

Evidence of the indirect hormonal activity of prohormones using liver S9 metabolic bioactivation and an androgen bioassay

J. C. W. Rijk · T. F. H. Bovee · M. J. Groot ·
A. A. C. M. Peijnenburg · M. W. F. Nielen

Received: 25 April 2008 / Revised: 24 June 2008 / Accepted: 1 July 2008 / Published online: 22 July 2008
© The Author(s) 2008

Abstract Prohormones such as dehydroepiandrosterone (DHEA) are steroid precursors that do not show hormonal activity by themselves. Abuse of these prohormones in cattle fattening is hard to prove because of strong in vivo metabolism and the difficulty to detect metabolites which are not significantly above endogenous levels. The aim of the present work was to develop an in vitro assay capable of detecting the indirect hormonal activity of prohormones that might be present in feed supplements and injection preparations. Sample extracts were incubated with a bovine liver S9 fraction in order to mimic the in vivo metabolic activation. Subsequently incubated extracts were exposed to a highly androgen-specific yeast bioassay to detect hormonal activity. Metabolic activation of DHEA, 4-androstene-3,17-dione (4-adione) and 5-androstene-3,17-diol (5-adial) resulted in an increased androgenic activity caused by the formation of the active androgen 17 β -testosterone (17 β -T), as shown by ultra-performance liquid chromatography and time-of-flight mass spectrometry with accurate mass measurement. The developed in vitro system successfully mimics the hydroxysteroid dehydrogenase (HSD)- and cytochrome P450-mediated in vivo metabolic transitions, thus allowing

assessment of both bioactivity and chemical identification without the use of animal experiments. Screening of unknown supplement samples claimed to contain DHEA resulted in successful bioactivation and positive screening results according to the androgen yeast biosensor.

Keywords Bioactivation · Prohormone · Steroid · Bovine · Liquid chromatography · Mass spectrometry

Introduction

Within the European Union the use of growth-promoting agents in cattle fattening is banned according to EC directive 96/22 [1]. Interestingly, there is not a black list of substances but EC 96/22 states that all substances having thyrostatic, estrogenic, androgenic or gestagenic activity are prohibited. A trend has been observed from the abuse of synthetic steroids towards natural steroid esters and prohormones. Feed supplements and preparations containing prohormones have the potential to enhance the levels of natural occurring steroids and can be misused in livestock production. After administration and uptake in the blood circulation, peripheral tissues are able to metabolize prohormones into more biologically active androgens and estrogens [2–4]. With respect to androgens this leads to anabolic action and subsequently to increased body weight, muscle strength and improved lean/fat ratios in farm animals [5].

Abuse of prohormones in livestock production is hard to prove: urinary metabolites are unknown or not significantly above highly fluctuating endogenous levels [6]. Chemical methods have the drawback of detecting only targeted compounds of interest. Biological transcription activation assays, however, have the advantage of detecting compounds based on bioactivity. For screening of hormones a wide range

Electronic supplementary material The online version of this article (doi:10.1007/s00216-008-2275-6) contains supplementary material, which is available to authorized users.

J. C. W. Rijk (✉) · T. F. H. Bovee · M. J. Groot ·
A. A. C. M. Peijnenburg · M. W. F. Nielen
RIKILT - Institute of Food Safety, Wageningen UR,
P.O. Box 230, 6700 AE Wageningen, The Netherlands
e-mail: jeroen.rijk@wur.nl

M. W. F. Nielen
Laboratory of Organic Chemistry,
Wageningen University,
Dreijenplein 8,
6703 HB Wageningen, The Netherlands

of mammalian or yeast cell-based bioassays have been developed [7–10]. These assays focus mainly on ligand–receptor interactions in which activation of a specific receptor is linked to a transcription reporter mechanism. Assays based on mammalian cell lines are in general more sensitive; however, the metabolic capacity of both mammalian and yeast cell-based assays is rather limited [11–13]. The latter are relatively easy to use and very robust, making them suitable for screening of samples from practice without complex sample cleanup procedures. In addition, yeast cells lack endogenous receptors and thus lack the potential cross talk from other receptor types [14]. As a result the signals obtained in the yeast androgen bioassay may be associated only with the androgenic properties of the compound or sample extract tested.

In vivo, hepatic first pass metabolism of exogenous and endogenous compounds normally leads to inactivation by phase I and phase II enzymes and the subsequent excretion of the deactivated compounds. However, metabolism can also result in an increased biological activity of a given compound [15]. Prohormonal compounds can be activated by hydroxysteroid dehydrogenases (HSDs). Prohormones such as dehydroepiandrosterone (DHEA), 4-androstene-3,17-dione (4-adione) and 5-androstene-3,17-diol (5-adione) are direct precursors of potent androgens like 17 β -testosterone (17 β -T) and dihydrotestosterone (DHT). The 3 β -hydroxysteroid dehydrogenase/isomerase (3 β -HSD) enzyme catalyzes the two-step conversion of 3 β -hydroxysteroids, like DHEA and 5-adione, into 4-adione and 17 β -T, respectively. 17 β -Hydroxysteroid dehydrogenase (17 β -HSD) catalyzes the conversion of 17-ketosteroids, like DHEA

and 4-adione, into their corresponding 17-hydroxysteroids, 5-adione and 17 β -T, respectively (Fig. 1). These conversions yield an increase of biological activity in vivo [2–4]. The action of biologically active androgens is mediated by the androgen receptor (AR). Upon ligand binding, the AR dissociates from its chaperone proteins and is translocated in its active state to the nucleus ready to bind to androgen-responsive elements (AREs) [16]. Binding results in recruitment of coactivators and enhanced transcription of target genes regulating androgenic-anabolic action.

