals displayed increases in finishing bodyweights relative to control animals, with greater gains observed in treated female calves. During the adult cattle study significant increases in live bodyweight were observed in control steer (412.0 ± 22.7 kg; $p < 0.05$) and heifer (417.0 ± 25.6 kg; $p < 0.01$) groups at Day 42 compared to weights at study initiation. However, in response to administrations, oestradiol-treated steers (454.0 ± 22.1 kg; $p < 0.01$) and nortestosterone-treated heifers (428.0 ± 19.3 kg; $p < 0.001$) displayed greater finishing bodyweight levels.

3.2. *Calve plasma SHBG binding capacity*

Fig. 2 illustrates the plasma SHBG binding capacities as measured by SPR biosensor assay of (a) male and (b) female control and growth-promoter treated calves on Days −4, 17, 28 and 37 of the experimental study. Initial baseline (Day −4) SHBG binding capacity levels were found to be similar in both male and female control and treatment groups. SHBG binding capacity levels of plasma from control male calves did not change significantly over the course of the study period. However, male calves exposed to oestradiol, nortestosterone and dexamethasone administrations demonstrated reduced levels at Day 17 ($p < 0.05$) and Day 37 ($p < 0.001$) relative to

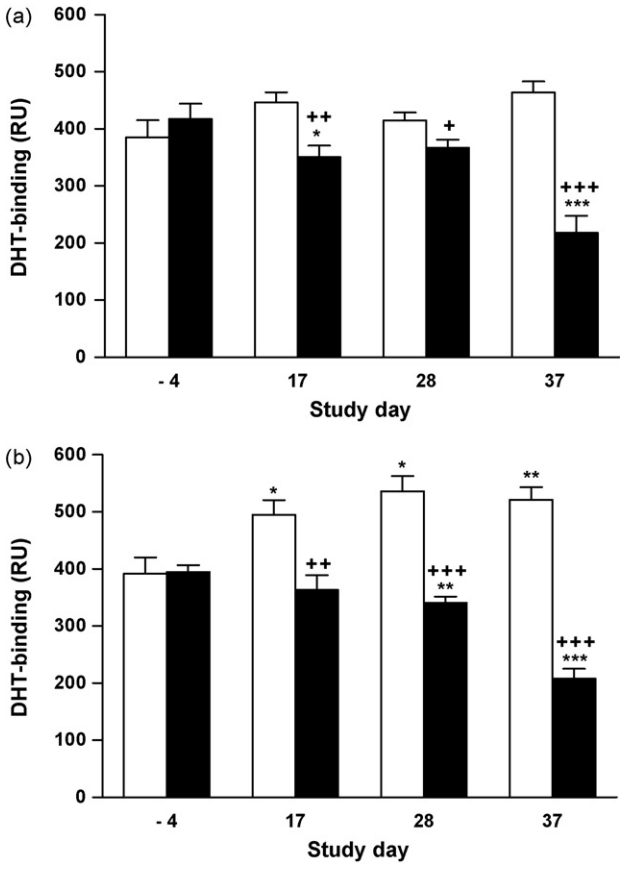

Fig. 2 – SHBG binding capacity levels in (a) male and (b) female control (□) and treated (■) calve plasma as measured by SPR biosensor assay detection of binding to 1α-DHT derivative. Values shown are mean ± S.E.M. ($n = 6$ per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. respective group baseline (Day −4) levels; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. control group on same study day.

initial baseline (Day −4) measurements. In addition, measured responses in treated male animals were significantly lower compared to matching control group animals at all time-points during the growth-promotion phase of the study.

In contrast to the stable levels observed in control male calves, control females demonstrated an elevation in measured SHBG binding capacities over the course of the study. SHBG binding capacities of plasma from treated female calves were significantly depressed after treatments, with calves within this group exhibiting lower binding capacity levels at Day 28 ($p < 0.01$) and Day 37 ($p < 0.001$) relative to starting baseline levels. Treated female animals also demonstrated lower SHBG binding capacity levels compared to control group animals on Days 17, 28 and 37.

