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Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays

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ABSTRACT

Anabolic androgenic steroids (AAS) are a class of steroid hormones related to the male hormone testosterone. They are frequently detected as drugs in sport doping control. Being similar to or derived from natural male hormones, AAS share the activation of the androgen receptor (AR) as common mechanism of action. The mammalian androgen responsive reporter gene assay (AR CALUX® bioassay), measuring compounds interacting with the AR can be used for the analysis of AAS without the necessity of knowing their chemical structure beforehand, whereas current chemical–analytical approaches may have difficulty in detecting compounds with unknown structures, such as designer steroids. This study demonstrated that AAS prohibited in sports and potential designer AAS can be detected with this AR reporter gene assay, but that also additional steroid activities of AAS could be found using additional mammalian bioassays for other types of steroid hormones. Mixtures of AAS were found to behave additively in the AR reporter gene assay showing that it is possible to use this method for complex mixtures as are found in doping control samples, including mixtures that are a result of multi drug use. To test if mammalian reporter gene assays could be used for the detection of AAS in urine samples, background steroidal activities were measured. AAS-spiked urine samples, mimicking doping positive samples, showed significantly higher androgenic activities than unspiked samples. GC–MS analysis of endogenous androgens and AR reporter gene assay analysis of urine samples showed how a combined chemical–analytical and bioassay approach can be used to identify samples containing AAS. The results indicate that the AR reporter gene assay, in addition to chemical–analytical methods, can be a valuable tool for the analysis of AAS for doping control purposes.

1. Introduction

Anabolic androgenic steroids (AAS) are the most frequently detected doping compounds in sports [1]. Athletes abuse them to increase muscle mass and performance. International sports federations such as the World Anti-Doping Agency (WADA), have prohibited the use of AAS to ensure fair play and to protect athletes from their possible adverse side effects [2].

An extensive program of doping control analyses carried out by WADA-accredited laboratories serves to safeguard

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sport competitions from the unwanted interference of performance enhancing doping compounds. Doping control of AAS is based on the detection of specific compounds, compound profiles and metabolites in urine and plasma samples from athletes. Typically, chemical–analytical approaches combining gas chromatography (GC) or liquid chromatography (LC) separations with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection are applied for doping detection. Such methods are able to specifically identify the use of particular drugs. Furthermore, they can also detect the use of rapidly metabolised AAS by the analysis of their metabolites [3]. GC–MS and LC–MS/MS target analysis methods use the acquisition of only a predetermined selection of ions and MS/MS transitions from known compounds [4]. Although the thus obtained sensitivity and specificity is excellent, the consequence of this approach is that compounds with similar biological activities as known AAS but with a different chemical structure might be overlooked. New designer steroids that are unknown to authorities could thus be abused without being detected in routine doping control. This scenario is exemplified by the case of tetrahydrogestrinone (THG), which for some time was not detectable with the standard doping analysis methods [5] and norbolethone [6] (now both included in the WADA List of Prohibited Compounds).

AAS are structurally related to the male sex steroid hormone testosterone [7]. Just as testosterone, AAS exert their action by binding and activating the androgen receptor [8]. This steroid receptor is a member of the nuclear hormone receptor superfamily that includes receptors for androgenic, estrogenic, progestagenic and glucocorticoid hormones, and receptors for retinoids and thyroid hormones [9,10]. Natural steroid hormones are essential for reproductive processes and can influence many other physiological processes. Activation of the androgen receptor leads to androgenic effects, such as development and maintenance of male secondary sex characteristics [11,12] and anabolic effects, such as promotion of muscular growth [13].

The fact that all AAS share a common mechanism of action opens the door to the application of bioactivity analysis tools (bioassays) for their detection. Bioassays use the biological endpoint of drug action, e.g. receptor activation, rather than the chemical itself as an endpoint. As such, they detect all compounds interacting with the endpoint of choice, independent of their chemical structure. Bioassays could be promising complementary techniques to the established systems used in doping analysis to ensure the detection of AAS with known as well as unknown structures. Examples of bioassays for androgenic compounds include the *in vivo* Hershberger assay and closely related *in vivo* assays [14,15], and more recently developed *in vitro* bioassays such as a receptor binding assay using androgen receptor ligand binding domains [16] and various yeast androgen reporter gene screens [4,17–20]. In addition, a whole range of mammalian reporter gene assays has been developed for compounds interacting with the androgen receptor [21–26]. One of the most sensitive, specific and biologically relevant assays for the assessment of androgenic compounds to date is the AR CALUX (Androgen Responsive Chemical Activated *LU*ciferase *eX*pression) bioassay [27]. This mammalian reporter gene assay is based on a human osteosarcoma cell line that is stably co-transfected with the human AR and a luciferase reporter gene of the firefly (*Photinus pyralis*) under transcriptional control of androgen responsive elements (AREs). Exposure of these AR reporter gene cells to androgenic compounds results in AR activation and subsequent synthesis of luciferase. The amount of luciferase produced is directly proportional to the amount and androgenic potency of the compound(s) the cells have been exposed to. The bioassay is highly representative for the *in vivo* situation, as it covers all events involved in receptor activation, from uptake of the compound by the human cell to synthesis of protein. This was reflected in the excellent correlations observed between androgenic activities in the AR reporter gene assay and other *in vivo* and *in vitro* screening models for androgenic activities [28]. The AR reporter gene assay is part of a panel of reporter gene assays with the same cellular background (human U2-OS cells) allowing efficient and sensitive measurement of androgen-, estrogen-, progesterone-, and glucocorticoid receptor interacting compounds [27,29,30].

In the present study, we investigated if the mammalian AR reporter gene assay [27,28] would be a valuable addition to chemical–analytical methods for the analysis of AAS in sport doping control. The first objective of this study was to investigate if the AR reporter gene assay could be applied for the detection of AAS. The steroid activities of all available AAS from the List of Doping compounds that are prohibited by WADA [31] and other synthetic steroids were tested in the AR reporter gene assay. Furthermore, the responses were compared to those of additional reporter gene assays specific for other steroids, such as estrogens (ERα and ERβ reporter gene assays), progestins (PR reporter gene assay) and glucocorticoids (GR reporter gene assay) in order to determine compound selective bioactivity profiling of AAS.

The second objective of our study was to evaluate the behaviour of mixtures of androgenic compounds in the AR reporter gene assay. Urine samples tested for doping control contain mixtures of various endogenous steroids and metabolites thereof, possibly supplemented with compounds resulting from doping administration. The response of such mixtures in the AR reporter gene assay is a result of the contributions of all compounds present in the mixture. A model that is often used for the description of combined behaviour of compounds acting by receptor activation (such as steroids) is the concept of concentration addition [32], as shown previously for estrogenic compounds [33,34]. Here we investigated if the combined behaviour of steroids is also valid for AAS using the AR reporter gene assay. This could be used to compare androgenic activity measured in a urine sample with the activity that is calculated based on chemically determined individual compounds in the sample. A higher measured than predicted activity would then indicate the presence of active androgens not looked for in the chemical analysis.

The third objective of our study was to investigate whether reporter gene assay analysis could be used to detect traces of anabolic steroids in urine samples. This was done by analysing human urine samples containing background levels of steroids and urine samples spiked with doping compounds and by comparison of reporter gene assay results in the samples with chemically determined concentrations (GC–MS) of endogenous androgenic steroids.