In this context we explored the combined use of a bovine liver S9-based bioactivation model and a previously developed highly androgen-specific yeast assay based on the constitutive expression of the human androgen receptor (hAR) in combination with an androgen-responsive element coupled to an enhanced green fluorescent protein (EGFP) reporter system. A promising in vitro model for evidence of the indirect hormonal activity of prohormones, in accordance with the bioactivity-based legislation [1], has been developed. The results obtained were supported by chemical identification of the metabolites using ultra-performance liquid chromatography (UPLC) and time-of-flight mass spectrometry (TOFMS).

Methods and materials

Chemicals

Dehydroepiandrosterone (DHEA), 5-androstene-3,17-diol (5-adione), 4-androstene-3,17-dione (4-adione), 17 β -testosterone

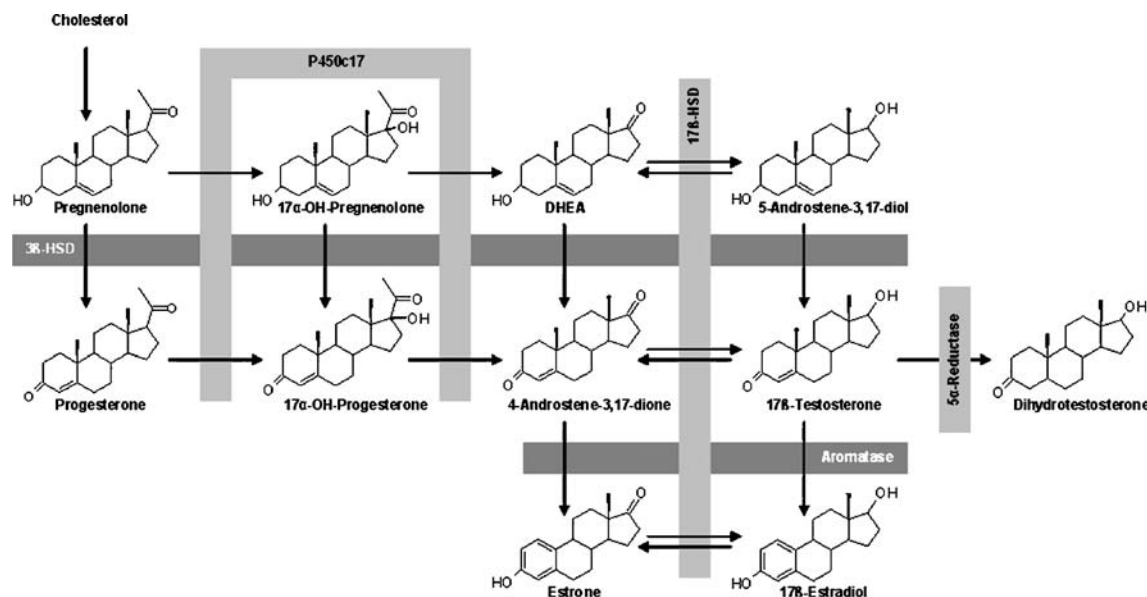


Fig. 1 In vivo steroid hormone biosynthesis: side chain cleavage of cholesterol results in pregnenolone which is metabolized into DHEA under P450c17 activity. From DHEA, conversions are catalyzed by 3 β -HSD and 17 β -HSD activity resulting in formation of potent

androgens like 17 β -T. Reversible reactions (marked by *double arrows*) depend on cofactor availability (e.g. NADP/NADPH ratios). Estrogens are formed from androgens (4-adione and 17 β -T) by aromatase activity

(17 β -T), 17 α -testosterone (17 α -T), 5 α -dihydrotestosterone (DHT), 7 α -hydroxy-DHEA (7 α -OH-DHEA), 7 β -hydroxy-DHEA (7 β -OH-DHEA), 16 α -hydroxy-DHEA (16 α -OH-DHEA), 16 β -hydroxy-DHEA (16 β -OH-DHEA), 11 β -hydroxy-DHEA (11 β -OH-DHEA) and 19-hydroxy-DHEA (19-OH-DHEA) were obtained from Steraloids (Newport, RI, USA). Sodium acetate, sodium carbonate, sodium chloride, di-sodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, ammonium sulphate, magnesium chloride, potassium chloride, tris (hydroxymethyl)aminomethane (Tris), dimethylsulfoxide (DMSO), hydrochloric acid and acetic acid were purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate, NADH disodium salt, NADP disodium salt and NADPH tetrasodium salt were from Roche Diagnostics (Almere, the Netherlands). Acetonitrile, methanol and isooctane were obtained from Biosolve (Valkenswaard, the Netherlands). Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were from Difco (Detroit, MI, USA). L-Leucine, bovine serum albumin (BSA), hydroxysteroid dehydrogenase originating from *Pseudomonas testosteronei* and NAD sodium salt were purchased from Sigma (St. Louis, MO, USA). Water used for LC/MS was purified using a Millipore (Bedford, MA, USA) Milli-Q system.

Preparation of liver S9 fractions

Four Frisian bovines (350–430 kg, 13–14 months old) that served as normally fed negative controls in a 6-week animal experiment were sacrificed at the end of the trial. At sacrifice, liver tissue was rinsed with ice cold 0.9% sodium chloride and snap-frozen in liquid nitrogen. Liver tissues were homogenized in twice their volume of Tris-HCl buffer (50 mM, pH 7.4, 1.15% KCl), using a blender. Homogenates were pooled and centrifuged at 10,000 *g* for 25 min at 4 °C. The supernatant was snap-frozen in liquid nitrogen and stored at –80 °C until use. Protein concentrations in this S9 fraction were determined according to Lowry [17], using the BioRad DC protein assay (BioRad, Veenendaal, the Netherlands) and BSA as a standard. The animal experiment referred to was approved by the Animal Ethics Committee of Gent University, Belgium, in accordance with local ethical requirements.