3.3. *Adult cattle plasma SHBG binding capacity*

Fig. 3 illustrates the SHBG binding capacity of plasma from (a) male and (b) female control and treated animals from the adult cattle treatment study. Pre-treatment (Day 0) levels within groups of both sexes were found not be statistically different. Measurement of SHBG binding capacities of plasma at the end of the study period (Day 39) revealed that levels in both control and treated steer animals, who had been subjected to repeated oestradiol administrations, had remaining unchanged over the course of the study (Fig. 3a). SHBG binding capacities within control heifer animals also did not vary significantly during the study (Fig. 3b), which was in contrast to treated heifer animals who had received nortestosterone decanoate and who were found to possess significantly reduced levels at Day 39 compared to both initial baseline measurements ($p < 0.05$) and matching control group animals ($p < 0.01$) on the same day.

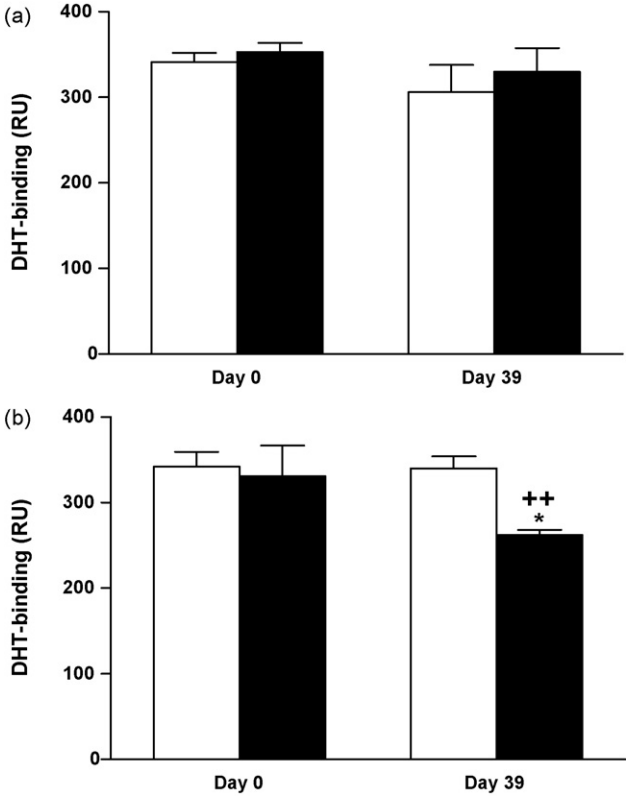

Fig. 3 – SHBG binding capacity levels in (a) male and (b) female control (□) and treated (■) adult cattle plasma as measured by SPR biosensor assay detection of binding to 1α-DHT derivative. Values shown are mean ± S.E.M. ($n = 5$ per group). $^{*}p < 0.05$ vs. respective group baseline (Day 0) levels; $^{++}p < 0.01$ vs. control group on same study day.

4. Discussion

This study has demonstrated for the first time the potential of using a biosensor-based assay to measure SHBG binding capacities in plasma to identify illicit treatment of animals with growth-promoting agents. Binding capacities were determined on a ligand-binding assay format using an immobilised 1α-DHT derivative to measure SHBG binding [17]. SHBG binding capacities in young male and female calves were shown to be significantly altered following exposure to a growth-promoter regime based on oestradiol, nortestosterone and dexamethasone administrations. SHBG binding levels of calves of both sexes were found to be approximately halved in response to treatments by the end of the study. By Day 37, treated male calves displayed binding levels approaching approximately 50% of that within age-matched control animals, whilst levels within treated female calves relative to control animals were comparatively lower (40%) due to increased binding levels within the control female group. These relative binding levels as measured by biosensor are comparable to that measured in these animals previously using a conventional radioligand-binding assay [9]. SHBG binding levels measured in adult male steer animals were found to be unaffected by repeated intramuscular oestradiol injections. However, adult female heifer animals administered with multiple nortestosterone doses demonstrated a significant reduction in binding capacities by the end of the treatment study. Levels within these animals at Day 39 were approximately 20% lower than both baseline levels measured at the start of the study and levels within matching control group animals.