2. Experimental

2.1. Chemicals

19-Norandrosterone and 19-noretiocholanolone were obtained from Cerilliant (Round Rock, TX, USA). Gestrinone, norbolethone, oxabolone and tetrahydrogestrinone (THG) were obtained from LGC Promochem GmbH (Wesel, Germany). 5α-Androstane-3α,17β-diol and 5α-androstane-3β,17β-diol were obtained from MRC steroid Reference Collection (Queen Mary's College, London, United Kingdom). Methyltrienolone (R1881) was obtained from PerkinElmer (Groningen, The Netherlands). Clostebol was obtained from Research plus (South Plainfield, New Jersey, USA). Mesterolone and methandriol were purchased from Schering (Berlin, Germany). 19-Norandrostenedione was obtained from Serva (Heidelberg, Germany). Aldosterone, bethamethasone, cholesterol, corticosterone, cortisol, cortisone, dehydroepiandrosterone (DHEA), 11-deoxycorticosterone, 11-deoxycortisol, dexamethasone (DEX), dihydrotestosterone-glucuronide (DHT-gluc), epiandosterone, 17α-estradiol, 17β-estradiol (E2), estrone (E1), 17α-ethynylestradiol (EE), 17β-estradiol-glucuronide (E2-gluc), levonorgestrel (LNG), mestanolone, 17α-methyltestosterone (MT), mifepristone (RU 486), norethynodrel (NE), prednisolone, progesterone, testosterone (T) and testosterone propionate (TP) were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Androst-5-ene-3β,17α-diol, 5-androstenediol, 4-androstenediol, 4-androstenedione, 5-androstenedione, androsterone, bolasterol, boldenone, boldione, calusterone, cyproterone acetate (CA), danazol, 5α-dihydrotestosterone (DHT), drostanolone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 4-hydroxysterone, medroxyprogesterone acetate (MPA), methandienone, methenolone, mibolerone, nandrolone, 19-norclostebol, norethandrolone, oxandrolone, pregnenolone, stanozolol, testosterone glucuronide and 17β-trenbolone were obtained from Steraloids Inc. (Newport, RI, USA). Fluoxymesterone was obtained from Upjohn (Kalamazoo, MI, USA). All other tested steroids were supplied by the Department of Medicinal Chemistry of Organon (Oss, The Netherlands).

Highly concentrated stock solutions (1×10^{-2} or 1×10^{-3} M) were prepared from the standards in dimethylsulfoxide (DMSO, spectrofotometric grade 99.9%, Acros, Geel, Belgium) and stored at −20 °C. Dilution series were prepared in DMSO for the measurement of steroidal activities.

2.2. Mixtures

Two mixtures were prepared to investigate the combined behaviour of androgens in the AR reporter gene assay, both containing compounds in millimolar concentrations. The first mixture consisted of the four androgens (testosterone, stanozolol, methandienone and nandrolone) that were most frequently detected in AAS positive samples in 2005 by WADA accredited laboratories [1] and was composed in such way that, according its individual androgenic potency, each component would contribute equally to the overall androgenic potency of the mixture (Table 1). The second mixture consisted of four endogenous androgenic steroids (epitestosterone, testosterone, etiocholanolone, androsterone) that are usually found in urine. The four steroids were mixed in realistic concentration ratios based on the concentrations reported in urine for a male reference group (Table 1; [35]). Both mixtures were prepared from stock solutions of the individual compounds in DMSO. Dilution series were prepared in DMSO for AR reporter gene assay measurements. In this way, the ratio of concentrations of individual compounds was similar for each dilution (so called fixed-ratio mixtures).

Table 1 – Composition of two mixtures of four AAS used to test their combined effect in the AR reporter gene assay and obtained EC50 and REP values of individual compounds and mixture

Compound	Concentration ratio in mixture	EC50 this exp. (M)[a]	REP this exp. DHT = 1	RTA this exp. DHT = 100	Contribution to effect (%)
Mixture 1					
Testosterone	1	8.62×10^{-10}	0.2135	103	24.1
Stanozolol	1.05	1.96×10^{-09}	0.1915	93	22.6
Methandienone	3.65	4.23×10^{-09}	0.072	77	29.6
Nandrolone	0.3	4.65×10^{-10}	0.6987	97	23.7
Mixture 1	–	1.94×10^{-10}	1.142	87	100
Mixture 2					
Epitestosterone	1	$>1.00 \times 10^{-6}$	$<4.1 \times 10^{-4}$	na	≤0.06
Testosterone	1.25	9.79×10^{-10}	0.1777[b]	92	≥31.46
Etiocholanolone	63.8	$>1.00 \times 10^{-6}$	$<2.7 \times 10^{-4}$	na	≤2.51
Androsterone	83.3	3.11×10^{-8}	0.0056	24	≥65.96[c]
Mixture 2	–	4.26×10^{-10}	1.403	72	100

na: not achieved.

[a] EC50 values of individual compounds were tested by exposure of AR reporter gene cells to concentration series of pure standards of single compounds and are expressed in M *compound*. Note that the EC50 value of the mixture is expressed in M DHT-equivalents.

[b] Due to interexperimental fluctuations, the REP of testosterone in the experiment with mixture 2 was slightly lower than in the experiment with mixture 1.

[c] Androsterone is only expected to contribute to the total response at response levels of the mixture lower than the RTA of androsterone (about 24% of the RTA of DHT).

2.3. Urine samples

Urine samples were provided by 10 adult volunteers (five males and five females) and collected in acetone rinsed bottles of brown glass and stored at −20 °C. Pooled urine samples of male and female volunteers were prepared and spiked with THG or nandrolone at levels of 10, 20 and 50 ng mL^{-1}. Furthermore, 10 human urine samples derived from Caucasian athletes competing at high level sports (collected in the European project ISOTRACE and provided by the Barcelona Doping laboratory, Spain) were analysed with GC–MS and AR reporter gene assay.

2.4. Deconjugation and extraction of urine samples

Portions of 2 mL of urine were deconjugated with 25 μL β-glucuronidase from *E. coli* (Sigma–Aldrich, Zwijndrecht, The Netherlands, dissolved in 50% glycerol to a concentration of 300 units mL^{-1}) after addition of 0.75 mL of a 0.8 M sodium phosphate buffer (pH 7.0). The deconjugation mixture was incubated at 50 °C for 1 h. After deconjugation, 0.5 mL of a 20% K_2CO_3/$KHCO_3$ buffer (1:1, pH 9) was added. Samples were extracted by liquid–liquid extraction with 5 mL of methyl-*tert*-butyl ether (MTBE; ultra resi analysed, J.T. Baker, Philipsburg, NJ, United States). The organic phase was collected, carefully evaporated under a gentle stream of nitrogen and taken up in 50 μL of DMSO.

To determine the recovery of the extraction, urine samples were spiked in triplicate with 400 ng DHT mL^{-1}, 20 ng E2 mL^{-1}and 21 ng Org 2058 mL^{-1}. The recovery of the sample preparation including the deconjugation was investigated by the analysis of extracts of urine samples that were spiked prior to deconjugation with DHT-glucuronide and E2-glucuronide leading to similar concentrations of DHT and E2 as in the determination of the recovery of the extraction only.

2.5. CALUX bioassays

Generation and maintenance of CALUX bioassays (AR, ERα, ERβ, PR and GR reporter gene assays) was described previously [27]. To perform experiments, reporter gene assay cells were plated in 96-well plates with phenol red-free DF medium supplemented with 5% dextran coated charcoal stripped FCS (DCC-FCS; [36]) at a volume of 100 μL per well. One day later, the medium was refreshed and cells were incubated with the compounds to be tested (dissolved in DMSO) in triplicate at a 1:1000 dilution. A concentration series of a reference compound (DHT for AR, E2 for ERα and ERβ, Org 2058 for PR and dexamethasone (dex) for GR reporter gene assay) was included on each plate. After 24 h, the medium was removed. Cells were lysed in 30 μL Triton-lysis buffer and measured for luciferase activity using a luminometer (Lucy2; Anthos Labtec Instruments, Wals, Austria) for 0.1 min well^{-1}. Luciferase activity per well was measured as relative light units (RLUs).

