Article

Bioanalytical Detection of Steroid Abuse in Sports Based on the Androgenic Activity Measurement

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Abstract: The anabolic androgenic steroids (AAS) are the most frequently consumed performance enhancing drugs (PED) in sports. In the anti-doping field, the detection of AAS is carried out by the analysis of the athlete's urine using methodologies based on liquid/gas chromatography-mass spectrometry. Unfortunately, the detection of unknown compounds is not possible. BDS's AR CALUX® bio detection technology was studied as an indirect method to detect the administration of a single dose of testosterone (T). Twelve T and placebo single dose administered men volunteers underwent a triple-blind crossover clinical trial. The UGT2B17 deletion was present among the volunteers and evenly distributed in heterozygous (ins/del), wild-type homozygous (ins/ins), and mutated homozygous (del/del) groups. A significant statistical difference in terms of bioluminescence was observed after the testosterone (T) administration for the three types of polymorphic groups. The ratio of means between the pre- and post-T administration periods, depending on the type of polymorphism, was in group ins/ins 3.31 (CI. 95%: 2.07–5.29), group ins/del 4.15 (CI 95%: 3.05–5.67), and group del/del 2.89 (CI 95%: 2.42–3.46). The results of the study are very promising, as they may offer us the possibility of designing a detection approach that, based on intra-individual monitoring of androgenic values, in the UGT2B17 deletion type.

Keywords: doping; UGT2B17 genotype; steroid profile; testosterone administration; sport; athlete biological passport; AR CALUX®; androgenic activity

1. Introduction

Sport plays an important role in society due to the values of effort, dedication, and excellence that it transmits. However, it is very often tainted by the fraudulent use of doping substances to enhance athletic performance and to perform under the pressures of success. This disrupts fair play and constitutes a serious health risk for athletes [1,2].

Today, steroids are the substances most widely detected by WADA accredited laboratories [3]. The use of AAS in sports began in 1950 [4]. At present, approaches for the detection of steroid abuse in the Anti-Doping field are standardized by the World Anti-Doping Agency through technical documents and guidelines [5–7].

The methods routinely used in the WADA accredited laboratories for steroid screening mainly focus on substances that are excreted as parent compounds or their metabolites, conjugated as glucuronides or sulphates. Common procedures of urine analysis include deconjugation using the β-glucuronidase enzyme, followed by extraction and concentration. Finally, the target analytes are submitted to chromatography mass spectrometry analysis, generally tandem MS–MS [8,9]. However, steroids of unknown composition will not be detected by these target testing procedures [10].

In the fight against doping in sport, the detection of testosterone administration is based on the quantification of a series of testosterone metabolites and their integration into the biological passport of the athlete to carry out a longitudinal assessment of the variations [11,12].

One of the factors that most affects the athlete's steroid profile is the genetic deletion of the UGT2B17 gene, with a prevalence of 9% in Caucasian populations and 67% in Asian populations [13,14]. This makes it difficult to detect T-doping, especially when the steroid module of the athlete's biological passport (ABP) is in the first stage [2,15–20].

Several studies demonstrated that one of the most impacting factors on steroid profiling is genetic deletion of the UGT2B17 gene that alters the glucuronidation of several T target metabolites, and consequently, the urinary excretion of such compounds is significantly reduced compared to the rest of the non-deleted sport population. Consequently, the detection of T misuse becomes more difficult [14,15], a conclusion that was ratified in other studies [16,17]. T administration detection is more complicated when the steroid module of the athlete's biological passport (ABP) is in the early days of athlete data processing [18–20].

Studies started in 2005 by Edwin Sonneveld et al., through assays with CALUX cell lines (Chemically Activated LUciferase eXpression) and specifically for androgens (AR CALUX)®, have shown a great potential for the detection of the abuse of steroids in urine, even without knowing their chemical structure, through androgen receptors. This gives it a competitive advantage over current chemical–analytical approaches that present difficulties in detecting compounds with unknown structures, such as designer steroids [21–24].

Previous studies have examined in detail the efficacy of the AR-CALUX® bioassay to detect the abuse of T and steroids [25–27]. The accuracy of AR CALUX® bioassay was evaluated by comparing the androgen measurement against the measurement of T by analytical methods in urine samples and by detailed characterization of the ability of AR CALUX® bioassay to detect steroids and long-term steroid metabolites monitored in urine by Anti-Doping laboratories.

The objective of this study was to evaluate the efficacy of the AR CALUX® bioassay to detect the administration of a single dose of 250 mg of testosterone cypionate through a triple-blind randomized trial and athletes with genetic deletion of UGT2B17. This research allows us to observe the performance of the methodology and to know if it is also useful in athletes with this genetic deletion.

2. Materials and Methods

2.1. Subjects

The study followed a triple-blind randomized placebo-controlled crossover design with 12 volunteers. The athletes who agreed to take part were informed in detail about the protocol to be followed, were submitted to a clinical evaluation, if they are suitable fit to undertake the study, and were requested to sign the informed consent form.

The study was approved by the Ethics Committee of the Hospital Clínico San Carlos and the Spanish Agency for Medicine and Health Products (AEMPS) and conducted according to the Helsinki Declaration. This trial study was registered in the European Clinical Trials Database (EudraCT) with the number 2013-005135-24. Details of the trial's design were previously published [17].

The mean age values of the twelve men athletes included in the study was 38.7 years ($\pm$10.9), the age distribution among the three evenly distributed UGT2B17 deletion groups was: $\bar{x}$:42.25 ($\pm$9.77 SD) ins/ins, $\bar{x}$:32 ($\pm$10.26 SD) ins/del, and $\bar{x}$:45.5 ($\pm$11.26 SD) del/del.

The volunteers who participated in the study were amateur athletes who trained for about two hours a day at least five days a week; none were included in the population of athletes who undergo doping controls in Spain.