Prohormone incubations

Incubations of 50 μ g (pro)hormones, or of 100 μ l final SPE eluent from supplement samples (see [Extraction of supplement samples](#)), were carried out in a glass tube containing 0.1 M sodium phosphate buffer (pH 7.4), 10 mM glucose-6-phosphate, 33 mM KCl, 8 mM MgCl₂, 2 mg/ml bovine S9 fraction and either 4 mM NAD, NADH, NADP or NADPH. The final volume was 1 ml and the mixtures were

incubated at 37 °C in a water bath for 6 h. Single incubations of prohormones and of SPE eluents were performed. Blanks without bovine liver S9 and blanks without cofactor were included to check for nonenzymatic reactions during the incubation period. Reactions were terminated at *t*=0 and *t*=6 h with 1 ml acetonitrile, and the reaction products were subsequently subjected to a cleanup, which is similar to a method described by Marwah et al. [18]. In summary, the mixture was centrifuged for 15 min at 3,000 *g*, and the supernatant was transferred to a fresh glass tube. The mixture was extracted again with 2 ml acetonitrile and centrifuged for 10 min at 3,000 *g*. The combined supernatants were evaporated under nitrogen at 45 °C to approximately 0.5 ml. Next, the extract was diluted with 3 ml methanol, centrifuged for 15 min at 3,000 *g* and evaporated at 45 °C under nitrogen until dryness. The residue was dissolved in 200 μ l methanol and, following the addition of 1.8 ml water, applied onto a preconditioned reversed-phase solid-phase extraction (SPE) cartridge (Waters Oasis™ HLB, 3 cc, 6 mg). The cartridge was washed twice with 2 ml water and eluted with 2 ml methanol. The SPE eluent was evaporated under nitrogen at 45 °C and reconstituted in 2 ml acetonitrile. Aliquots of 200 μ l of this final acetonitrile extract plus 50 μ l 4% DMSO were pipetted in a conical 96-well plate (Greiner Bio-One, Germany) and evaporated overnight in a fume cupboard to leave only DMSO the next day.

Extraction of supplement samples

Two authentic supplement samples were extracted. According to the labels, each capsule of supplement A contained 50 mg DHEA and 10 mg vitamin C in a base of rice flour, and each capsule of supplement B contained 500 mg *Tribulus terrestris*, 100 mg 4-androstenedione, 100 mg DHEA, 100 mg lysine and 15 mg zinc amine acid chelate. A 100-mg aliquot of each capsule was extracted according to a method described by Bovee et al. [14]. In summary, sample aliquots were mixed with 4 ml methanol and 4 ml sodium acetate buffer (0.25 M, pH 4.8), sonicated for 10 min and mixed for 15 min in a head over head apparatus. Samples were then centrifuged for 10 min at 3,000 rpm, and 4 ml of the supernatant was collected. The pH of this supernatant was adjusted to pH 4.8 with 4 M acetic acid and applied onto an SPE cartridge (Varian, Bond Elut, C18, 500 mg, 3 ml), previously activated with 3 ml methanol and 3 ml sodium acetate buffer. Next, the SPE cartridge was washed with respectively 1.5 ml sodium acetate buffer, 2 ml MilliQ water, 1.5 ml sodium carbonate (10% w/v), 2 ml MilliQ water and 2 ml methanol/water (50:50 v/v). The SPE cartridge was dried and eluted with 4 ml acetonitrile. The SPE eluent thus obtained was applied onto an NH₂ SPE cartridge (Isolute, 100 mg, 3 ml)

previously activated with 4 ml acetonitrile. The run through was collected and evaporated at 45 °C under nitrogen and reconstituted in 4 ml acetonitrile. At this point the SPE eluent is either measured directly in the androgen yeast biosensor or bioactivated according to the procedure as described in [Prohormone incubations](#) and then measured by the androgen biosensor. Two sets of spiked supplement samples were prepared to monitor the extraction recovery (“spike before samples”) and the potential presence of AR antagonists in the supplements (“spike after samples”). 17 β -Testosterone was added to “spike before samples” (5 μ g/g) before the sample preparation step and to “spike after samples” (30 μ M in DMSO, 2 μ l) prior to biosensor exposure.

Recombinant yeast androgen bioassay

Transformants of a *Saccharomyces cerevisiae* strain that express the human androgen receptor (hAR) and an yEGFP reporter system [10] were grown on selective minimal medium plates supplemented with L-leucine. Minimal medium consisted of yeast nitrogen base without ammonium sulphate or amino acids (1.7 g/l), dextrose (20 g/l), ammonium sulphate (5 g/l) and was supplemented with L-leucine (6 mg/l). At day 1, 10 ml minimal medium supplemented with L-leucine (MM/L) was inoculated with a single colony and cultured overnight at 30 °C, in a shaking incubator at 125 rpm. The next day, the overnight culture was diluted in MM/L to an optical density (OD) value between 0.04 and 0.06 at 630 nm. Aliquots of 200 μ l yeast suspension were added to each well of a 96-well plate, already containing the DMSO extract of samples and

controls as described in [Prohormone incubations](#). A standard dose–response curve of 17 β -testosterone was included in each experiment. Plates were incubated at 30 °C for 24 h in a shaking incubator (125 rpm), and fluorescence was measured (485-nm excitation, 530-nm emission) using a Synergy™ HT multi-detection microplate reader (BioTek Instruments Inc., USA). The OD of the yeast was measured at 630 nm after 24 h to monitor for any cytotoxic effects on the yeast cells.

