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A specific yeast androgen bioassay for the detection of androgens, anti-androgens and pro-androgens in supplements and urine

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1. Introduction

In order to achieve fair play and to fight doping, the World Anti-Doping Agency (WADA) prohibited the use of anabolic steroids in sports. This list of prohibited steroids has grown continuously. The use and misuse of these substances has been reviewed by Van Eenoo and Delbeke in 2006 and starts with the subcutaneous injections of a liquid obtained from the testis of animals in 1889 and finds its tentative culmination in the discovery of the designer steroid tetrahydrogestrinone (THG) in 2004 [1-3]. The rapid development of mass spectrometry resulted in specific screening methods for anabolic steroids in the 1980's, but the abuse of the endogenous steroid testosterone could not be detected until the testosterone to epitestosterone (T/E) ratio was introduced as a biomarker [4]. In the 1990's several new steroids were commercialised as nutritional supplements. Initially these new steroids were precursors of testosterone, commonly referred to as prohormones. Later, a range of prohormones from other steroids, like 19-nortestosterone, boldenone and 17α-alkylated steroids, became available as over-the-counter preparations. Since 2005 there is a ban on dietary supplements containing prohormones in the U.S., but in order to improve their performance athletes are often tempted to use these kinds of nutritional supplements. Moreover, there is a tendency to switch to novel unknown compounds to circumvent doping control. Due to the great variety of chemicals with hormone-like activity we start to realise that methods based on analytical chemistry only detect target compounds and are not able to determine biological activity of unknown compounds and their metabolites, this in contrast to biological assays. Recently we constructed recombinant yeast cells that express the human androgen receptor (hAR) and yeast enhanced green fluorescent protein (yEGFP), the latter in

response to androgens. When exposed to testosterone, the concentration where half-maximal activation is reached (EC50) was 50 nM [5]. In this paper we demonstrate that this yeast androgen bioassay is capable of detecting both compounds with an androgenic and anti-androgenic mode of action and that the assay is suited to detect androgen receptor agonists in supplements. Additionally, prohormones with an indirect androgenic mode of action, e.g. dehydroepiandrosterone and steroid esters such as testosterone decanoate, can be detected after an enzymatic bioactivation using liver S9 fractions and an esterase, respectively.

2. Materials and Methods

Supplement samples were grinded and two portions of 100 mg were weighted. One of the 100 mg portions was spiked with 10 µl of a 50 µg ml^{-1} testosterone stock solution, resulting in a final spiked amount of 5 µg/g. Samples were extracted and subsequently subjected to solid phase extraction (SPE) on a C18 and NH_2-column, similar as described previously for urine and feed samples [6]. Aliquots of 200 µl of the obtained acetonitrile eluate and ten fold dilutions in acetonitrile were transferred to a V-shaped 96 well plate in triplicate and 50 µl of a 4% DMSO solution was added to each well. Spike after controls were performed by adding 50 µl water and 2 µl of a 30 µM testosterone stock solution in DMSO. The plate was dried overnight in a fume cupboard and was then ready to be screened on androgenic activities with the yeast androgen bioassay. Exposure and fluorescent measurements were similar as described before for the yeast estrogen bioassay [6].

3. Results

Typical androgen bioassay dose-response curves for a few natural and synthetic androgens are shown in figure 1. It can be seen that testosterone, 19-nortestosterone and boldenone are potent androgens. Figure 2 shows the anti-androgenic activity of the known antagonist flutamide, when co-exposed with a dose of 5α-dihydrotestosterone (DHT): the near maximal response of DHT declines rapidly at increasing dose of the anti-androgen.

Figure 3 shows the bioassay result from a dietary supplement that was screened negative by a liquid chromatography tandem mass spectrometry method (LC-MS/MS) for the presence of 49 anabolic steroids [7]. In contrast, the androgen activity histogram in figure 3 shows that there is at least one potent agonist present in this supplement. The extract of this supplement can be diluted more than 100 times before the response declines (black bars). There is no evidence for the presence of any anti-androgenic or inhibiting compound as the “spike after” control is not influenced by diluting the extract (white bars).

Figure 1

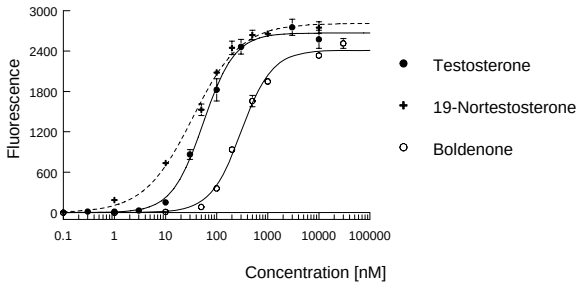
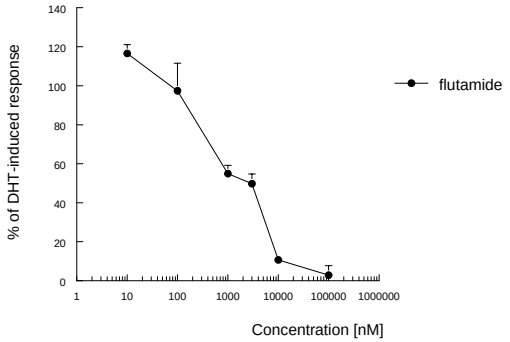
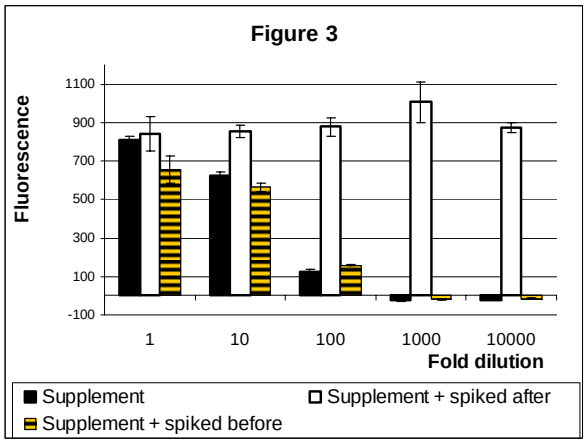