2.2. Samples Collected

Initially 24 samples from each volunteer were planned to be collected, 10 of them from the first period (coded C1, placebo administration), another 10 after T administration (coded C2), and finally, four more samples from the period C3, that included samples collected after 15, 20, 25, and 30 days of the T administration, so in total 288 samples were expected to be collected. All the samples had to be collected daily in the early morning just after the volunteer woke up. The final number of samples collected was 282, because in six cases the sample needed to be rejected for insufficient volume.

For the androgen activity measurement, 5 mL aliquots from each sample were prepared. Once the aliquots were packed, they were shipped under refrigeration and temperature-controlled transport to the reference BioDetection Systems BV Laboratory, Science Park 406, 1098XH Amsterdam, The Netherlands.

The androgenic signal level measured in the samples was not corrected according to the urine specific gravity or creatine concentration. The mean specific gravity of the samples was 1.022 ($\pm$0.005), and the specific gravity for each UGT2B17 deletion group was: 1.023 ($\pm$0.005) ins/ins, 1.022 ($\pm$0.005), ins/del, and 1.020 ($\pm$0.006) del/del.

Given the fact that the compounds under study, even being conjugated with glucuronic acid in its sulphated form, have androgenic activity, although reduced, it was planned to measure the androgenic activity modification before and after the T administration with and without the hydrolysis. Avoiding hydrolysis may be simple in the future with the development of a quick screening analysis method to apply "in situ" during sample collection sessions.

2.3. Sample Preparation

Prior to the androgenic activity measurement, it was necessary to carry out a sample preparation process that included acid hydrolysis, liquid–liquid phase extraction with terbutyl methyl ether (MTBE), evaporation, and resuspension of the final extract in dimethyl sulfoxide (DMSO). In summary, the process starts with the thawing of the urine samples at room temperature and vortexing until completely resuspended. Each sample was split into two aliquots of 2.5 mL. One of the aliquots was submitted to acid hydrolysis, the other was reserved. For hydrolysis, 4 mL 0.25 M HCl (0.25 M in methanol containing 8% water) were added and swirled carefully, followed by incubation of the tubes for one hour at 40 °C (heated water bath) under gentle shaking. The mild acidic pre-treatment conditions used similar to [28–31] resulted in deconjugation of both androgen sulphates and androgen glucuronides that are present in human urine.

After hydrolysis, the tubes were allowed to rest until they reached room temperature. Then together with the non-hydrolyzed tubes, the extraction was continued by liquid phase. Eight ml of MTBE were added, followed by vortexing for at least two minutes, and then the organic phase was transferred to a new test tube. This process was repeated two more times. The total of 24 mL of MBTE was evaporated under a gentle stream of nitrogen at a maximum temperature of 45 °C until approximately 0.5 mL was left. The 0.5 mL were transferred to a conical vial and further evaporated to dryness. The tubes were rinsed twice with a small amount of MBTE, transferred to the conical vial and evaporated to dryness, and the final residue was dissolved in 25 µL of DMSO [7,16,17].

2.4. AR CALUX®

The AR CALUX® bio-analyses were performed using human U2-OS cell lines stably transfected with an androgen-controlled luciferase reporter gene construct, see Figure 1 [21–28]. AR CALUX® cells that are exposed to compound that bind to the androgen receptor (AR) will result in induction (agonist) or suppression (antagonist) of AR-based gene expression.

In the AR-CALUX® cells, the compound-AR complex binds to AR-specific response elements (RE), which are incorporated in the upstream regulatory regions of the firefly gene luciferase. By addition of the appropriate substrate for the expressed luciferase, light is emitted. The amount of light produced is proportional to the amount of ligand-specific receptor binding, which is benchmarked against the relevant reference compound, dihydrotestosterone (DHT). AR CALUX® cells were cultured in a DMEM/F12-medium (with phenol red) supplemented with 7.5% FCS, non-essential amino-acids, antibiotics (penicillin, streptomycin), and G418 under standard conditions (37 °C, 5% CO_2, 100% humidity). For exposure, AR CALUX® cells were plated in 96-well plates (10,000 cells/well) in DMEM/F12 medium (without phenol red) supplemented with 5% dextran-coated charcoal-stripped FCS (DCC-FCS), non-essential amino-acids and antibiotics (penicillin, streptomycin) at a volume of 100 μL per well. After 24 h of incubation, the medium was refreshed, and cells were exposed to serial dilution of the final extracts in DMSO and procedure blanks in triplicate (0.1% DMSO). Each 96-well microtiter plate also contained the standard dihydrotestosterone (DHT) calibration range. After 24 h, the medium was removed, and cells were lysed in 30 μL Triton-lysis buffer and measured for luciferase activity using a luminometer. Validity of analysis results was checked (R2 calibration series >0.98; z-factor calibration series >0.6; EC50 reference compound between present limits; SD triplicate analysis <15%; recovery reference compounds >75%), after which sample analysis results were interpolated in the DHT calibration curves and expressed in ng DHT equivalents per ml of urine (EEQ: ng DHT eq./mL urine). AR CALUX® bioassay has been extensively validated [23–25] and has been approved by the OECD [24,25] as an official method for determination of natural, synthetic, and environmental (anti)androgens and compounds with AR-like activity. The steroid activity of compound and its relationship with luciferase activity is widely documented in different articles [26].
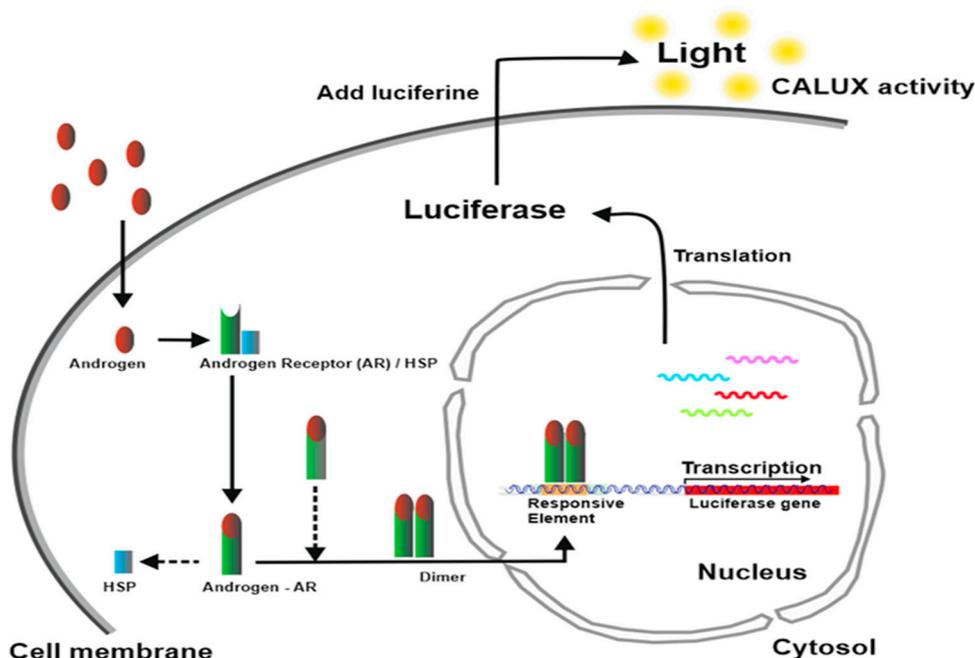
Figure 1. Diagram of the AR CALUX® bio-analyses performed using human U2-OS cell lines stably transfected with an androgen-controlled luciferase reporter gene construct.