UPLC–TOFMS assignment of substances formed in metabolic bioactivation

Ultra-performance liquid chromatography (UPLC) was performed on a Waters (Milford, MA, USA) Acquity system containing a Waters Acquity BEH C₁₈ 1.7 μ m, 2.1 $\times$ 50-mm column, with mobile phases (A) acetonitrile/water/formic acid (10:90:0.2, v/v/v) and (B) acetonitrile/water/formic acid (90:10:0.2, v/v/v), linearly increasing from 20 to 46% B in 5 min at 0.7 ml/min. The column temperature was 45 °C, and the injection volume 20 μ l. The column effluent was split 1:1 prior to mass spectrometry.

Mass spectrometry was performed on a Waters QTOF micro instrument equipped with a dual electrospray ionization (ESI) probe and operated in the positive ion mode (ESI) at a source temperature of 120 °C, desolvation temperature 350 °C, desolvation gas flow 700 l/h, ESI capillary voltage of 3,000 V and a cone voltage of 30 V. Phosphoric acid in acetonitrile/water (0.01:50:50, v/v/v) was used as reference solution in the LockSpray™ at a flow rate of 10 μ l/min. TOF data were collected between m/z 80 and 1,200 and processed using Masslynx v 4.0 software.

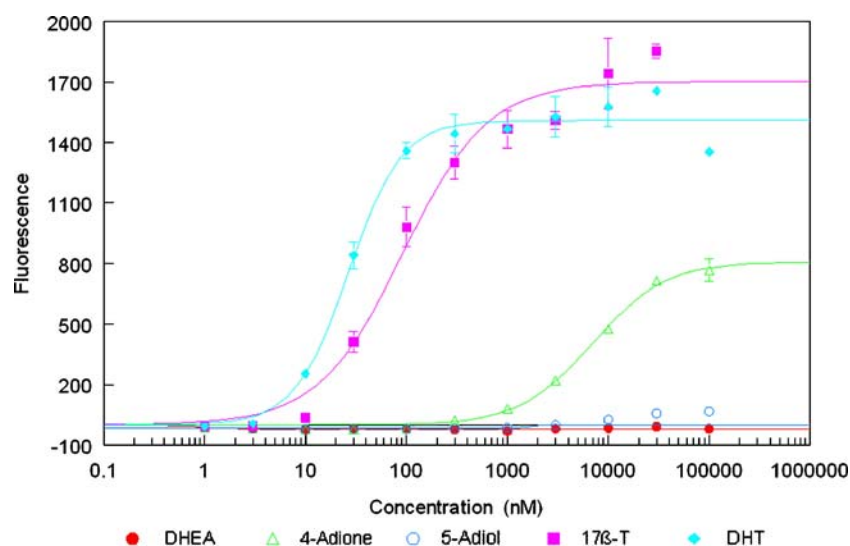


Fig. 2 Dose–response curves of DHEA, 4-adione, 5-adiol, 17 β -T and DHT after 24 h obtained in the androgen yeast biosensor. Fluorescence signals are the mean of an assay triplicate ($\pm$ SD) and corrected for the signal at $t=0$ and the blank DMSO

Results and discussion

Direct androgen bioassay screening

Androgen bioassay dose–response curves of DHEA and known *in vivo* metabolites 4-androstene-3,17-dione (4-adione), 5-androstene-3,17-diol (5-adiol), 17 β -testosterone (17 β -T) and 5 α -dihydrotestosterone (DHT) are shown in Fig. 2. Nonlinear regression curves were fitted through the data points, and the concentration giving half the maximum (EC₅₀) was calculated. Exposure of the androgen reporter-gene yeast bioassay to DHEA and 5-adiol did not result in a response of the cells, i.e. no direct hormonal activity was observed *in vitro* in accordance with expectations. In contrast a direct androgenic activity was observed for 4-adione, 17 β -T and DHT, with EC₅₀ values of 6,900 nM, 92 nM and 26 nM, respectively. It should be noted that the obtained dose–response curve of 4-adione was also caused by a 17 β -T impurity (data not shown), apart from the androgenic activity of 4-adione itself.

Incubation of prohormones with bovine liver S9 followed by androgen bioassay screening

To study the bioactivation of prohormones, 50 μ g of DHEA, 4-adione and 5-adiol were incubated with a bovine liver S9 mix in the presence of either NAD, NADH, NADP or NADPH. Initially, trials were performed with incubations of DHEA with NAD and pure hydroxysteroid dehydrogenase originating from *Pseudomonas testosteroni* containing 3 α - and 3 β -HSD activity. Incubation of DHEA for 1 h resulted in an increase of response in the androgen yeast assay, caused by formation of 4-adione and 17 β -T (results not shown). However, further work was with bovine liver S9 to include the entire species specific liver metabolism.

Results for sample extracts incubated with the S9 mix for 0 and 6 h in the presence of 4 mM cofactor and subsequently screened for androgenic activity in the yeast androgen bioassay are shown in Fig. 3. The androgen assay showed no signal for DHEA at *t*=0, but after metabolic activation of DHEA with S9 in combination with either NAD, NADP or NADPH, an androgenic activity was measured (Fig. 3a). Application of NADH as a cofactor, however, caused only a slight increase in the bioactivity signal.