2.6. Analysis of steroid activity of pure compounds

Steroid potencies of at least eight different concentrations of individual compounds were tested in triplicate in at least two independent experiments. Fold induction was calculated by dividing the mean value of light units of each triplicate of exposed and non-exposed (solvent control) wells. Luciferase induction was expressed as percentage of the maximum activity of the reference compound. Dose–response curves were fitted using the sigmoid fit

$$y = \frac{a_0 + a_1}{(1 + \exp(-(x - a_2)/a_3))} \quad (1)$$

with y representing the luciferase activity and *x* the concentration and plotted in GraphPad Prism (version 4.00 for Windows, GraphPad Software, San Diego, CA). For comparison of steroid potencies between reference compounds and the other compounds, the steroid activity of compound A was expressed relative to that of reference compound R by calculation of the relative potencies (REP) as

$$\mathrm{REP_A} = \frac{\mathrm{EC50_R}}{\mathrm{EC50_A}}. \quad (2)$$

The relative transactivation activity (RTA, the maximum luciferase induction caused by a compound) of each compound tested was calculated as the ratio of maximum luciferase induction values of each compound and the maximum luciferase induction value of reference compound of each specific reporter gene assay according to

$$\mathrm{RTA} = \frac{\mathrm{ymax_A}}{\mathrm{ymax_R}} \times 100\%. \quad (3)$$

Two-dimensional hierarchical clustering on the base 10 logarithm of EC50 data was performed using the correlation option within the clustergram function from the bioinformatics toolbox in Mathlab (The Mathworks, The Netherlands).

2.7. Calculation of mixture effects

For the mathematical representation of CA, toxic units (TU) can be used. The TU_i of compound (or mixture) *i* is the ratio of the actual concentration *c* of *i* and of the concentration needed to cause a certain effect *x* (ECx_i), according to

$$\mathrm{TU_i} = \frac{c_i}{\mathrm{ECx_i}}. \quad (4)$$

According to CA, the overall TU of a mixture TU_{mix} is equal to the sum of all *n*TU_is in the mixture

$$\sum_{i=1}^{n} \mathrm{TU_i} = \mathrm{TU_{mix}}. \quad (5)$$

In case of androgenic steroids, individual potencies of compounds are expressed relative to that of DHT as the REP. To minimise the influence of inter-experimental fluctuations, REP values were used that were derived from concentration–response series tested in the same experiment as the mixture. The concentration of compound *i* expressed in DHT-equivalents is then given by Eqs. (6) and (7):

$$\mathrm{DHT\text{-}eq_i} = c_i \times \mathrm{EC50_{DHT}} \quad (6)$$

$$\mathrm{DHT\text{-}eq_i} = \mathrm{TU_i} \times \mathrm{EC50_{DHT}}. \quad (7)$$

According to CA, the total effective concentration of the mixture can be calculated solely based on the individual concentrations and potencies of the mixture components as

$$\sum_{i=1}^{n} \text{DHT-eq}_i = \text{DHT-eq}_{mix}. \quad (8)$$

Concentration–response curves of individual compounds and mixtures in the AR reporter gene assay and their 95% confidence limits were used to visually assess the similarity between predicted and observed mixture concentration response curves.

2.8. Quantification of steroid activities in urine samples

Steroid activities caused by urine extracts were interpolated in the standard curve of the reference compound. Extract dilutions generating a response between the limit of quantification (LOQ) and the EC50 were used for quantification. Activities were expressed as ng reference compound equivalents per mL urine.

2.9. Chemical analysis of endogenous androgenic steroids

Urine samples destined for chemical analysis (6 mL) were extracted as described above and taken up in 3 mL of methanol. Portions of 10 μL and 500 μL of the methanol extract were evaporated with nitrogen at 60 °C and derivatised with 25 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA++) at 60 °C for 1 h. After evaporation, the residue was dissolved in 50 μL iso-octane and 5 μL was injected on a GC–MS with a Varian VF-1MS column (60 m × 0.25 mm i.d., 0.25 μm film thickness) and a constant flow of 1.3 mL min^{-1}. The temperature program was:

$$80\,^{\circ}\text{C}\,(3\,\text{min}) \xrightarrow{50\,^{\circ}\text{C/min}} 290\,^{\circ}\text{C}\,(0\,\text{min}) \xrightarrow{2\,^{\circ}\text{C/min}}$$
$$306\,^{\circ}\text{C}\,(1\,\text{min}) \xrightarrow{50\,^{\circ}\text{C/min}} 325\,^{\circ}\text{C}\,(5\,\text{min}).$$

Ions for acquisition in single ion mode were *m*/*z* 434.1 (DHT, epi-DHT, etiocholanolone and androsterone) and *m*/*z* 432.1 (epi-testosterone and testosterone).

3. Results and discussion

Anabolic androgenic steroids (AAS) are frequently detected drugs in sport doping control. Being similar to or derived from natural male hormones, AAS share a common mechanism of action, i.e. activation of the androgen receptor (AR). As a mechanism-based bio-analytical approach, reporter gene bioassays can be used for the analysis of AAS without the necessity of knowing their chemical structure beforehand, as for chemical–analytical approaches. Several androgen reporter gene assays have been developed the last decade, either yeast-derived [4,18–20] being rapid and easy of use, or mammalian cell line-derived [21–27] having an origin close to the organism of main concern (human) and improved sensitivity compared with yeast-based androgen assays [23]. However, most of these systems still have several drawbacks, since they either have a low responsiveness, use slowly growing prostatic cell lines, or are not selective in their response because of expression of other nuclear hormone receptors, activating the reporter gene through non-AR-mediated mechanisms. Therefore a new androgen reporter cell line was generated using the human U2-OS cell line (AR CALUX bioassay), combining high specificity, sensitivity and ease of handling [27,28].

3.1. Steroid activities of individual compounds

The list of AAS that are prohibited by WADA contains exogenous AAS, endogenous AAS, metabolites and isomers thereof and other substances with a similar chemical structure or similar biological effect [31]. It was investigated if, due to their androgenic activity, these compounds could be detected with the mammalian AR reporter gene assay. Most compounds indeed activated the androgen receptor and induced luciferase activity in a concentration-dependent manner. Typical dose–response curves for several endogenous AAS (DHT, testosterone) as well as exogenous AAS (stanozolol, methandienone, nandrolone) are shown in Fig. 2a. In total, 55 AAS prohibited by WADA were tested. Their half maximum effect concentrations (expressed as ^{10}log transformed EC50-values), relative potencies (REP) values (calculated with Eq. (2)) and highest induction levels (relative transactivation activities (RTA), calculated with Eq. (3)) are given in Table I of Supplementary Data section. Highest potency (lowest EC50) was found for the exogenous AAS mibolerone (EC50 77 pM) and the lowest potency for methandriol (EC50 4 μM). The endogenous AAS DHEA and 19-noretiocholanolone and some diol metabolites did not generate a measurable response at the tested concentrations (EC50 > 10 μM).

Norbolethone and THG, designer drugs used in the past to circumvent detection in routine doping analysis, both showed high activity in the AR reporter gene assay (Table I of Supplementary Data section). Chemical modification of existing AAS could serve to synthesise new potential designer steroids. Alkylated and hydrogenated congeners of nandrolone and norethisterone (NET) were tested as examples of such compounds. Their androgenic activities ranged from high (EC50 = 90 pM; MENT) to moderate (EC50 = 76 nM; 11β-ethenyl-NET) (see Table I of Supplementary Data section, section other exogenous androgens). These results demonstrate that the AR reporter gene assay is capable of detecting the androgenic activities of these designer steroids.