2.5. Statistical Analysis

Qualitative variables are provided with their frequency distributions. Quantitative variables are expressed as their mean and standard deviation (SD) and variables not showing a normal distribution as medians and interquartile ranges (IQR = P25–P75).

The AR-dependent induction of luciferase activity in AR CALUX® cells was selected as the dependent variable. The luciferase enzymatic activity was measured in a luminometer and expressed in relative light units (RLUs). The fold induction of luciferase activity was calculated by dividing the mean value of RLUs obtained when cells were exposed to the model substrate, dihydrotestosterone (DHT), and the mean value of RLUs generated when the cells were exposed to the solvent control sample. The luciferase induction was expressed as percentage of the maximal response induced by DHT in the AR CALUX® cells. Polymorphism and the testosterone (T) administration were used as independent variables in this study.

The effect of administration related with genotype was analyzed with multiple linear regression models through generalized estimating equations (GEE). The results obtained from the same individuals were grouped into clusters, the responses of which represent an intra-group correlation, but are independent of the distinct groups. As the dependent variables (androgenic activity) were not normally distributed, the data were log-transformed. To present the results the model coefficients and its 95% confidence intervals are displayed in original scale by inverse transformation and interpreted as the ratio of means between polymorphism groups in period C1 and also between post-pre administration values. To assess whether the increase in androgenic activity after T administration differs between the polymorphic groups the interaction term was introduced in the model.

A *p*-value of 5% was accepted for all tests. Processing and data analysis were performed using the STATA 15.0 statistical package.

3. Results

As indicated above, 12 individuals participated in the study, but due to the inconsistency and dispersion of the results compared to the rest, it was found that most of the samples of individual number 01 were seriously affected by degradation; therefore, they were excluded. Therefore, finally, the total number of samples for the conjugated fraction was 258. In the case of the samples that were not submitted to hydrolysis, due to the fact that the androgenic response is always much weaker, six samples from two individuals had to be discarded due to the practical absence of signal; thus, for the non-hydrolyzed fraction, the total number of samples available was 252.

Table 1 shows the descriptive analysis of the medians and the Inter quartile range (IQR) of the androgenic response (DHT equivalent), with and without hydrolysis, for the samples collected during the periods C1, C2, and C3.

According to the results, the administration of T, independently of the polymorphic group to which the volunteer belonged, increased the androgenic response; this effect was observed with or without hydrolysis. Performing hydrolysis results in deconjugation of the steroids and therefore permits increasing the signal, because more active steroids are generated, leading to more efficient interaction so that more activity can be detected. For example, in the case of the (ins/ins) group, the median value of the androgenic response went from 0.2 to 0.6 without hydrolysis and from 0.9 to 3.2 with hydrolysis, during the periods C1 and C2, respectively (see Table 1).

Figure 2 shows the descriptive distribution of androgen activity expressed in DHT equivalents measured by AR CALUX® for each polymorphic population with and without hydrolysis during period C1.

Table 1. Descriptive analysis of the number of urine samples analyzed by AR CALUX®, including median and the interquartile range (IQR) of the androgenic response (DHT equivalent), with and without hydrolysis, for samples collected during the periods C1, C2, and C3.