SHBG binds endogenously produced testosterone, oestradiol and dihydrotestosterone in plasma with high affinity enabling the control of the bioavailability of these hormones by altering their clearance rates. The precise basis for the detection of reduced SHBG binding capacities in response to exogenous growth-promoter administrations still has to be elucidated and will require further investigation. The fact that there is not an immediate reduction in SHBG binding levels in treated veal animals in response to administrations but rather a gradual reduction over the study period culminating in the lowest binding levels been detected at Day 37, does appear to exclude the possibility that exogenously administered compounds are competing directly for the steroid binding sites of circulating SHBG thereby reducing binding to the immobilised 1α-DHT derivative. Rather it suggests that administered compounds are directly or indirectly reducing hepatic SHBG synthesis through complex feedback mechanisms. Exogenous anabolic steroids [18,19] and growth hormone [20] have been shown to reduce SHBG levels in humans, with levels remaining depressed for significant lengths of time after treatments

have been terminated. The observation that oestradiol-treated adult male animals did not display alterations to SHBG binding responses suggests that the nortestosterone component of the growth-promoter regime used in the veal calve study is primarily responsible for detected reduced binding levels in these animals. This may be due to the fact that although oestradiol and testosterone bind to the same binding site on SHBG, the binding affinity for testosterone is much higher than that for oestradiol [21], thereby minimising potential oestrogen-mediated long term effects. In humans, exogenously administered oestrogen has been shown to increase SHBG levels [22,23] but this effect may be dependent on the route of administration with peripherally delivered oestrogen having no impact on circulating SHBG levels [24]. In contrast to oestrogen administered via the systemic circulation, oral oestrogens are delivered to the liver once absorbed and thus have an increased ability to affect SHBG synthesis directly [25]. Both oral and intramuscular oestrogen administrations have been reported to have no effect on SHBG levels in sheep [26] suggesting that species specific effects should not be excluded.

Alterations to the underlying metabolic homeostasis of bovine animals in response to treatments could also indirectly impact on circulating SHBG levels. Insulin and insulin-like growth factor-1 (IGF-1), both factors which play key roles in anabolic responses, have been demonstrated to inhibit hepatic SHBG synthesis both *in vitro* [27] and *in vivo* [19,28]. Glucocorticoid administrations have also been shown to decrease SHBG levels in humans [29]. However, it is unlikely that the dexamethasone administered towards the end of the veal calve study played a significant role in the decreased SHBG binding levels detected in these animals as dexamethasone affects SHBG levels by indirect means and only following repeated administrations over a sustained period of time [30].

5. Conclusions

This study has provided evidence that an assay, established on a biosensor platform, can be used to measure significantly reduced SHBG binding capacities within plasma of growth-promoter treated animals. This finding suggests that a biomarker-based approach centred on SHBG binding capacity measurements, either alone or in a multiplex system with a combination of other such biomarker assays, is a feasible additional routine screening tool which may be used to provide indirect biological evidence of growth-promoter use. The ease with which blood required for such an analysis can be obtained throughout the animal rearing process and the potential to use this assay in a high-throughput format makes such an approach potentially preferable to existing testing methodology. The increased testing capacity would also improve the sensitivity of testing procedures allowing for the identification of suspect herds rather than isolated treated animals. The use of a SHBG-related parameter as a biomarker of growth-promoter abuse is promising due to the extended half-life of this glycoprotein and it's relatively stable circulating levels [31] even through stages of the bovine reproductive cycle [32]. Additional work will be required to determine the normal range of SHBG binding capacities in animals of various breeds, age and nutritional status, and who have been subjected to various forms of hormone administrations, so as to enable accurate identification of treated animals.

Acknowledgements

This work was financially supported by grants from the European Commission project 'New technologies to screen multiple chemical contaminants in foods' (project acronym BioCop, contract FOOD-CT-2004-06988).

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