The compound 4-adione is already androgenic without metabolic activation. Assuming 100% recovery, the 50 μ g 4-adione (containing the 17 β -T impurity) without activation would result in a final concentration of 87,000 nM in the well, and in accordance with Fig. 2 this resulted in a maximal fluorescence response of about 800. After S9 treatment the signal increased up to maximal 1,200, suggesting that metabolites are formed that are more active than 4-adione itself (Fig. 3b). Figure 3c shows that 5-adiol can be activated by the S9/NAD combination, but no changes in response were found after using NADH, NADP and NADPH as a cofactor. Whether the differences in response found between NAD and NADP are caused by the high level of specificity of HSDs for either NAD or NADP cannot be stated from the present study. The blanks, without bovine liver S9 or cofactor, showed no increase in response after 6-h incubation (results not shown).

Bioactivation plus androgen bioassay screening of supplement samples

Two supplements, A and B, were extracted and screened directly in the yeast androgen bioassay (Fig. 4a and b). In addition, aliquots of both supplements were spiked before extraction with 5 μ g 17 β -T per gram sample in order to

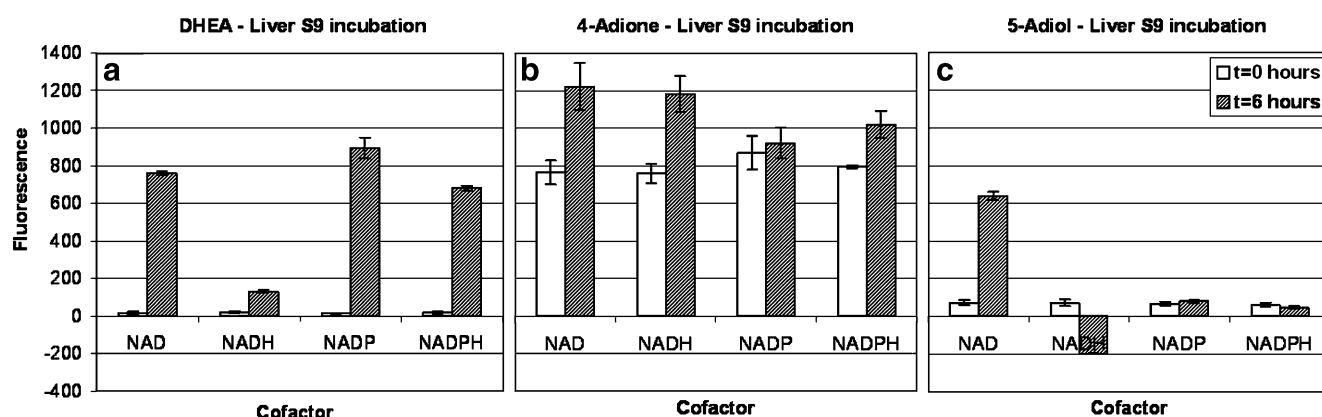


Fig. 3 Androgen bioassay responses of *a* DHEA, *b* 4-adione and *c* 5-adiol, before (*t*=0) and after (*t*=6) incubation with bovine liver S9 in the presence of different cofactors. Fluorescence signals are the mean

of an assay triplicate ($\pm$ SD) and corrected for the signal at *t*=0 and the reagent blank

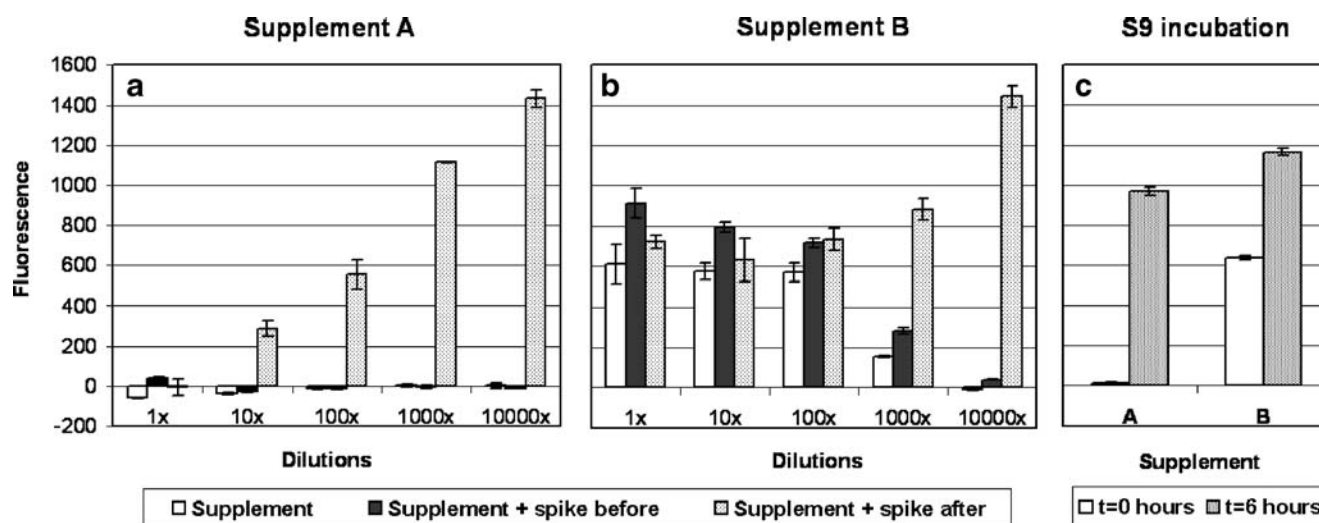


Fig. 4 Androgen bioassay responses of *a* supplement A and *b* supplement B as well as spiked with 17β -T before (217 nM in the well, calculated assuming 100% recovery) and after (300 nM added to the well) extraction and cleanup procedure. Fluorescence signals are