|			c1	c2	c3
With Hydrolysis	ins/ins	n	30	30	10
		median	0.9	3.2	1.0
		IQR	0.6–1.5	2.3–4.4	0.4–1.7
	ins/del	n	40	40	13
		median	0.8	5.4	1.3
		IQR	0.5–2.6	3.2–10	1.3–2.0
	del/del	n	40	39	16
		median	0.6	2.1	0.7
		IQR	0.4–1.5	1.5–2.9	0.4–1.0
Without Hydrolysis	ins/ins	n	27	30	10
		Median	0.2	0.6	0.2
		IQR	0.1–0.4	0.5–1.3	0.2–0.5
	ins/del	n	40	40	13
		Median	0.3	0.8	0.3
		IQR	0.1–1.7	0.3–1.8	0.1–0.8
	del/del	n	37	39	16
		Median	0.1	0.4	0.2
		IQR	0.1–0.2	0.3–0.6	0.1–0.3
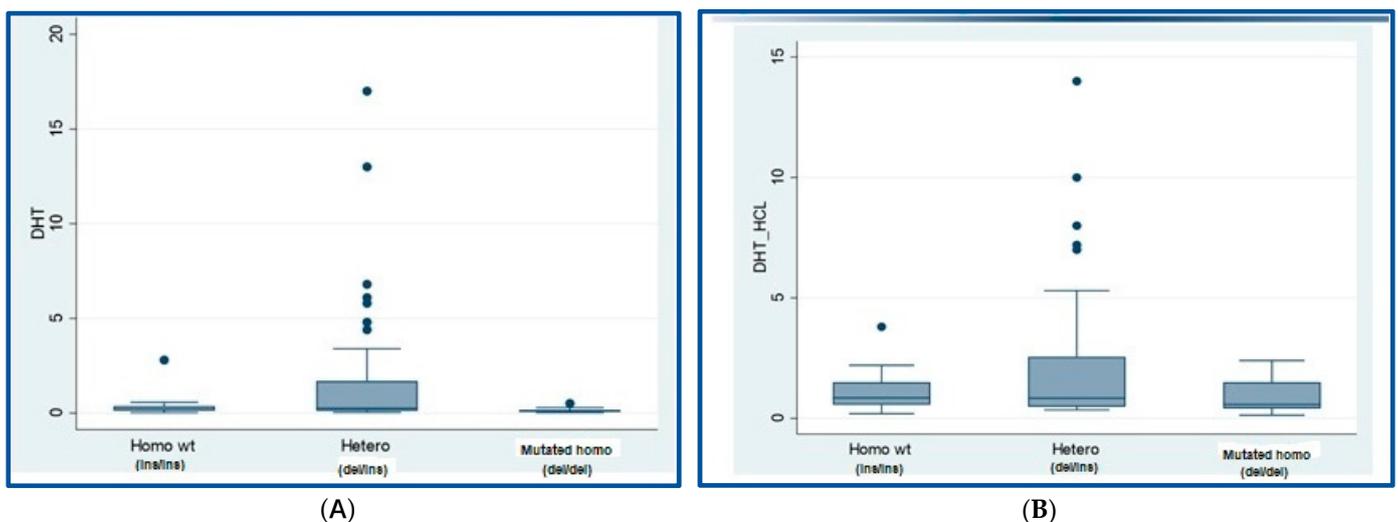
Figure 2. Distribution of the androgenic activity expressed in DHT equivalents in urine measured by AR CALUX® for each polymorphic population (**A**) without and (**B**) with hydrolysis. In all cases, the data correspond to the period C1.

Table 2 shows the androgen responses in urine, by AR CALUX®, with and without hydrolysis, and expressed as the values of ratio of means. For the interpretation of the ratio of means, the homozygous polymorphism wild type (ins/ins) group was taken as the reference compared to the other two groups, heterozygous (ins/del) and homozygous mutated (del/del).

According to the results (Table 2), there were no statistically significant differences in DHT values in period C1 among the three groups of genetic polymorphisms.

Again, the fact of applying a hydrolysis step allows increasing the signal independently of the polymorphic group. In general, a greater dispersion of the equivalent values of DHT was observed when hydrolysis was applied.

Table 2. Androgenic responses in urine, analyzed by AR CALUX® with and without hydrolysis presented as values of the ratio of means. For the interpretation of the ratios of means, the homozygous polymorphism wild type (ins/ins) group has been taken as the reference compared to the other two groups, heterozygous (ins/del) and homozygous mutated (del/del). For the comparison, the ratio of means, the inter quartile range, and the *p* value are presented.

With Hydrolysis	Ratio of Means	Inter Quartile 95% Range		*p*
(ins/del) vs. (ins/ins)	1.37	0.44	4.28	0.578
(del/del) vs. (ins/ins)	0.79	0.26	2.47	0.690
Without Hydrolysis				
(ins/del) vs. (ins/ins)	2.21	0.41	12.03	0.355
(del/del) vs. (ins/ins)	0.53	0.10	2.89	0.464

To evaluate the impact of T administration in each of the three polymorphic groups the ratio of means between pre-T (C1) and post-T (C2) administration was calculated. Table 3 shows that there was a significant increase in DHT values after T administration (both with and without HCl), the ratio of means increased significantly after T administration. When we evaluated the interaction, with and without HCl, to assess whether the increase in DHT values that occurred after T administration was independent of the polymorphic group, we observed that with HCl hydrolysis, there were no significant differences in signal increase among the three polymorphisms (p = 0.2504). However, without HCl, the interaction term was statistically significant (p = 0.0019). Differences were found in the pairwise comparison between groups (ins/ins) vs. (ins/del), p = 0.003 and (ins/del) vs. (del/del), p = 0.002.

Table 3. Androgenic activity in urine of athletes measured by AR CALUX® with and without hydrolysis for each of the three different types of genotypes expressed as the values of the ratio of means between period C1 and C2 (pre and post T adm.), the inter quartile range, and the *p* value.

