PROJECT REVIEW

"*Testosterone misuse: evaluation of metabolites conjugated with glucuronic acid stable to hydrolysis wit b-glucuronidase to improve detection capabilities*"

Dr. Ventura, Dr. Segura, Dr. Matabosch, Dr. Gome (Fundacio IMIM, Spain)

Anabolic androgenic steroids and, among them, testosterone are the most important group of forbidden substances detected in antidoping control analyses. The existing marker to detect testosterone misuse (ratio between testosterone and epitestosterone excreted as glucuronides) present some limitations and it is necessary to develop analytical approaches to improve the detection of testosterone misuse. In the last years, LC-MS systems have demonstrated wide possibilities for the elucidation of new phase I and phase II metabolites and the use of this technology has resulted in the detection of previously unreported metabolites.

The objective of the project will be the evaluation of testosterone metabolites conjugated with glucuronic acid resistant to hydrolysis with enzymes with -glucuronidase activity, that may include mono, bis and diglucuronides. Open screening methods for the detection of glucuronoconjugated metabolites by LC-MS/MS will be developed. The methods will be applied to samples obtained before and after testosterone administration for the identification of the new metabolites. The structure of the metabolites will be characterized using mass spectrometric techniques. The excretion profiles of the identified metabolites in samples obtained after administration of testosterone by different routes (oral, intramuscular, topic) will be compared with those of other markers of testosterone misuse targeted in conventional screening procedures, in order to evaluate their interest as long-term markers of testosterone misuse.

Finally, a methodology addressed to the reliable detection of identified metabolites in routine antidoping analysis will be developed.

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Results and Conclusion:

The objective of the project was to evaluate testosterone metabolites conjugated with glucuronic acid resistant to hydrolysis with β-glucuronidase enzymes. Two testosterone glucuronide metabolites which remain conjugated in urine after hydrolysis with β-glucuronidase enzymes were detected (G1 and G2) by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a neutral loss scan method based on losses of 176, 194, 211 and 229 Da, characteristic of steroids conjugated with glucuronic acid. The metabolites were characterized using mass spectrometric techniques. The structure of the phase I metabolites released after chemical hydrolysis of G1 and G2 was confirmed using reference standards as 3α,6β-dihydroxy-5α-androstan-17-one (6β-hydroxy-androsterone) and 3α,6β-dihydroxy-5β-androstan-17-one (6β-hydroxy-etiocholanolone), respectively.

The hydrolysis in urine of G1 and G2 using different amounts of β-glucuronidase showed that almost no cleavage of these compounds was achieved in conventional analysis conditions. Therefore, due to their resistance to hydrolysis with β-glucuronidases, these metabolites have been underestimated in previous works using conventional analysis conditions. In contrast to previously published results, an important increase in G1 and G2 concentrations (between 50-300 folds) was observed after testosterone undecanoate administration when using direct detection of G1 and G2 by LC-MS/MS. The concentrations remained elevated for longer times compared with the current marker (T/E ratio).

The results show the need to quantify G1 and G2 in samples obtained after administration of testosterone and other endogenous steroids, and in samples from athletes to gather additional information to evaluate the usefulness of G1 and G2 to detect the administration of endogenous steroids and their inclusion in the steroidal module of the athlete´s biological passport for longitudinal monitoring.

Presentations in congress:

A Fabregat, OJ Pozo, J Marcos, J Segura, R Ventura. *Exploring novel alternatives for the detection of*
testosterone misuse. Is there still something hidden by a naked eye? 30th Cologne Workshop on Dope Analysis, Cologne (Germany), 2012.

A Fabregat, OJ Pozo, A Kotronoulas, J Joglar, J Marcos, J Segura, R Ventura. *Analytical strategies to*
detect and elucidate steroid metabolites conjugated with glucuronic acid resistant to enzymatic
hydrolysis. Application to doping control analysis. VI Meeting of the Spanish Society of Mass
Spectrometry, Úbeda (Spain), 2013

Publications:

A Fabregat, OJ Pozo, J Marcos, J Segura, R Ventura. *Use of LC-MS/MS for the open detection of steroid metabolites conjugated with glucuronic acid.* Analytical Chemistry 2013;85:5005-5014.

C Gómez, A Fabregat, OJ Pozo, J Marcos, J Segura, R Ventura. *Analytical strategies based on mass spectrometric techniques for the study of steroid metabolism.* Trends in Analytical Chemistry 2014;53:106-16.