the mean of an assay triplicate ($\pm$ SD) and corrected for the signal at $t = 0$ and the reagent blank. *c* Androgen bioassay results after incubation using bovine liver S9 and cofactor NAD

check the recovery. Assuming no losses, the 17β -T spike would result in a calculated concentration of 217 nM 17β -T in the well. After sample cleanup but prior to the androgen bioassay nonspiked sample aliquots were also spiked with 300 nM 17β -T in the well in order to investigate whether there are any interfering or antagonistic compounds in the extract. According to the dose–response curve shown in Fig. 2, both 17β -T spikes are expected to give maximal bioassay response. Supplement A, stated to contain mainly DHEA, did not give a direct androgen bioassay response, but also the 17β -T spike before and spike after controls failed to give a response. Dilution of the extract, however, resulted in an increase of the spike after control, suggesting that the extract of this preparation contains an antagonist, which could be DHEA. This is in line with earlier performed quantitative structure–activity relationship (QSAR) modeling approaches, where calculations of the free energy after ligand docking and energy minimization of the ligand–receptor complex were plotted against the relative androgenic potency (RAP) [13]. DHEA showed no androgenic activity, but the calculated free energy is low, suggesting a good binding to the androgen receptor. Thus, DHEA shows affinity for the androgen receptor and might compete with bioactive androgens explaining its antagonistic properties. These antagonistic properties of DHEA were confirmed in the androgen yeast bioassay by coexposure of two concentrations of 17β -T, one concentration (70 nM) at half maximum and a concentration (1,000 nM) at full androgen bioassay response (data not shown).

Figure 4b shows that supplement B gives a direct androgen bioassay response in accordance with the declared ingredient 4-andione. This response remains at the

same level up to a 100-fold dilution of the extract, but at a 1,000-fold dilution the bioassay signal decreases. It should be noted that the “spike after” control does not reach its maximum fluorescence response of about 1,400 and that following dilution of the samples the signal of the “spike after” in the well increases. Again this is due to the antagonistic effects of DHEA, which is also a declared main compound in supplement B. Figure 4c shows the androgen bioassay responses of supplement A and B in the yeast androgen bioassay without and after the metabolic activation with the S9/NAD combination. Supplement A does not give a signal without the activation; however, after 6 h of incubation with bovine liver S9/NAD the signal reaches near maximal response, indicating that bioactive androgenic metabolites have been formed. As mentioned before, supplement B already shows a response; however, after the S9/NAD treatment the signal increases from about 600 to a near maximal response, again indicating that androgenic metabolites have been formed. From these results it can be concluded that supplement A contains proandrogens showing antiandrogenic properties and supplement B contains both androgens and proandrogens containing androgenic and antiandrogenic properties.

UPLC–TOFMS analysis

UPLC–TOFMS analysis with accurate mass measurement was used for the identification of the metabolites formed after bovine S9 incubation. The metabolites were identified by exact mass measurement and elemental composition calculations thereof, followed by comparison of retention times and mass spectra with the data obtained from

commercially available standards. Fifty-mDa window reconstructed ion chromatograms were used, being an appropriate window for elemental composition elucidation with a limited chance of false negative results due to mass

shifts caused by coeluting isobaric compounds and/or detector saturation [19]. After metabolic activation of DHEA with bovine liver S9 and cofactor NAD (Fig. 5) the main metabolites of DHEA (X) appeared to be 4-adione