		Ratio of Means	Inter Quartile Range C 95%		*p*
	ins/ins	3.31	2.07	5.29	<0.001
With Hydrolysis	ins/del	4.15	3.05	5.67	<0.001
	del/del	2.89	2.42	3.46	<0.001
	ins/ins	4.10	2.96	5.67	<0.001
Without Hydrolysis	ins/del	2.02	1.43	2.84	<0.001
	del/del	3.95	3.01	5.19	<0.001

Figure 3 shows the mean and standard deviation for the increase in androgenic activity (ng DHT-eq mL^{-1}) pre- and post-T administration to the volunteers, (A) without hydrolysis and (B) with hydrolysis. According to the results, it was noted that the T administration generated an important increase in androgenic activity, especially during the first 10 days post-administration, in comparison with the basal values (placebo). The effect was more evident when hydrolysis was used, in any case after 10 days the effect of a T single dose administration was no longer detectable.
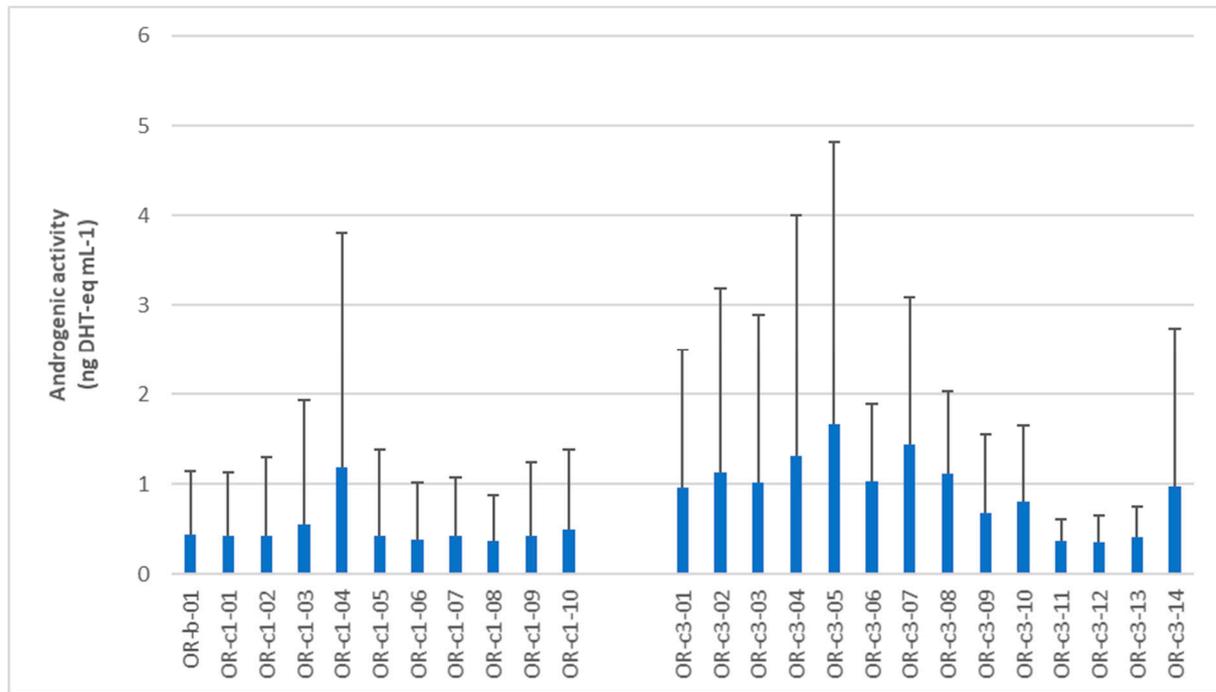
(**A**)
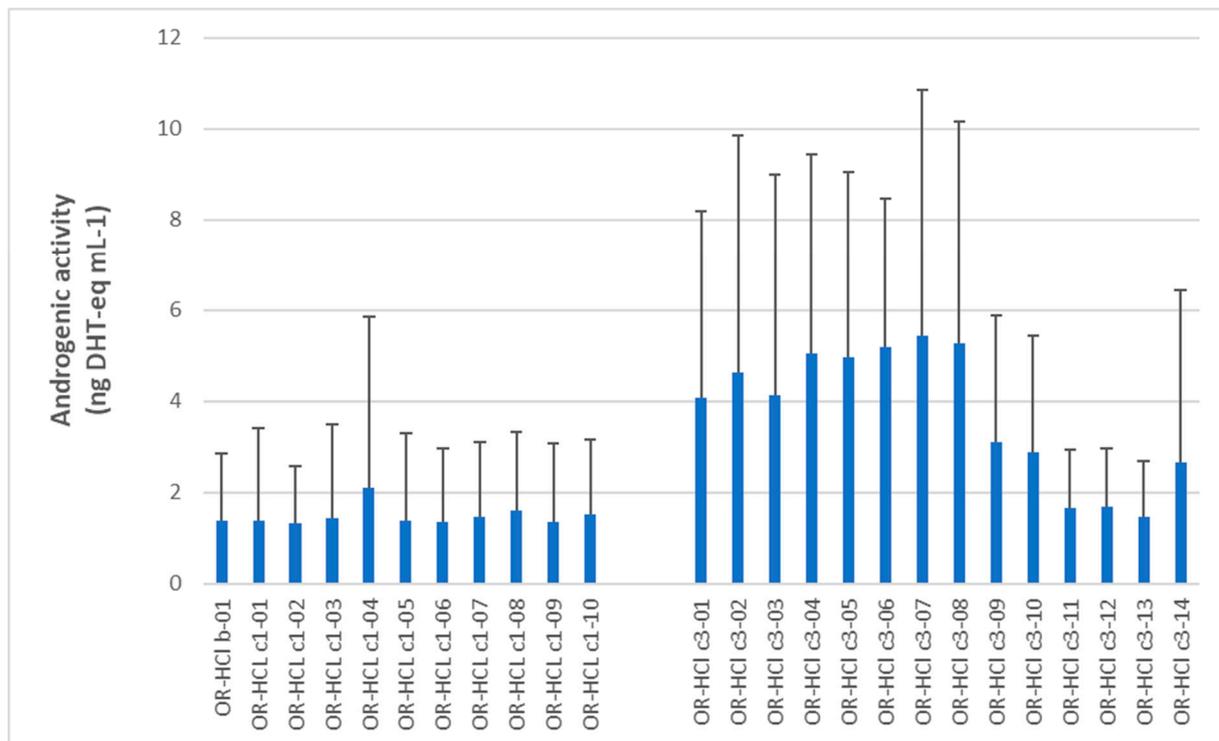
(**B**)

Figure 3. Profile of the increase of androgenic activity (ng DHT-eq mL^{-1}), mean, and standard deviation pre- and post-T administration shown for the 11 volunteers (**A**) without hydrolysis and (**B**) with hydrolysis.