To determine the selectivity of the AR reporter gene assay for compounds activating the androgen receptor also non-androgenic (pre-)steroid hormones, i.e. corticosteroids, estrogens and progestins (e.g. cortisol, 17β-estradiol, progesterone, aldosterone, and a range of synthetic glucocorticoids, progestins and estrogens) were tested. In general, these compounds did not evoke a response in the AR reporter gene assay with the exception of dexamethasone, E2 and some synthetic progestins [20,28] (Table I of Supplementary Data section). The observed androgenic activity of the synthetic progestins levonorgestrel, norethisterone, norethynodrel and medroxyprogesterone acetate confirms the androgenic activity in an

in vivo rat model that was found previously for these compounds [28]. Furthermore, androgenic activities for some of these progestins were reported using a yeast-based *in vitro* androgen bioassay, suggesting AR binding and subsequent reporter gene transcription instead of metabolism [20].

The potency of the tested compounds to activate other steroid receptors was investigated using the ERα, ERβ, PR and GR reporter gene assays measuring estrogenic, progestagenic and glucocorticoidal activities (see Table I of Supplementary Data section). Several AAS with profound androgenic activity in the AR reporter gene assay (mibolerone, methyltrienolone, trenbolone, norbolethone, norethandrolone, THG, gestrinone, nandrolone and methandienone), showed activity in the PR reporter gene assay, indicating their capability to also activate the progesterone receptor. This phenomenon was shown before for THG and gestrinone [37] and several other compounds [20,28,38]. Other AAS prohibited by WADA that did not generate an androgenic response in the AR reporter gene assay, such as DHEA and dihydroxylated 5α-androstane-, androst-4-ene and androst-5-ene compounds, were found to be active estrogens in the ERα and ERβ reporter gene assays (Table I of Supplementary Data section). Fluoxymesterone was

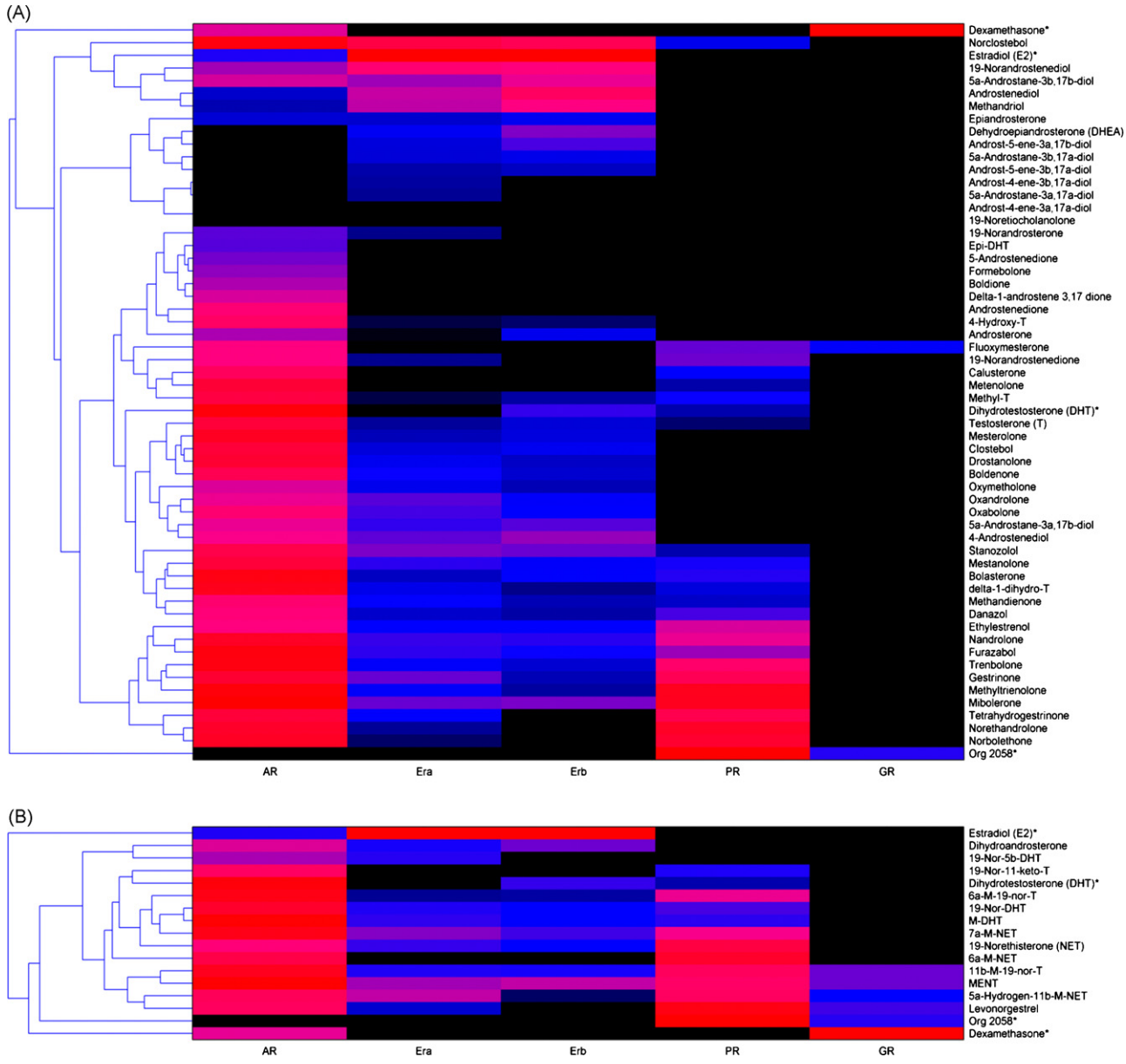

Fig. 1 – Profiling of androgenic compounds from the WADA prohibited list (A) and potential other designer AAS (B) by means of hierarchical clustering. Activities of compounds were determined in reporter gene assays for androgenic (AR), progestagenic (PR), estrogenic (ERα and ERβ) and glucocorticoid (GR) activities (see Supplementary Data section). Reference compounds for respective reporter gene assays (DHT for AR, Org 2058 for PR, E2 for ERα and ERβ, and dex for GR reporter gene assays) are marked with an asterisk. Activities (expressed as logarithm of median effective concentrations (LogEC50)) were clustered according to the hierarchical clustering method. The intensity of the red colour represents the activity of the compound. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

the only compound on the WADA list for which a weak glucocorticoid activity was observed.

The steroid compound data set obtained with various reporter gene assays (see Table I of Supplementary Data section) allows the cross-bioassay bioactivity profiling of compounds. By means of hierarchical clustering, bioactivity relationships between both compounds and between various bioassays were determined on the base of 10 logarithm of EC50 values. Furthermore, these relationships can be visualised easily by colouring the activity of a compound; the higher the activity of the compound in a certain reporter gene assay, the more intense the colour. Fig. 1a shows the bioactivity relationships of the androgenic anabolic steroids from the WADA 2005 prohibited list. Fig. 1b shows the bioactivity relationships of various potential new AAS (designer steroids) as yet not present on the WADA prohibited list, having strong androgenic activities, but also strong progestagenic activities. Examples of such new potential AAS are 7α-methyl-19-nor-testosterone (MENT; trestolone), and several methylated 19-norethisterone modifications.

In summary, AAS relevant in the field of doping control can be measured with mammalian reporter gene assays. Almost all AAS prohibited by WADA and a series of other synthetic AAS could be detected with reporter gene assays (89 out of 92 compounds: 97%). Most compounds showed androgenic activity in the AR reporter gene assay (82 out of 92 compounds: 89%), whereas the seven compounds that were not able to activate the androgen receptor at the tested concentrations, e.g. metabolites of potent androgens appeared to activate the estrogen receptors α and β and/or the progesterone receptor and could thus be detected in the ERα, ERβ and/or PR reporter gene assays. To our knowledge there are no (synthetic) AAS to date which need *in vivo* metabolic activation to become active AAS able to bind and activate the AR, which would be classified as false negative in the AR reporter gene assay due to limited *in vitro* metabolism. Exceptions are the natural pro-hormones DHEA and 5-androstenediol which show no or hardly activity in the AR reporter gene assay. However, they do show activities in the ERα and ERβ reporter gene assays (Table I).

