

PROJECT REVIEW

“The Development and application of a tight bioassay-based control system for steroids and other prohibited substances in sport doping.”

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Currently, chemical-analytical methods are the only methods used to detect specific compounds, compound profiles and metabolites in urine and serum samples from athletes as well as in natural- and synthetic ingredients in sport supplements and in food. Such methods have the advantage of being sensitive and highly selective, being able to fingerprint the use of particular drugs. They have the disadvantage in that the compounds being looked for are defined single chemical substances. Substances with slightly different chemical structure but with similar biological activity can be missed as their specific analytical detection parameters are not defined. In contrast, bioassays can directly probe the biological pathway of drug action rather than individual compound detection. Such methods have the advantage that bypassing detection is hardly possible since they will detect all compounds interacting with the endpoint of choice. BioDetection Systems' CALUX[®] (Chemical Activated LUciferase eXpression) bioassays, being mechanism based, highly selective and extremely sensitive, have potential to be employed as broad screening tools, being able to identify the presence of hormone levels outside of established population normal ranges. These stable cell lines have incorporated the firefly luciferase gene coupled to specific hormone responsive elements together with selective receptors for the hormonal class of interest. Exposure of the designed CALUX[®] cells to their respective class of hormones induces the production of luciferase and the consequent emission of light. The amount of light produced is proportional to the amount of specific hormonal activity present. We have recently developed a panel of highly sensitive and selective CALUX[®] cell lines allowing sensitive, rapid, cost-effective and straightforward measurement of not only androgen-, but also estrogen-, progesterone-, and glucocorticoid receptor interacting compounds. As 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 norms. A system consisting of screening for known compounds using sensitive chemical-analytical methods in conjunction with broader, effect-based CALUX[®] bioassays is expected to establish a robust system able to detect the use of almost any chemical compound that interferes with normal steroid hormone action. In this project suitable CALUX[®] bioassays will be established to detect

relevant anabolic agents (with androgenic, estrogenic, progestagenic or glucocorticoid activity) as well as agents with anti-estrogenic activity capturing all steroidal compounds from WADA prohibited list.

Development of a tight bioassay-based control system for steroids and other prohibited substances in sport doping

Results and Conclusions

Currently, chemical-analytical methods are used to detect specific compounds, compound profiles and metabolites in urine and serum samples from athletes, as well as natural- and synthetic ingredients in sport supplements and in food. Such methods have the advantage of being sensitive and highly selective, being able to specifically detect the use of particular compounds. The disadvantage of these chemical-analytical methods is that they can only detect a pre-defined list of chemical substances, or their metabolites. Substances with slightly different chemical structure but with similar biological activity can be missed as their specific analytical detection parameters are not defined. In contrast, bioassays can directly probe the biological mechanism of drug action rather than individual compound detection. Such methods have the advantage that bypassing detection is hardly possible since bioassays will detect all compounds interacting with the endpoint of consideration.

BDS' CALUX[®] (Chemical Activated LUCiferase eXpression) bioassays, being highly selective and extremely sensitive, have potential to be employed as broad screening tools, are able to identify hormonally active compounds irrespective of their mode of action. These stable cell lines have incorporated the firefly luciferase gene coupled to specific hormone responsive elements together with selective receptors for the hormonal class of interest. Exposure of CALUX cells to their respective class of hormones induces the production of luciferase and the consequent emission of light. The amount of light produced is proportional to the amount of specific hormonal activity present. We have recently developed a panel of highly sensitive and selective CALUX cell lines allowing sensitive, rapid, cost-effective and straightforward measurement of not only androgen-, but also estrogen-, progesterone-, and glucocorticoid receptor interacting compounds.

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 known compounds using effect-based CALUX bioassays in conjunction with more specific sensitive and identifying chemical-analytical methods such as GC-MS is expected to establish a robust system able to detect the use of almost any chemical compound that interferes with normal steroid hormone action.