4. Discussion

Steroids are the most commonly used doping substances among professional and amateur athletes [3]. Their use has dramatically increased in recent years [27,31]. However,

the use of steroids is hazardous, and can lead to serious health problems for the professional or amateur athlete [29,31].

Detection in doping control laboratories has been based mainly on the testosterone/epitestosterone ratio (T/E) >4 in urine and the presence of other metabolites [28,29]. Nevertheless, in recent years it has been shown that in people who have a genetic deletion of the UGT2B17 gene, which modifies glucuronidation, commonly present a T/E <2, it could allow them to use micro dose steroids and avoid detection [28,32].

In contrast to common procedure among anti-doping laboratories, *E. coli* ß glucuronidase hydrolyzed with dilute HCl was applied in order to deconjugate the sulphate metabolites together with glucuronide forms [30,31].

At present, additional approaches and markers are still required to detect exogenous testosterone administration, especially in sports populations with UGT2B17 mutations, where their determination at low doses is more difficult.

On the other hand, with the intention of strengthening the steroidal module of ABP, through this project the hypothesis was proposed that longitudinal monitoring of androgenic activity in urine could obtain complementary and additional information [19].

Given our results, a great potential for using the androgen receptor as an indirect way of detecting abuse of steroids in urine can be confirmed. Conducting bioluminescence assays applied to modified mammalian cell lines with the receptor promises to be one of the cornerstones of detecting the substances in urine. This detection method could be utilized for any type of steroid, dosage, and route of administration [10,24–26,33–36].

Bioassays have an advantage compared to mass spectrometry, since they allow the detection of any compound that includes new substances that interact with AR, regardless of the chemical structure, route of administration, and dispensation [25,37–39].

These data support the fact that androgen receptor (AR) assays are useful as a complement to the doping detection as an indirect marker of any substance with an androgenic character, and in this specific case, improve the detection of T administration based on the measurement of the T/E ratio and steroid profile, which is highly influenced by the polymorphism of the UGT2B17 gene [13,14,28,40].

As BDS' CALUX® bioassays also respond to endogenous hormones, we envisage determination of normal values within a specific population or sub-population followed by screening for deviations from these normal values. A system consisting of screening for unknown compounds using effect-based CALUX® bioassays in conjunction with more specific sensitive and identifying chemical-analytical methods such as GC-MS is expected to provide a robust protocol that can be useful for detecting the use of almost any chemical compound that interferes with normal steroid hormone action [33].

The comparison of AR CALUX® bioassay activities with the main endogenous steroids detected with GC-MS in human urine samples showed an excellent correlation, providing proof of principle of the compatibility of both techniques [33], as has been observed in previous experiments [21].

These results support the fact that the AR CALUX® bioassay, in addition to chemical–analytical methods, can be a valuable tool for the analysis of steroids for doping control purposes [27].

5. Conclusions

The results obtained allow us to conclude that the AR CALUX® bioassay analysis was able to detect the exogenous administration of testosterone independently of the genotype for the three types of polymorphism. A statistically significant change in bioluminescence induced by anabolic androgenic steroids ($p < 0.001$) was observed after T intramuscular administration.

The analysis method developed based on AR CALUX® bioassay allows measurement of the variations of the androgenic signal in a reproducible and consistent way without the need to carry out hydrolysis, although the signal without hydrolysis is always significantly

weaker, which can facilitate the development of a simple and fast screening method for the future.

With the results obtained, it is not possible to define a global cut-off value that allows deducing that with a single measure there could have been administration of some compound with androgenic activity, the method is effective with a longitudinal follow-up of each individual.

Unlike what happens with the routine methods of analysis in anti-doping that are based on the structure of the analytes, the developed method measures an effect and therefore makes it capable of detecting the possible administration of compounds with an unknown structure, this can be used for the development of rapid screening methods not only applicable to physiological samples, but also to the possible analysis of nutritional supplements.

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Institutional Review Board Statement: The study was approved by the Ethics Committee of the Hospital Clínico San Carlos, Madrid (Spain) and the Spanish Agency for Medicine and Health Products (AEMPS) and conducted according to the Helsinki Declaration. This trial study was registered in the European Clinical Trials Database (EudraCT) with the number 2013-005135-24.

Informed Consent Statement: In this study Informed consent was obtained from all subjects involved in the study, we are following the indications of Ethics Committee of the Hospital Clínico San Carlos, Madrid (Spain) and the Spanish Agency for Medicine and Health Products (AEMPS).

Data Availability Statement: The data obtained for this study were stored on the server where the SPORT MEDICINE database is hosted, in turn hosted on a server at the Complutense University of Madrid (Spain). This server is managed by a data protection officer from this University. The supervision and the purpose are the management is the correct storage of the results obtained by the medical examinations and investigations, the legitimation is a mission of public interest / essential; execution, the recipients will not transfer their data to third parties, patients have the right to rectify their data. Information on data protection can be found at www.ucm.es/file/info-adic-medicina-deporte, accessed on 26 March 2021.

Conflicts of Interest: The authors declare no conflict of interest.

References

1.　Lissavetzky, J. Química y deporte: La lucha contra el dopaje en el horizonte del siglo XXI. *Arbor* **2011**, *187*, 105–112. [CrossRef]
2.　Reardon, C.L.; Creado, S. Drug abuse in athletes. *Subst. Abus. Rehabil.* **2014**, *5*, 95–105. [CrossRef]
3.　Aguilar, M.; Muñoz-Guerra, J.; Plata, M.D.M.; Del Coso, J. Thirteen years of the fight against doping in figures. *Drug Test. Anal.* **2017**, *9*, 866–869. [CrossRef] [PubMed]
4.　Baron, D.; Martin, D.; Magd, S. El dopaje en el deporte y su propagación a las poblaciones en riesgo: Una revisión internacional. *Rev. Of. De La Asoc. Mund. De Psiquiatr. (WPA)* **2007**, *5*, 118–123.