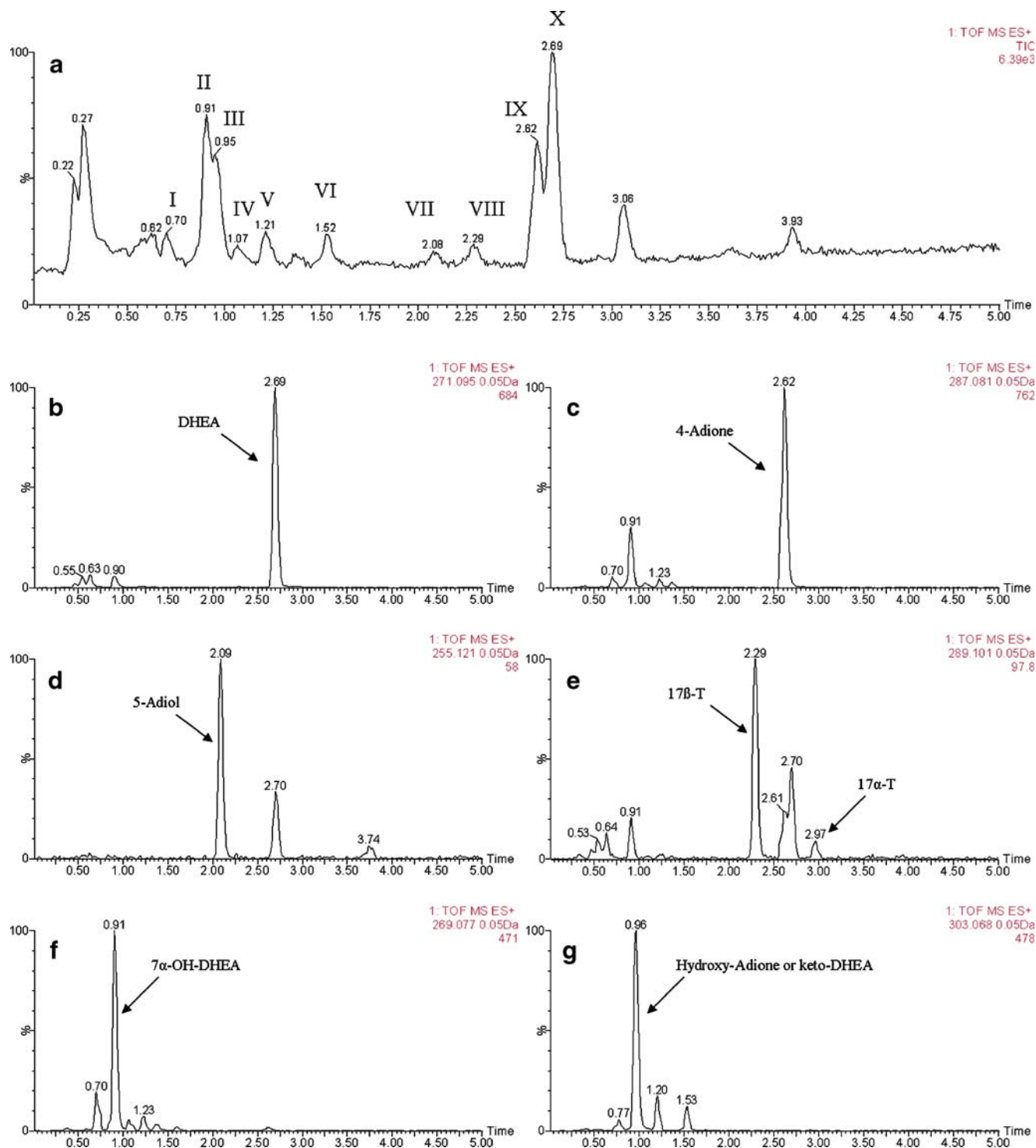


Fig. 5 UPLC-TOFMS total ion current (a) and reconstructed accurate mass chromatograms of DHEA (b), 4-adione (c), 5-adiol (d), 17 α - and 17 β -T (e), 7 α -OH-DHEA (f) and a possible hydroxy-adione or keto-DHEA metabolite (g) using the accurate mass of the

[M+H]⁺ or [M+H-H₂O]⁺ ion and a mass window of 0.05 Da. Conditions: bovine liver S9 bioactivation using cofactor NAD and an incubation time of 6 h

(IX), 7 α -OH-DHEA (II) and metabolite III at a retention time of 0.95 min having an abundant ion at m/z 303 and minor ions at m/z 285 and m/z 267 suggesting a keto-metabolite of DHEA (e.g. 7-oxo-DHEA) or a hydroxy-metabolite of 4-adione. Several minor abundant metabolites eluted after 0.70, 1.07, 1.21, 1.52, 2.09 and 2.29 min of which the last two appeared to be 5-adiol (VII) and 17 β -T (VIII), respectively. The identification of 17 β -T and 4-adione confirm the bioactivation of DHEA into androgenic substances observed in the androgen bioassay. Metabolites I and IV are most likely hydroxy-metabolites of DHEA resulting in $[M+H]^+$ ions at m/z 305 and $[M-H_2O+H]^+$ ions at m/z 287. V and VI most likely are keto-metabolites of DHEA or hydroxylated metabolites of 4-adione, showing $[M+H]^+$ ions at m/z 303 and $[M-H_2O+H]^+$ ions at m/z 285. According to ref. [20] metabolism of DHEA employing human liver S9 for 20 min (instead of bovine liver S9 for 6 h) resulted mainly in 7 α -OH-DHEA, 7 β -OH-DHEA, 7-oxo-DHEA, 16 α -OH-DHEA and 5-adiol depending on the cofactor used; no androgenic metabolites like 4-adione and 17 β -T were observed. The retention times of DHEA and its metabolites with corresponding exact masses have been summarized in Table 1. UPLC–TOFMS analysis showed that the spectra of 5-adiol, DHEA and hydroxy-metabolites generally were dominated by $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ ions, whereas the base peak in the mass spectra of 4-adione and 17 β -T was the $[M+H]^+$ ion, as expected for 3-keto-4-ene steroids [21].

Metabolic activation of 4-adione, containing a 17 β -T impurity, with bovine liver S9 resulted in an increase of 17 β -T, thereby confirming the increase in androgen bioactivity as observed in the bioassay. Interestingly 17 α -T was also formed. For 5-adiol, only metabolic activation using NAD resulted in formation of small amounts 17 β -T, in accordance with the bioassay results in Fig. 3b. The fact

that DHEA is converted into 4-adione and 5-adiol and the latter two into 17 β -T supports that bovine liver S9 contains 3 β -HSD/isomerase and 17 β -HSD activity. The observed hydroxy-metabolites of DHEA are due to P450 enzyme activity. Indeed in vitro bovine S9 treatment can mimic the in vivo conversions shown in Fig. 1.

UPLC–TOFMS analysis showed that supplement A contained only DHEA which is in accordance with what is declared on the label. Apart from the claimed DHEA and 4-adione, supplement B contained 17 β -T and traces of 17 α -T, which are possibly by-products formed during production or storage. Metabolic activation of the supplements showed similar metabolite profiles as obtained during activation of DHEA and 4-adione standards, resulting in higher levels of 5-adiol and the more potent androgens 4-adione and 17 β -T (see [Electronic Supplementary Material](#)).

