

“Bis-Conjugates in the Endogenous Profile of Steroids (BICEPS)”

Dr. O. Pozo, Dr. J. Marcos (IMIM, Spain)

Project Overview

Screening for endogenous androgenic anabolic steroid (EAAS) misuse remains one of the main challenges in doping control. Currently, this challenge is addressed by quantification of the seven markers forming the steroid profile included in the steroid module of the Athlete Biological Passport (ABP). The common approach for this quantification involves enzymatic hydrolysis, TMS-derivatization and GC-MS(/MS) analysis. However, several markers might be either lost or underestimated by this approach.

Preliminary experiments performed by the project team show the occurrence of EAAS excreted as bis-sulfates and highlight their potential usefulness for the detection of testosterone misuse.

The Bis-Conjugates in the Endogenous Profile of Steroids (BICEPS) project aims to evaluate the potential of steroid bis-conjugates for the detection of EAAS misuse. For this purpose, the project will be divided in two parts.

In Part I, the usefulness of bis-sulfates for the detection of EAAS misuse will be evaluated. Bis-sulfate reference materials will be quantitatively synthesized and characterized, and an analytical approach for the quantification of urinary EAAS bis-sulfates will be developed and validated. The validated method will be applied to several samples from available drug administration excretion studies.

In Part II, the occurrence and usefulness of other EAAS bis-conjugates will be evaluated. Libraries of steroid bis-glucuronides and glucuronide-sulfates will be synthesized. From these available reference materials an open screening strategy will be developed based on MS behavior of the synthesized compounds enabling the detection of EAAS bis-conjugates. The detection of these bis-conjugates in selected postadministration samples will be evaluated.

Taken together, the BICEPS project will reveal which bis-conjugate metabolites are useful for screening of EAAS misuse. Further, the project will deliver a range of characterised reference materials derived by chemical synthesis for their further study and quantification.

Results and Conclusions:

Several important EAAS might remain undetectable with the current approach used for the determination of the steroid profile i.e. gas chromatography-mass spectrometry (GC-MS) analysis after an enzymatic hydrolysis with *E. coli* β -glucuronidase, and the silylation of the steroids. Among them steroid bis-conjugates remain unexplored. The main goal of BICEPS is to evaluate the potential of steroid bis-conjugates for the detection of EAAS misuse.

Firstly, we have characterized the MS behavior of steroid bis-sulfates and we have developed an open screening method for their detection in urine. We have quantitatively synthesized 12 steroid bis-sulfates and we have developed and validated a quantitative method for their determination in urine. The method has been applied to samples collected after oral administration of testosterone undecanoate. We have evaluated several ratios between the validated analytes and we found that they have limited applicability for doping control purposes. However, we found two additional steroid bis-sulfates which allowed for the screening of the misuse with promising results. We hypothesize that these markers are two isomeric forms of the compound 3 \square ,16 \square -dihydroxy-5 \square -androstane-17-one bis sulfate. Synthesis of reference material is required to confirm the identity. Using these two markers, we obtained results comparable with those obtained with the best retrospective markers for oral misuse (resistant glucuronides and cysteinyl conjugates).

In a second part of the project we have synthesized some steroid bis-glucuronides and steroid glucuronide-sulfates. We have demonstrated the occurrence of some of them in human urine samples. Preliminary results showed that one of them (5 α -androstane-3 β ,17 β -diol 3-sulfate 17-glucuronide) clearly increased after oral testosterone administration supporting its potential usefulness for doping control.

Taken together, the results of BICEPS provide the first evidence about the potential usefulness of bis-conjugates in the doping control field.

The main results of BICEPS have been published at :

1.- McLeod MD, Waller CC, Esquivel A, Balcells G, Ventura R, Segura J, Pozo OJ*. A constant ion loss method for the untargeted detection of bisulfate metabolites. *Anal Chem* 2017; 89(3): 1602-1609.

2.- Pranata A, Fitzgerald CC, Khymenets O, Westley E, Anderson NJ, Ma P, Pozo O J, McLeod MD*. Synthesis of Steroid Bisglucuronide and Sulfate Glucuronide Reference Materials: Unearthing Neglected Treasures of Steroid Metabolism. *Steroids* 2019: doi 10.1016/j.steroids.2018.11.017.

Some results have been presented at: 1.- "Steroid bis-sulfates: a forgotten minority" Oral presentation at SUPA2017, Targeting Steroid Sulfation Pathways, Birmingham April 2017