5. WADA. Technical Document for the Harmonization of Analysis and Reporting of 19-Norsteroids Related to Nandrolone. WADA Technical Document—TD2019NA. March 2019. Available online: https://www.wada-ama.org/en/resources/science-medicine/td2019na-0. (accessed on 17 November 2020).

6. WADA. Detection of Synthetic Forms of Endogenous Anabolic Androgenic Steroids by GC/C/IRMS. 2019. Available online: https://www.wada-ama.org/sites/default/files/td2019irms_final_eng_clean.pdf. (accessed on 17 November 2020).

7. WADA. Endogenous Anabolic Androgenic Steroids: Measurement and Reporting. Technical Document TD2018EAAS. 2018. Available online: https://www.wada-ama.org/sites/default/files/resources/files/td2018eaas_final_eng.pdf (accessed on 17 November 2020).

8. Parr, M.K.; Schänzer, W. Detection of the misuse of steroids in doping control. *J. Steroid Biochem. Mol. Biol.* **2010**, *121*, 528–537. [CrossRef]

9. Thevis, M.; Walpurgis, K.; Thomas, A. Analytical Approaches in Human Sports Drug Testing Recent Advances, Challenges, and Solutions. *Anal. Chem.* **2019**, *92*, 506–523. [CrossRef] [PubMed]

10. Bailey, K.; Yazdi, T.; Masharani, U.; Tyrrell, B.; Butch, A.; Schaufele, F. Advantages and limitations of androgen receptor-based methods for detecting anabolic androgenic steroid abuse as performance enhancing drugs. *PLoS ONE* **2016**, *11*, e0151860. [CrossRef] [PubMed]

11. Mareck, U.; Geyer, H.; Opfermann, G.; Thevis, M.; Schänzer, W. Factors influencing the steroid profile in doping control analysis. *J. Mass Spectrom.* **2008**, *43*, 877–891. [CrossRef]

12. Baume, N.; Geyer, H.; Vouillamoz, M.; Grisdale, R.; Earl, M.; Aguilera, R.; Cowan, D.A.; Ericsson, M.; Gmeiner, G.; Kwiatkowska, D.; et al. Evaluation of longitudinal steroid profiles from male football players in UEFA competitions between 2008 and 2013. *Drug Test. Anal.* **2016**, *8*, 603–612. [CrossRef]

13. Jakobsson, J.; Ekström, L.; Inotsume, N.; Garle, M.; Lorentzon, M.; Ohlsson, C.; Roh, H.-K.; Carlström, K.; Rane, A. Large differences in testosterone excretion in Korean and Swedish men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 687–693. [CrossRef]

14. Ekström, L.; Cevenini, L.; Michelini, E.; Schulze, J.; Thörngren, J.-O.; Belanger, A.; Guillemette, C.; Garle, M.; Roda, A.; Rane, A. Testosterone challenge and androgen receptor activity in relation to UGT 2B17 genotypes. *Eur. J. Clin. Investig.* **2013**, *43*, 248–255. [CrossRef] [PubMed]

15. Strahm, E.; Mullen, J.E.; Ericsson, M.; Schulze, J.J.; Rane, A.; Gårevik, N.; Ekstrom, L. Dose-dependent testosterone sensitivity of the steroidal passport and GC-C-IRMS analysis in relation to the UGT2B17 deletion polymorphism. *Drug Test. Anal.* **2015**, *7*, 1063–1070. [CrossRef] [PubMed]

16. Martín-Escudero, P.; Muñoz-Guerra, J.; Del Prado, N.; Canales, M.G.; Ferrer, M.F.; Vargas, S.; Soldevilla, A.B.; Serrano-Garde, E.; Miguel-Tobal, F.; Casas, M.M.D.L.; et al. Impact of UGT 2B17 gene deletion on the steroid profile of an athlete. *Physiol. Rep.* **2015**, *3*, e12645. [CrossRef]

17. Martín-Escudero, P.; Muñoz-Guerra, J.A.; García-Tenorio, S.V.; Garde, E.S.; Soldevilla-Navarro, A.B.; Canales, M.G.; Prado, N.; Ferrer, M.E.F.; Pérez, C.F. Impact of the UGT2B17 polymorphism on the steroid profile. Results of a crossover clinical trial in athletes submitted to testosterone administration. *Steroids* **2018**, *141*, 104–113. [CrossRef] [PubMed]

18. Kuuranne, T.; Saugy, M.; Baume, N. Confounding factors, and genetic polymorphism in the evaluation of individual steroid profiling. *Br. J. Sports Med.* **2014**, *48*, 848–855. [CrossRef] [PubMed]

19. Ponzetto, F.; Baume, N.; Schweizer, C.; Saugy, M.; Kuuranne, T. Steroidal module of the athlete biological passport. *Curr. Opin. Endocr. Metab. Res.* **2019**, *9*, 14–21. [CrossRef]

20. Juul, A.; Sørensen, K.; Aksglaede, L.; Garn, I.; Meyts, E.R.-D.; Hullstein, I.; Hemmersbach, P.; Ottesen, A.M. A common deletion in the uridine diphosphate glucuronyltransferase (UGT) 2B17 gene is a strong determinant of androgen excretion in healthy pubertal boys. *J. Clin. Endocrinol. Metab.* **2009**, *94*, 1005–1011. [CrossRef]