Conclusions

This work has outlined the concept of a bioactivity screening assay for prohormones using a combination of bovine liver S9 bioactivation with androgen bioactivity detection. The prohormone DHEA shows no direct androgenic activity in the androgen yeast biosensor but antiandrogenic properties. On the other hand, DHEA, but also 4-adione and 5-adiol, can be converted by bovine S9 into more potent androgens, resulting in an indirect androgenic hormonal activity. The developed in vitro bioactivation system successfully mimics the hydroxysteroid dehydrogenase (HSD)- and cytochrome P450-mediated in vivo metabolic transitions, thus allowing assessment of the indirect bioactivity and chemical identification without the use of animal experiments. UPLC–TOFMS analysis con-

Table 1 UPLC–TOFMS analysis data of DHEA incubated for 6 hours with bovine liver S9/NAD

	Retention time (min) ^a	m/z^b			Elemental composition	Identified metabolites ^c
		$[M+H]^+$	$[M-H_2O+H]^+$	$[M-2H_2O+H]^+$		
I	0.70	305.2117 (−0.3)	287.2023 (1.2)	269.1928 (2.3)	C ₁₉ H ₂₉ O ₃	–
II	0.91	–	287.2010 (−0.1)	269.1905 (0.0)	C ₁₉ H ₂₉ O ₃	7 α -OH-DHEA
III	0.95	303.1945 (−1.5)	285.1864 (0.9)	267.1781 (0.9)	C ₁₉ H ₂₇ O ₃	–
IV	1.07	305.2077 (−4.0)	287.2038 (2.7)	269.1889 (−1.6)	C ₁₉ H ₂₉ O ₃	–
V	1.21	303.1974 (1.4)	285.1855 (0.0)	–	C ₁₉ H ₂₇ O ₃	–
VI	1.52	303.1966 (0.6)	285.1858 (0.3)	267.1768 (1.9)	C ₁₉ H ₂₇ O ₃	–
VII	2.08	–	273.2220 (0.2)	255.2126 (1.3)	C ₁₉ H ₃₁ O ₂	5-Androstene-3,17-diol
VIII	2.29	289.2197 (2.9)	–	–	C ₁₉ H ₂₉ O ₂	17 β -Testosterone
IX	2.62	287.1981 (−3.0)	–	–	C ₁₉ H ₂₇ O ₂	4-Androstene-3,17-dione
X	2.69	289.2160 (−0.8)	271.2049 (−1.3)	253.1947 (−0.9)	C ₁₉ H ₂₉ O ₂	DHEA

^a Experimental retention times

^b Accurate masses of observed ions, with mass error versus the theoretical masses in mDa (in parenthesis)

^c Compounds confirmed by comparison with a standard

firmed that the tested prohormones are metabolized into the androgenic active steroids 4-adione and 17 β -T. In the same manner other prohormones of androgenic bioactive compounds requiring transformation by HSDs at the 3-position and 17-position can be screened for e.g. the prohormones 19-norandrostenedione and 19-norandrostenediol are expected to be converted into the potent anabolic androgenic steroid nandrolone by 3 β -HSD/isomerase and 17 β -HSD activity. In conclusion we can state that the androgen bioassay can be used for the screening of supplements for the presence of androgens, antiandrogens and prohormones, the last of these following liver S9 bioactivation. The system developed is expected to be equally applicable to prohormone preparations from sports doping.

Acknowledgements This project was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

1. EEC Directive 96/22 (1996) Official J Eur Communities L125:3–9
2. Labrie F (1991) Mol Cell Endocrinol 78:C113–C118
3. Labrie F, Belanger A, Luu-The V, Labrie C, Simard J, Cusan L, Gomez J-L, Candas B (1998) Steroids 63:322–328
4. Lukacik P, Kavanagh KL, Oppermann U (2006) Mol Cell Endocrinol 248:61–71
5. Meyer HHD (2001) APMIS 109:1–8
6. Angeletti R, Contiero L, Gallina G, Montesissa C (2006) Vet Res Commun 30:127–131
7. Blankvoort BMG, de Groene EM, van Meeteren-Kreikamp AP, Witkamp RF, Rodenburg RJT, Aarts JMMJG (2001) Anal Biochem 298:93–102
8. Willemsen P, Scippo M, Maghuin-Rogister G, Martial JA, Muller M (2005) Anal Bioanal Chem 382:894–905
9. Bovee TFH, Helsdingen RJR, Koks PD, Kuiper HA, Hoogenboom LAP, Keijer J (2004) Gene 325:187–200
10. Bovee TFH, Helsdingen RJR, Hamers ARM, van Duursen MBM, Nielen MWF, Hoogenboom LAP (2007) Anal Bioanal Chem 389:1549–1558
11. Soto AM, Maffini MV, Schaeberle CM, Sonnenschein C (2006) Best Pract Res Clin Endocrinol 20:15–33
12. Binda D, Lasserre-Bigot D, Bonet A, Thomassin M, Come MP, Guinchard C, Bars R, Jacqueson A, Richert L (2003) Toxicol In Vitro 17:59–67
13. Bovee TFH, Lommerse JPM, Peijnenburg AACM, Antunes Fernandes E, Nielen MWF (2008) J Steroid Biochem 108:121–131
14. Bovee TFH, Bor G, Heskamp HH, Hoogenboom LAP, Nielen MWF (2006) Food Addit Contam 23:556–568
15. Coldham NG, Horton R, Byford MF, Sauer MJ (2002) Food Addit Contam 19:1138–1147
16. Gelmann EP (2002) J Clin Oncol 20:3001–3015
17. Lowry OH, Farr Randall RJ (1951) J Biol Chem 193:265–275
18. Marwah A, Marwah P, Lardy H (2002) J Chromatogr B 767:285–299
19. Nielen MWF, van Engelen MC, Zuiderent R, Ramaker R (2007) Anal Chim Acta 586:122–129
20. Chalbot S, Morfin R (2005) Drug Metab Dispos 33:563–569
21. Pozo OJ, Van Eenoo P, Deventer K, Delbeke FT (2007) J Mass Spectrom 42:497–516