"Improving detection of anabolic steroids: new screening based on the direct analysis of phase II metabolites using LC-Q-HRMS"

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Current screening methods for exogenous anabolic androgenic steroids (AAS) are based on the hydrolysis of the urine samples using β-glucuronidase enzymes and the measurement of the released phase I metabolites using gas and liquid chromatography coupled to mass spectrometric techniques. In recent years, new long-term phase II metabolites of AAS, not detectable using the current screening conditions, have been identified using liquid chromatography-mass spectrometry technology, including glucuronoconjugated metabolites not readily hydrolysed with β-glucuronidase enzymes and sulfoconjugated metabolites.

The hypothesis of the project is that significant information is missed in current AAS screening methods and the aim of the project is to develop a screening method based on the direct detection of the metabolites excreted in the urine, including phase II metabolites (glucuronides and sulfates) and relevant unconjugated metabolites using liquid chromatography coupled to tandem mass spectrometry with high resolution instruments. The method will improve the detection capabilities of AAS through the monitoring of important long-term metabolites, not detectable using the currently used screening methods. The approach will also facilitate the incorporation of new phase II metabolites. In addition, the possibility of acquiring full scan data will allow the retrospective reprocessing of all results for the detection of unknown metabolites, without the need of repeating the analysis. Moreover, the successful outcome of the project will represent a cost-effective simple approach, that can readily be shared amongst WADA accredited laboratories.

Results and Conclusions:

Anabolic androgenic steroids (AAS) are the most frequently reported group of prohibited substances detected in doping controls. In recent years, new phase II metabolites of these compounds have been identified by LC-MS/MS and their usefulness improving the detection capabilities of the AAS misuse was demonstrated. However, the detection of some analytes was compromised due to the co-elution of endogenous interferences sharing the same ion transitions. The aim of this work was to evaluate the possibility of high-resolution mass spectrometry (HRMS), performed with LC-Q-TOF MS and LC-Q-Orbitrap MS systems, to overcome these limitations, thanks to the increased selectivity provided by the determination of the exact mass.

The study of the ionization and fragmentation behaviour of unconjugated, sulfates and glucuronides steroids was performed with both Q-TOF and Q-Orbitrap instruments, acquiring fullscan and product ion spectra at different collision energies in positive and negative electrospray modes. The chromatographic conditions were optimized to achieve the best baseline separation of the isobaric compounds and to limit matrix interferences. The LC separation was finally performed with an Acquity UPLC BEH C18 column using H2O and CH3CN:H2O 95:5 (v/v) (0.01% formic acid, 1 mM ammonium formate) as mobile phase, with a total run time of 23 min. Solid-phase extraction (SPE) with Bond Elut C18 cartridges was used for sample clean-up and pre-concentration. Limits of detection (LODs), estimated with both HRMS systems, showed values below the 50% of the MRPL for sulfates and most of the glucuronides tested. Lower ionization efficiencies were obtained for some unconjugated steroids, producing unsatisfactory LODs with respect to WADA requirements.

The Q-TOF-HRMS method was applied to urine samples obtained from oral and/or intramuscular administration of stanozolol, clostebol, methyltestosterone and metandienone, with the purpose to include in the study markers of these compounds that had been identified in previous works and for which standards were not available. Most of the main metabolites were identified on the basis of the accurate mass, relative retention time and characteristic fragmentation.

Metabolomics strategies and statistical data analysis were used with the excretion studies after stanozolol and clostebol administrations to try to identify new metabolites. The most important metabolites already reported in the literature were identified using PLS-DA, but unfortunately, no new metabolites were discovered. Nevertheless, the results demonstrate the usefulness of the metabolomics strategy for the study of new metabolites.

Overall, non-targeted screening methods based on HRMS instrumentation are a very useful tool in routine anti-doping analysis, with comparable applicability with respect to targeted MRM methods.

Presentations and Publications:

Presentations to congresses/meetings:
Esquivel A, Alechaga E, Monfort N, Ventura R
Evaluation of endogenous steroid sulfates as markers of testosterone oral misuse.
36th Cologne Workshop on Dope Analysis, Cologne (Germany), 2018.

Oral communications
Esquivel A, Alechaga E, Monfort N, Ventura R
LC-MS/MS quantitation of steroid sulfate metabolites to evaluate its potential as markers of testosterone administration.
XVIII Reunión de la Sociedad Española de Cromatografía y Tecnicas Afines, Granada (Spain), 2018

Oral presentation
Esquivel A, Alechaga E, Monfort N, Ventura R
Potential of endogenous steroid sulfates as markers of intramuscular administration of testosterone in Caucasian and Asian population.
37th Cologne Workshop on Dope Analysis, Cologne (Germany), 2019.

Publications
Esquivel A, Alechaga É, Monfort N, Ventura R.
Direct quantitation of endogenous steroid sulfates in human urine by liquid chromatography-electrospray tandem mass spectrometry.
Drug Test Anal 2018;10(11-12):1734-1743. doi: 10.1002/dta.2413.

Doctoral Thesis
Some of the results of the project have been part of the following doctoral thesis:
Title: Control of anabolic steroids misuse in sports: potential of direct detection of phase II metabolites.
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