Figure 2





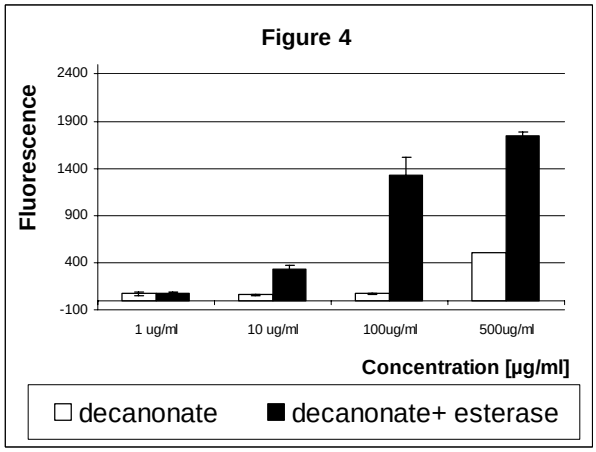


Figure 4 shows the androgen activity histogram of testosterone decanoate. The histogram demonstrates that testosterone decanoate is activated by esterase (black bars) in accordance with expectations. The white bars in figure 4 suggest that testosterone decanoate is already active without the esterase treatment. However, it was proven that this activity was due to a low testosterone contamination in the testosterone decanoate standard.

4. Discussion and conclusions

The new yeast androgen bioassay is sensitive and highly specific for compounds with an androgenic mode of action. This assay was found to be a good tool to check and optimise QSAR model approaches to predict activities of different isomers and designer steroids [8]. Moreover, the yeast androgen bioassay has a surplus value in comparison with LC-MS/MS for the screening of anabolic steroids in dietary supplements. We identified the androgen receptor agonist in the supplement of figure 3 that was screened negative by LC-MS/MS, using a similar LC-fractionation-bioassay-TOFMS approach as described before for the identification of THG in human urine [9]. Results revealed that 1-testosterone is most likely the responsible compound.

Prohormones are compounds that need *in vivo* biotransformation before they become hormonally active. There are several methods for the *in vitro* bioactivation of prohormones. Pure enzymes like an esterase, liver S9 fractions or methods based on whole cells, e.g. liver or intestine slices, are often used [10]. The example of the *in vitro* bioactivation of testosterone decanoate using an esterase shows that bioactivation in combination with the yeast androgen bioassay is a powerful tool for detecting prohormones.

References

[1] Van Eenoo P., Delbeke F.T. (2006) Metabolism and excretion of anabolic steroids in doping control – New steroids and insights. *JSBMB* **101**, 161-178.
[2] Brown-Sequard C.E. (1889) The effects produced on man by subcutaneous injections of a liquid obtained from testis of animals. *Lancet* **2**, 105-107.
[3] Catlin D.H., Sekera M.H., Ahrens B.D., Starcevic B., Chang Y.C., Hatton C.K. (2004) Tetrahydrogestrinone: discovery, synthesis, and detection in urine. *Rapid Commun. Mass Spectrom.* **18**, 1245-1249.
[4] Donike M., Adamietz B., Opfermann G., Schänzer W., Zimmermann J., Mandel F. (1985) Die Normbereiche für Testosteron und Epitestosteron Urinspiegel sowie des Testosteron/Epitestosteron Quotienten, in Mellerowicz, F.H., Noack, W. (Eds.), *Training und Sport zur Prävention und Rehabilitation in der technisierten Umwelt*, Springer Verlag, Berlin, Heidelberg, pp 503-507.
[5] Bovee T.F.H., Helsdingen J.R., Hamers A.R.M., Van Duursen M.B.M., Nielen M.W.F., Hoogenboom L.A.P. (2007) A new highly specific and robust yeast androgen bioassay for the detection of agonists and antagonists. *Anal. Bioanal. Chem.* **389**, 1549-1558.
[6] Bovee T.F.H., Bor G., Heskamp H.H., Hoogenboom L.A.P., Nielen M.W.F. (2006) Validation and application of a robust yeast estrogen bioassay for the screening of estrogenic activity in animal feed. *Food Add. Contam.* **23**, 556-568.
[7] Van Poucke C., Detavernier C., Van Cauwenberghe R., Van Peteghem C. (2007) Determination of anabolic steroids in dietary supplements by liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta* **586**, 35-42.
[8] Bovee T.F.H., Lommerse J.P.M., Peijnenburg A.A.C.M., Antunes Fernandes E., Nielen M.W.F. (2007) A new highly androgen specific yeast biosensor, enabling optimisation of (Q)SAR model approaches. *JSMBM*, on line available at doi:10.1016/j.jsbmb.2007.05.035.
[9] Nielen M.W.F., Bovee T.F.H., Van Engelen M.C., Rutgers P., Hamers A.R.M., Van Rhijn J.A., Hoogenboom L.A.P. (2006) Urine testing for designer steroids by liquid chromatography with androgen bioassay detection and electrospray quadrupole time-of-flight mass spectrometry identification. *Ana.l Chem.* **78**, 424-431.
[10] Rijk J., Groot M., Peijnenburg A., Bovee T., Van Engelen M., Nielen M. (2006) Metabolic activation and detection of dehydroepiandrosterone (DHEA) using bovine liver S9 in combination with a sensitive yeast androgen assay. Abstract book of the 5th International symposium on Hormone and Veterinary Drug residue Analysis, pp 193-194.