21. Sonneveld, E.; Jansen, H.J.; Riteco, J.A.C.; Brouwer, A.; Van Der Burg, B. Development of androgen-and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. *Toxicol. Sci.* **2004**, *83*, 136–148. [CrossRef] [PubMed]

22. Sonneveld, E.; Riteco, J.A.C.; Jansen, H.J.; Pieterse, B.; Brouwer, A.; Schoonen, W.G.; Van Der Burg, B. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. *Toxicol. Sci.* **2005**, *89*, 173–187. [CrossRef]

23. Van der Burg, B.; Winter, R.; Man, H.; Vangenechten, C.; Berckmans, P.; Weimer, M.; Witters, H.; van der Lindena, S. Optimization and prevalidation of the in vitro AR CALUX® method to test androgenic and antiandrogenic activity of compounds. *Reprod. Toxicol.* **2010**, *30*, 18–24. [CrossRef]

24. Cooper, E.; McGrath, K.C.Y.; Li, X.; Akram, O.; Kasz, R.; Kazlauskas, R.; McLeod, M.D.; Handelsman, D.J.; Heather, A.K. The use of tandem yeast and mammalian cell in vitro androgen bioassays to detect androgens in internet-sourced sport supplements. *Drug Test. Anal.* **2016**, *9*, 545–552. [CrossRef]

25. Cooper, E.; McGrath, K.; Heather, A. In vitro androgen bioassays as a detection method for designer androgens. *Sensors* **2013**, *13*, 2148–2163. [CrossRef]

26. Houtman, C.; Sterk, S.S.; van de Heijning, M.P.M.; Brouwer, A.; Stephany, R.W.; van der Burg, B.; Sonnevelda, E. Detection of anabolic androgenic steroid abuse in doping control using mammalian reporter gene bioassays. *Anal. Chim. Acta* **2009**, *637*, 247–258. [CrossRef]

27. Bird, S.R.; Goebel, C.; Burke, L.M.; Greaves, R.F. Doping in sport and exercise: Anabolic, ergogenic, health and clinical issues. *Ann. Clin. Biochem.* **2016**, *53*, 196–221. [CrossRef]

28. Schulze, J.J.; Lundmark, J.; Garle, M.; Skilving, I.; Ekstroöm, L.; Rane, A. Doping test results dependent on genotype of uridine diphospho-glucuronosyl transferase 2B17, the major enzyme for testosterone glucuronidation. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 2500–2506. [CrossRef]

29. Strano-Rossi, S.; Fiore, C.; Chiarotti, M.; Centini, F. Analytical techniques in androgen anabolic steroids (AASs) analysis for antidoping and forensic purposes. *Mini Rev. Med. Chem.* **2011**, *11*, 451–458. [CrossRef] [PubMed]

30. Bao, S.; Peng, Y.; Sheng, S.; Lin, Q. Assessment of Urinary Total Testosterone Production by a Highly Sensitive Time-Resolved Fluorescence Immunoassay. *J. Clin. Lab. Anal.* **2008**, *22*, 403–408. [CrossRef] [PubMed]

31. Duntas, L.H.; Popovic, V. Hormones as doping in sports. *Endocrine* **2013**, *43*, 303–313. [CrossRef] [PubMed]

32. Durward-Akhurst, S.A.; Schultz, N.E.; Norton, E.M.; Rendahl, A.; McCue, M.E.; Mickelson, J.R.; Geor, R.J.; Brouwer, A.E.; Behnisch, P.A.; Besselink, H.T. Associations between endocrine disrupting chemicals and equine metabolic syndrome phenotypes. *Chemosphere* **2019**, *218*, 652–661. [CrossRef]

33. Sonneveld, E.; Van der Burg, B.; Brouwer, A.; Stephany, R.; Sterk, S. The Development and application of a tight bioassay-based control system for steroids and other prohibited substances in sport doping. *R. WADA* **2015**. Available online: https://www.wada-ama.org/en/resources/science-medicine/development-and-application-of-a-tight-bioassay-based-control-system-for (accessed on 30 November 2020).

34. Cadwallader, A.B.; Lim, C.S.; Rollins, U.E.; Botrè, F. The androgen receptor and its use in biological assays: Looking toward effect-based testing and its applications. *J. Anal. Toxicol.* **2011**, *35*, 594–607. [CrossRef]

35. Calabretta, M.M.; Lopreside, A.; Montali, L.; Cevenini, L.; Roda, A.; Michelini, E.A. Genetically Encoded Bioluminescence Intracellular Nanosensor for Androgen Receptor Activation Monitoring in 3D Cell Models. *Sensors* **2021**, *21*, 893. [CrossRef] [PubMed]

36. Aqai, P.; Cevik, E.; Gerssen, A.; Haasnoot, W.; Nielen, M.W.F. High-throughput bioaffinity mass spectrometry for screening and identification of designer anabolic steroids in dietary supplements. *Anal. Chem.* **2013**, *85*, 3255–3262. [CrossRef] [PubMed]

37. Beato, M.; Herrlich, P.; Schütz, G. Steroid hormone receptors: Many actors in search of a plot. *Cell* **1995**, *83*, 851–857. [CrossRef]

38. McKenna, N.J.; O'Malley, B.W. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **2002**, *108*, 465–474. [CrossRef]

39. Sato, T.; Matsumoto, T.; Yamada, T.; Watanabe, T.; Kawano, H.; Kato, S. Late onset of obesity in male androgen receptor-deficient (AR KO) mice. *Biochem. Biophys. Res. Commun.* **2003**, *300*, 167–171. [CrossRef]

40. Ekstrom, L.; Schulze, J.J.; Guillemette, C.; Belanger, A.; Rane, A. Bioavailability of testosterone enanthate dependent on genetic variation in the phosphodiesterase 7B but not on the uridine 5′-diphospho-glucuronosyltransferase (UGT2B17) gene. *Pharm. Genom.* **2011**, *21*, 325–332. [CrossRef]