3.2. *Androgenic activity of mixtures*

The combined effect of mixtures of androgenic compounds in the AR reporter gene assay was investigated using two fixed-ratio mixtures. The first mixture contained the four AAS most frequently detected by WADA laboratories: testosterone, stanozolol, methandienone and nandrolone. Full sigmoid concentration response curves of the individual compounds are shown in Fig. 2a. Obtained REP values (Table 1, fourth column) were used to predict the total androgenic activity of the mixture. Because the mixture concentration is expressed in pM DHT-eq, mixture behaviour should give rise to visual overlap

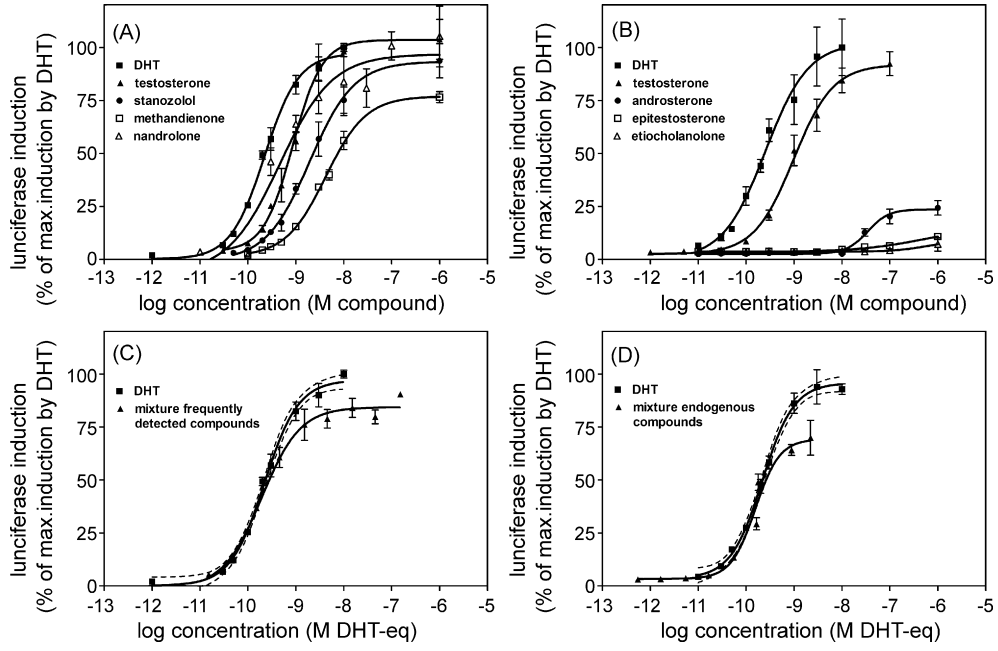

Fig. 2 – Concentration–response curves of individual standards and mixtures of steroids in the AR reporter gene assay, showing (A) individual standards of dihydrotestosterone (DHT, reference compound), and the four anabolic androgenic steroids most frequently detected in doping analysis testosterone, stanazolone, methandione and nandrolone; (B) individual standards of DHT and four steroids that are endogenously found in urine (testosterone, androsterone, epitestosterone and etiocholanolone); (C) concentration–response curves of DHT and an equipotent mixture of testosterone, stanazolol, methandienone and nandrolone; and (D) DHT and a mixture of testosterone, androsterone, epitestosterone and etiocholanolone mixed to their endogenous concentration ratios. The overlap between the mixture curves and the DHT curves in the same panes demonstrates that the combined behaviour of both mixtures is properly described with the concept of concentration addition. Error bars represent standard deviations of triplicate exposures. Dashed lines represent 95% confidence values. Please note that concentrations of individual compounds are expressed in M *compound*, whereas mixture concentrations are expressed in M *DHT-equivalents* (DHT-eq).

between the mixture curve and the DHT curve. Indeed, the mixture curve (partially) overlaps with the curve of reference compound DHT (Fig. 2c). The second mixture consisted of four endogenous androgenic steroids usually present in urine. The four androgens were mixed in concentration ratios based on the concentrations reported for a reference group of men [35]. As can be seen from the concentration–response curves of the individual compounds (Fig. 2b), mixture 2 contains a potent full agonist (testosterone), a partial agonist (androsterone, with a RTA of only about 24% of that of the reference compound DHT), and two compounds (epitestosterone and etiocholanolone) for which no or hardly any androgenic activity was observed at the tested concentrations. According to their REP values, testosterone and androsterone contribute most to the androgenic activity of the mixture, whereas only negligible contributions are expected from the other compounds (Table 1, fifth column). Fig. 2d shows that also for mixture 2 an overlap between the mixture curve and the predicted curve (represented by the curve of DHT) is obtained, demonstrating that the mixture behaviour is predicted correctly, as expected for compounds with a similar mechanism of action (i.e. activation of the androgen receptor).

The additive behaviour of androgens shown in Fig. 2 can be used to compare androgenic activity in a urine sample with activity that can be calculated based on chemically determined individual compounds in the sample. Furthermore, low concentrations of compounds that are only slightly active or even undetectable at an individual basis together might generate a considerable response in a bioassay. Measurement of the total androgenic activity using the AR reporter gene assay could, e.g. be helpful for the analysis of effects of mixtures of AAS that may be present after multi drug use [39]. As an example, we tested subnanomolar concentrations of five potent AAS that were most frequently detected in positive samples by WADA accredited laboratories [1] at concentrations evoking only a small response around or below 10% of the maximum response reached by the positive control DHT at 10 nM (Fig. 3) in the AR bioassay. Mixed in the same concentrations, however, the five compounds generated a considerable response (48.7 ± 3.3% of maximum induction by DHT). After multiplication with their respective REPs, the concentrations were summed and interpolated in the DHT curve to obtain the predicted mixture effect. The observed combined effect agreed very well with the predicted effect (94 ± 6% of the predicted effect; 52.1% of maximum induction by DHT; Fig. 3). This example illustrates that low concentrations of AAS (at or below 0.1 ng mL^{-1}) can cause significant androgenic effects that can be considerably larger when present in a mixture.

3.3. *Steroid activities in urine samples*

Excretion in urine is a major elimination route for (conjugates of) steroids from the human body. For this reason, and because urine collection is less inconvenient than that of, e.g. blood, urine is the preferred matrix for the chemical analysis of AAS in doping control. In this study, mammalian reporter gene assays were used to analyse androgenic, estrogenic, progestagenic and glucocorticoid activities in human urine samples. Recoveries of deconjugation and extraction procedures were high and reproducible as was determined by analysis in the

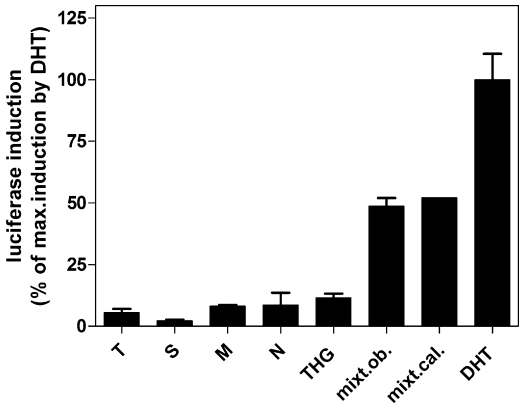

Fig. 3 – Androgenic responses of five relatively potent anabolic androgenic steroids at low concentrations (T = 0.2 nM testosterone; S = 0.2 nM stanazolol; M = 0.9 nM methandienone; N = 0.1 nM nandrolone; THG = 0.4 nM tetrahydrogestrinone) tested individually and as mixture in the AR reporter gene assay. The observed mixture (mixt.ob.) effect agreed very well with the effect calculated (mixt.cal.) according to the concept of concentration addition. Androgenic activity is expressed as a percentage of the maximal induction reached by the reference androgen DHT (at 10 nM).