Almost all anabolic androgenic steroids (AAS) from the 2005 List of Prohibited Compounds were tested for bioactivity using CALUX bioassays. Also other potential anabolic designer steroids were included. Testing of AAS from the prohibited list demonstrated that anabolic androgenic steroids relevant in the field of doping control can be measured with CALUX bioassays. 98% of the tested compounds were active in one or more of the bioassays, and most compounds showed androgenic activity (88%). Only certain urinary metabolites of androgenic steroids on the list of the prohibited compounds, did not generate a measurable response in the AR CALUX bioassay. However, also estrogenic and progestagenic activities were demonstrated for many compounds. The AR CALUX bioassay can be used for determining the presence of bioactive compounds with known chemical structures as well as bioactive compounds with unknown chemical structures, the latter being especially helpful for the analysis of new designer steroids. Various potential AAS as yet not present on the WADA prohibited list, showed strong androgenic activities, as well as progestagenic activities.

Methods were developed for the sample pre-treatment of urine and serum for analysis with CALUX bioassays. Steroidal activities in serum and urine samples could be analyzed both in extracts and un-extracted samples. Human urine samples had to be deconjugated prior to analysis. Extremely low limits of detection were obtained for the developed methods (for androgenic activity 0.02-0.04 ng DHT-equivalents/ml urine). Shake solvent extraction and solid phase extraction both proved to be suitable methods for the clean-up and concentration of urine and plasma samples for AAS analysis using CALUX bioassays. Analysis was also possible after direct exposure of CALUX cells to intact urine and plasma samples. All methods gave comparably high and reproducible recoveries of androgenic, estrogenic and progestagenic activity in spiked samples.

Mixtures of AAS behaved additively in the AR CALUX bioassay and the total sum of androgenic effects could be correctly described with the concept of concentration addition. Comparison between the sum activity (total equivalent) of mixtures and chemical analysis of individual compounds can be facilitated by multiplying the concentration times the relative potency in the bioassay and summing them all up. This can be used in the development of an integrated system for doping analysis. One of the consequences of additive behaviour of mixtures of androgens is that low concentrations of compounds that are undetectable on an individual basis, together may generate a detectable response. This will e.g. be extremely helpful in the detection of multi drug abuse.

CALUX bioassays were successfully applied to determine endogenous levels of steroidal activities in human urine samples collected from volunteers and from athletes competing at high level sports. Almost all samples showed activities several orders higher than the limit of detection. Androgenic, progestagenic and glucocorticoidal activities appeared to be significantly related with gender. Correlations between e.g. ethnical background and androgenic activity, and between contraception pill use and progestagenic activity were observed as well. By analyzing steroidal activities in this large set of samples normal values for endogenous levels of steroidal activities in urine could be determined.

Comparison of AR CALUX bioassay results with GC-MS analysis of these human urine samples revealed that the endogenous androgens testosterone and DHT are the main contributors to normal endogenous steroidal activities measured by the AR CALUX bioassay. 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. Since in the current project positive AAS doping samples were not available, the WADA proficiency test (PT) containing human urine samples of unknown composition was used to re-create a doping control setting. Very high androgenic activities were observed in some of the WADA PT samples, deviating from the "normal range". To determine if the high androgenic (and progestagenic) activities of certain WADA PT samples are due to exogenous bioactive AAS or to high (supra-physiological) endogenous androgen levels, also GC-MS analysis for the main endogenous androgens in urine was applied on these samples, revealing a clear difference between endogenous androgenic activities (GC-MS derived) and AR CALUX derived androgenic activities. This suggests the presence of exogenous bioactive AAS in these samples. Parallel, human urine samples spiked with different concentrations of two AAS (THG and nandrolone) were analysed to obtain an impression of the capability of the AR CALUX bioassay to discriminate between AAS "positive" and "negative" samples. Indeed the AR CALUX bioassay could detect the presence of

these active exogenous compounds above endogenous androgen levels and therefore was able to discriminate between doping negative and positive urine samples.

In conclusion, in the strategy of an integrated system design, all experiments performed in the current project indicate that CALUX bioassays and chemical analytical analysis are complementary methods. These first experiments indicate that GC-MS is the method of choice for identification purposes and CALUX is the method of choice for screening unknown active compounds and mixture effects.

Publications

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