AR, ERα and PR reporter gene assays of urine samples spiked with DHT-glucuronide (116 ± 9%), E2-glucuronide (152 ± 18%), DHT (93 ± 11%), E2 (107 ± 16%) and Org 2058 (121 ± 10%) (data not shown). Furthermore, high sensitivities were achieved for the analysis of steroid activities in extracts of deconjugated urine, reflected by very low limits of detection (AR reporter gene assay: 0.02 ng DHT-eq mL^{-1}; ERα reporter gene assay: 0.03 ng E2-eq mL^{-1}; ERβ reporter gene assay 0.02 ng E2-eq mL^{-1}; PR reporter gene assay 0.01 ng Org 2058-eq mL^{-1}; and GR reporter gene assay: 0.08 ng dexamethasone (dex)-eq mL^{-1}).

Steroid activities were assessed in urine samples of 10 adult volunteers (Fig. 4). Androgenic activity was found in all samples and ranged between 0.19 ± 0.03 and 18.1 ± 0.6 ng DHT-eq mL^{-1} (Fig. 4). Androgenic activities in male urine were significantly higher than those in female urine ($p < 0.04$). On the contrary, no significant differences between male and female samples were found in estrogenic activity in the ERα and ERβ reporter gene assays. Concentrations of activity on the estrogen receptor α ranged between 0.07 ± 0.01 and 3.1 ± 0.1 ng E2-eq mL^{-1} (Fig. 4). Concentrations of activity on the estrogen receptor β varied between 0.5 ± 0.2 and 11.9 ± 0.0 ng E2-eq mL^{-1}. Progestagenic activities could not be quantified in all samples. Activities ranged from less than 0.01 (the LOD) to 1.97 ± 0.01 ng Org 2058-eq mL^{-1} urine (Fig. 4). Glucocorticoid activities ranged between <0.08 and 285 ± 7 ng dex-eq mL^{-1}.

The volunteers from whom the urine samples were obtained were not suspected for doping abuse. Steroid activities in their urine samples are therefore most likely due to the presence of natural steroid hormones and metabolites thereof. These metabolites include phase I metabolites, metabolically activated prohormones and inactivated phase II metabolites

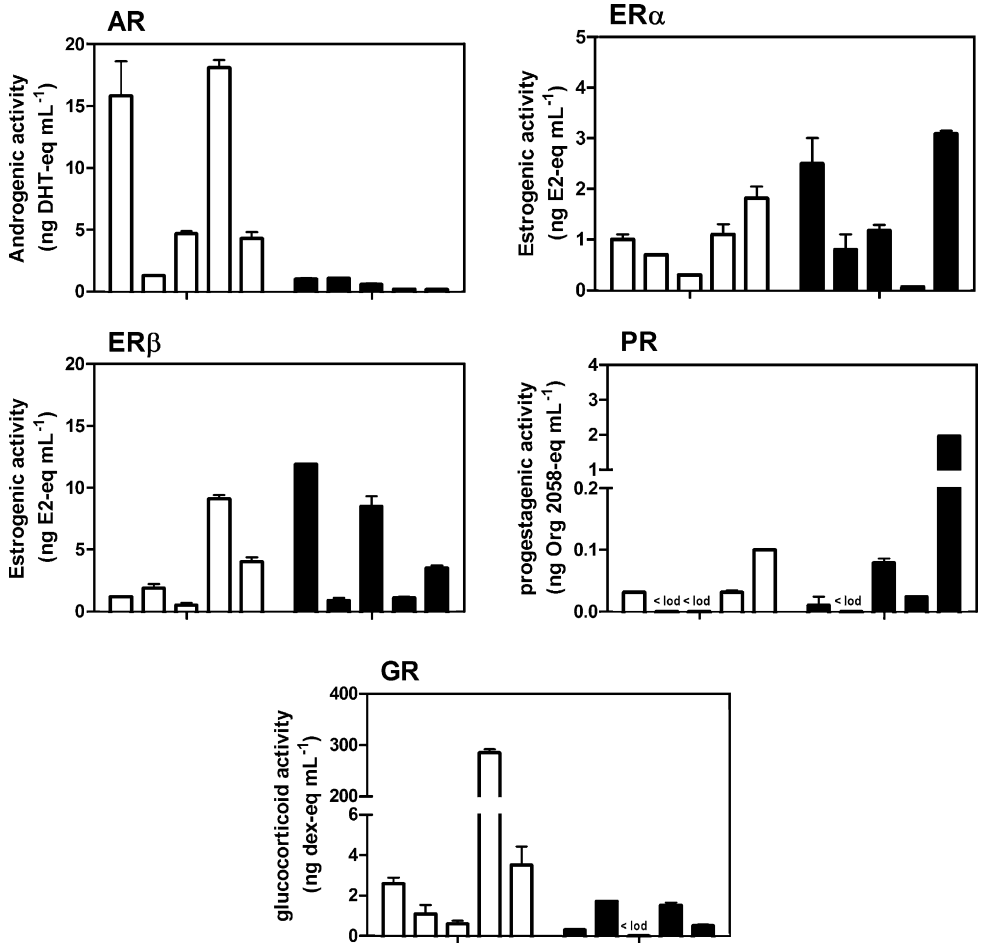

Fig. 4 – Androgenic, estrogenic, progestagenic and glucocorticoid activities in urine extracts of male and female healthy volunteers, analysed with AR, ERα ERβ, PR and GR reporter gene assays. Open bars: male urine samples; closed bars: female urine samples. lod = limit of detection.

that were reconverted to active steroids during the deconjugation step of the sample preparation. In the case of volunteer m4 and f5, prescribed steroids probably strongly contributed to the observed activities. The high progestagenic activity in the sample of volunteer f5 could be explained by a progestagen containing contraceptive pill taken by this volunteer. The remarkably high activity in the sample of volunteer m4 was probably due to the fact that this volunteer was medically treated with the synthetic glucocorticoid prednisone. In summary, reporter gene assays were successfully applied to determine endogenous levels of steroidal activities in human urine samples. Almost all samples showed activities several orders higher than the limit of detection.

3.4. *Androgenic activity in urine samples spiked with AAS*

The capability of the AR reporter gene assay to discriminate between AAS "positive" and "negative" urine samples was investigated by analysing urine samples spiked with AAS. The question was if the presence of AAS in urine would elevate androgenic activities above endogenous levels. Urine samples were spiked with different concentrations of THG or nandrolone and subsequently deconjugated, extracted and tested in the AR reporter gene assay (Fig. 5). The presence of THG or nandrolone elevated the androgenic activity in the sample in a concentration-dependent manner. The increase of androgenic activity compared with that in unspiked samples was statistically significant for both compounds and all concentrations tested ($p < 0.05$). This shows that the AR reporter gene assay can detect the presence of higher than endogenous levels of androgenic activity in urine samples, indicating the possible presence of AAS. The detectability of exogenous androgenic compounds is determined by their concentrations and their androgenic potencies (REPs). This implies that potent androgens at effective concentrations (such as the ones used in the spiking experiment) can be readily detected whereas less potent compounds in low concentrations are more difficult to detect. Also the background level of (endogenous) androgenic activity influences the chance to positively identify positive samples. Therefore, ideally a threshold level of endogenous androgenic activity, perhaps even individually assessed for each athlete, would be set above which a sample would be suspect for containing AAS.

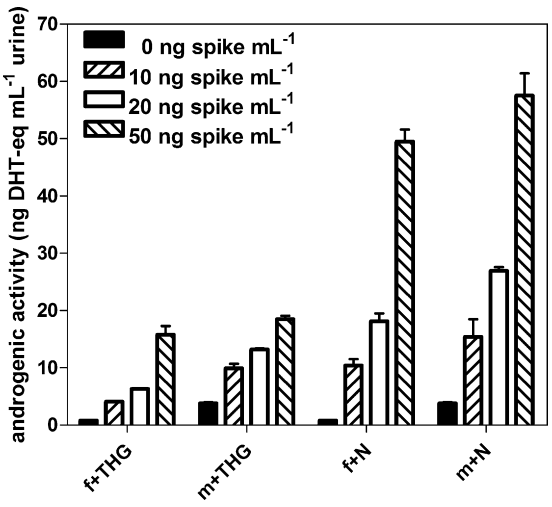

Fig. 5 – Androgenic activity (expressed as dihydrotestosterone (DHT)-equivalents) in pooled urine samples of female and male volunteers analysed in the AR reporter gene assay. Spiking of the samples with the anabolic androgenic steroids tetrahydrogestrinone (THG) or nandrolone (N) caused a concentration-dependent increase in the observed androgenic activity. This increase was significant ($p < 0.05$) in all cases. F = females; m = males.

3.5. *Detection of androgenic compounds using chemical and bioassay analysis*

Combining chemical analysis and identification of AAS with bioassay analysis of androgenic activity would provide a tight system for doping control able to detect compounds with known as well as unknown structures. As an example of how chemical and bioassay analysis could be combined, 10 urine samples from athletes competing at high level sports were firstly analysed by GC–MS for the main endogenous androgens present in human urine (testosterone, DHT, epi-testosterone, epi-DHT, androsterone and etiocholanolone). This set of human urine samples contains both male and female urines.

To be able to directly compare the GC–MS derived individual concentrations of the 6 androgens within the urine extracts with the total androgenic activity measured with the AR reporter gene assay in the same urine extracts, we make use of a well accepted method in the dioxin analysis field to express the toxic potency of mixtures of dioxins and dioxin-like compounds such as furans and PCBs. So-called WHO-TEF values are used, which are toxic equivalent factors for dioxin, furan and dioxin-like PCB congeners, derived from both *in vivo* and *in vitro* studies [40]. The WHO-TEF values for these congeners are highly comparable with the relative potencies (REPs) of individual dioxin and furan congeners to activate the aryl hydrocarbon receptor pathway as measured with the DR CALUX bioassay [41]. In line with this, HRGC–MS-derived concentrations of individual congeners in a certain matrix are multiplied by their corresponding TEF values, and the sum of these HRGC–MS-TEQs (which are in WHO-TEQs) can be compared directly with the DR CALUX TEQs.

To make direct comparison of GC–MS analysis with the AR reporter gene assay possible, the GC–MS concentrations of the 6 individual androgens analysed were multiplied by their corresponding relative potency (REP) to activate the androgen receptor (REP testosterone: 0.2135; REPs epi-testosterone and etiocholanolone: 0.0000; REP DHT: 1; REP epi-DHT: 0.0006; REP androsterone: 0.0056) resulting in so-called DHT equivalents. The sum DHT equivalents of the 6 individual androgens (the total GC–MS DHT equivalents, calculated according to formulae (6) and (8)) were compared with the corresponding DHT equivalents directly measured with the AR reporter gene assay. Measured concentrations of testosterone, DHT, epi-DHT and androsterone, together with associated calculated and measured androgenic activities are given in Table 2. Due to their low REPs, measured concentrations of etiocholanolone and epitestosterone were too low to contribute significantly to the androgenic activity. The analysis results for these compounds are provided in a more extensive version of Table 2 of Supplementary Data section (as Table II).

Table 2 – Levels of endogenous androgenic steroids in urine samples measured with GC–MS and associated calculated and measured androgenic activities

Sample	T (REP = 0.2135) (ng mL^{-1})	DHT (REP = 1) (ng mL^{-1})	epi-DHT (REP = 0.0006) (ng mL^{-1})	Androsterone (REP = 0.0056) (ng mL^{-1})	Total GC–MS (ng DHT-eq mL^{-1})	AR CALUX (ng DHT-eq Ml^{-1})
1-f	0.59	0.28	0.49	7.5	0.45	0.25
2-f	2.7	0.58	0.89	8.1	1.2	0.71
3-f	7.6	1.4	<0.05	61.2	3.4	2.9
4-f	4.7	4	18.3	67.4	5.4	3.5
5-f	3.2	1.50	2.0	50.6	2.4	1.7
6-m	49.5	15.0	11.1	383	27.7	26.1
7-m	61.1	11.3	7.8	249	25.7	18.7
8-m	28.2	4.7	5.1	20.9	10.8	9.2
9-m	41.5	3.5	<0.05	9.2	12.4	11.8
10-m	65.4	6.1	11.9	363	22.1	18.6

An extended version of this table also showing concentrations of etiocholanolone and epi-testosterone determined by GC–MS, and androgenic activities determined by the AR reporter gene assay is provided in Supplementary Data section. *Abbreviations*: T: testosterone, DHT: dihydrotestosterone, REP: relative potency; eq.: equivalents; f: female, m: male.

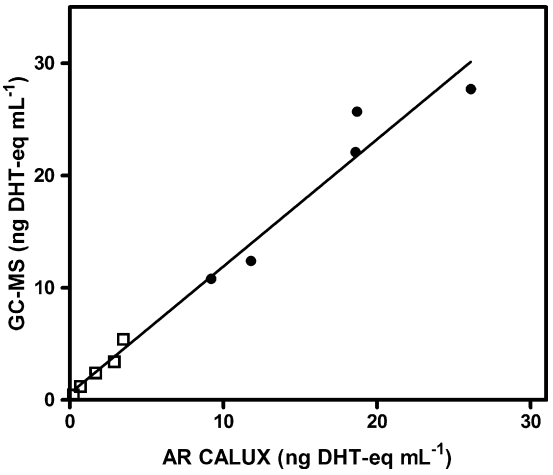

Fig. 6 – Correlation between androgenic activities measured in the AR reporter gene assay and activities calculated using GC–MS determined concentrations of endogenous androgens (data derived from Table 2; 10 urine samples tested; $y = 1.13x + 0.56$; $r^2 = 0.97$; $p < 0.0001$). Female urine samples (shown in open squares) deviate significant from male urine samples (closed circles) for both types of analyses.

As shown in Table 2 and Fig. 6, the GC–MS DHT equivalents correspond very well with the AR reporter gene assay equivalents ($y = 1.13x + 0.56$; $r^2 = 0.97$; $p < 0.0001$), with significant higher active androgens in male urine samples (9.2–26.1 ng DHT eq mL^{-1} in the AR reporter gene assay versus 10.8–27.7 ng DHT eq mL^{-1} in GC–MS) than female urine samples (0.25–3.5 ng DHT eq mL^{-1} in the AR reporter gene assay versus 0.45–5.4 ng DHT eq mL^{-1} in GC–MS). The high correspondence between GC–MS DHT-equivalents and AR reporter gene assay equivalents indicates that the measured activity is explained by the presence of endogenous steroids, with the highest contributions (in terms of GC–MS DHT equivalents) being due to testosterone and DHT. This shows that mammalian reporter gene assays can be used to determine normal values of bioactivities in samples, and even more important, can determine deviations from these normal values as shown by the spiking experiments shown in Fig. 5.

4. Conclusions

This study showed that mammalian reporter gene assays can measure the androgenic, estrogenic, progestagenic and glucocorticoid activities of (anabolic) steroids and their (bioactive) metabolites. Most of the tested compounds were active androgens in the AR reporter gene assay. As the AR reporter gene assay responds to all compounds able to activate the androgen receptor, this bioassay can be used for determining the presence of bioactive compounds with known as well as unknown chemical structures, such as designer steroids.

Endogenous steroidal activities in human urine samples were very sensitively measured with low limits of detection of, e.g. 0.02 ng DHT-eq mL^{-1} urine. Mixtures of AAS were shown to behave additively in the AR reporter gene assay, which can be used to predict the androgenic activity of a sample in the AR reporter gene assay that is caused by the androgenic compounds present.

Spiking of "doping negative" urine samples with AAS resulted in significantly higher androgenic activities in the AR reporter gene assay. The experiments indicate that CALUX reporter gene assays, especially the AR reporter gene assay, are promising tools for the detection of AAS in doping control. They can be applied to characterise steroidal activities of new compounds, and to screen urine samples for androgenic activities. Furthermore, combining the strengths of chemical analytical techniques (i.e. power of identification) with that of bioassays (detection based on biological activity) will, as earlier shown for the yeast bioassay combined with GC–MS [4], provide a tight control system able to detect compounds with known as well as unknown structures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2008.09.037.

REFERENCES

[1] World Anti-Doping Agency, 2005, Adverse Analytical Findings Reported by Accredited Laboratories, http://www.wada-ama.org/rtecontent/LABSTATS_2005.pdf and/LABSTATS_2004.pdf, 2006.
[2] F. Botrè, Toxicol. in Vitro 17 (2003) 509.
[3] W. Schaenzer, Clin. Chem. 42 (1996) 1001.
[4] M.W.F. Nielen, T.F.H. Bovee, M.C. van Engelen, P. Rutgers, A.R.M. Hamers, J.A. van Rijn, L.A.P. Hoogenboom, Anal. Chem. 78 (2006) 424.
[5] D.H. Catlin, M.H. Sekera, B.D. Ahrens, B. Starcevic, Y.C. Chang, C.K. Hatton, Rapid Commun. Mass Spectrom. 18 (2004) 1245.
[6] D.H. Catlin, B.D. Ahrens, Y. Kucherova, Rapid Commun. Mass Spectrom. 16 (2002) 1273.
[7] E. Marshall, Science 242 (1988) 183.
[8] M. Beato, P. Herrlich, G. Schutz, Cell 83 (1995) 851.
[9] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, Cell 83 (1995) 835.
[10] N.J. McKenna, B.W. O'Malley, Cell 108 (2002) 465.
[11] A. Brinkmann, G. Jenster, C. Ris-Stalpers, H. van der Korput, H. Bruggenwirth, A. Boehmer, A.J. Trapman, Steroids 61 (1996) 172.
[12] T. Sato, T. Matsumoto, T. Yamada, T. Watanabe, H. Kawano, S. Sato, Biochem. Biophys. Res. Commun. 300 (2003) 167.
[13] I. Thiblin, A. Petterson, Fundam. Clin. Pharmacol. 19 (2005) 27.
[14] L.G. Hershberger, E.G. Shipley, R.K. Meyer, Proc. Soc. Exp. Biol. Med. 83 (1953) 175.
[15] J. van der Vies, J. de Visser, Arzneim. -Forsch. 33 (1983) 231.

[16] X. Yuan, B.M. Forman, Nucl. Rec. Signal. 3 (2005) 1.
[17] K.W. Gaido, L.S. Leonard, S. Lovell, J.C. Gould, D. Babai, C.J. Portier, D.P. McDonnel, Toxicol. Appl. Pharmacol. 143 (1997) 205.
[18] P. Sohoni, J.P. Sumpter, J. Endocrinol. 158 (1998) 327.
[19] H.J. Lee, Y.S. Lee, H.B. Kwon, K. Lee, Toxicol. in Vitro 17 (2003) 237.
[20] L. McRobb, D.J. Handelsman, R. Kazlauskas, S. Wilkinson, M.D. McLeod, A.K. Heather, J. Steroid Biochem. Mol. Biol. 110 (2008) 39.
[21] B. Térouanne, B. Tahiri, V. Georget, C. Belon, N. Poujol, C. Avances, F. Orio Jr., P. Balaguer, C. Sultan, Mol. Cell. Endocrinol. 160 (2000) 39.
[22] B.M. Blankvoort, E.M. de Groene, A.P. van Meeteren-Kreikamp, R.F. Witkamp, R.J. Rodenburg, J.M. Aarts, Anal. Biochem. 298 (2001) 93.
[23] F. Paris, N. Servant, B. Térouanne, C. Sultan, Mol. Cell. Endocrinol. 198 (2002) 123.
[24] V.S. Wilson, K. Bobseine, C.R. Lambright, L.E. Gray Jr., Toxicol. Sci. 66 (2002) 69.
[25] J. Chen, M.R. Sowers, F.M. Moran, D.S. McConnell, N.A. Gee, G.A. Greendale, C. Whitehead, S.E. Kasim-Karakas, B.L. Lasley, J. Clin. Endocrinol. Metab. 91 (2006) 4387.
[26] P. Roy, S. Franks, M. Read, I.T. Huhtaniemi, J. Steroid Biochem. Mol. Biol. 101 (2006) 68.
[27] E. Sonneveld, J.H.J. Jansen, J.A.C. Riteco, A. Brouwer, B. van der Burg, Toxicol. Sci. 83 (2005) 136.
[28] E. Sonneveld, J.A.C. Riteco, J.H.J. Jansen, B. Pieterse, A. Brouwer, W.G. Schoonen, B. Van der Burg, Toxicol. Sci. 89 (2006) 173.
[29] M.E. Quaedackers, C.E. Van den Brink, S. Wissink, R.H.M.M. Schreurs, J.A. Gustafsson, P.T. Van der Saag, B. Van der Burg, Endocrinology 142 (2001) 1156.
[30] S.C. van der Linden, M.B. Heringa, H. Man, E. Sonneveld, L.M. Puijker, B. Brouwer, B. van der Burg, Environ. Sci. Technol. 42 (2008) 5814.
[31] World Anti-Doping Agency, The 2005 Prohibited List, http://www.wada-ama.org/rtecontent/document/list_2005.pdf, 2004.
[32] S. Loewe, H. Muischnek, Naunyn-Schmiedelbergs Archiv fuer Experimentelle Pathologie und Pharmakologie 114 (1926) 313.
[33] E. Silva, N. Rajapakse, A. Kortenkamp, Environ. Sci. Technol. 36 (2002) 1751.
[34] C.J. Houtman, Y.K. van Houten, P.E.G. Leonards, A. Brouwer, M.H. Lamoree, J. Legler, Environ. Sci. Technol. 40 (2006) 2455.
[35] H. Geyer, U. Mareck-Engelke, W. Schänzer, M. Donike, in: W. Schänzer, H. Geyer, U. Gotzmann, U. Mareck-Engelke (Eds.), Recent Advances in Doping Analysis (4), Sport und Buch Strauss, Koeln, 1997, p. 107.
[36] B. van der Burg, G.R. Rutteman, M.A. Blankenstein, S.W. de Laat, E.J.J. van Zoelen, J. Cell Physiol. 134 (1988) 101.
[37] A.K. Death, K.C. McGrath, R. Kazlauskas, D.J. Handelsman, J. Clin. Endocrinol. Metabol. 89 (2004) 2498.
[38] W.G. Schoonen, G. Deckers, M.E. De Gooijer, R. de Ries, G. Mathijssen-Mommers, H. Hamersma, H.J. Kloosterboer, J. Steroid Biochem. Mol. Biol. 74 (2000) 213.
[39] F. Hartgens, H. Kuipers, Sports Med. 34 (2004) 513.
[40] M. van den Berg, L.S. Birnbaum, M. Denison, M. De Vito, W. Farland, M. Feeley, H. Fiedler, H. Hakansson, A. Hanberg, L. Haws, M. Rose, S. Safe, D. Schrenk, C. Tohyama, A. Tritscher, J. Tuomisto, M. Tysklind, N. Walker, R.E. Peterson, Toxicol. Sci. 93 (2006) 223.
[41] P.A. Behnisch, K. Hosoe, S. Sakai, Environ. Int. 29 (2003) 861.