"*Development of an analytical method for 19-Norandrosterone, Boldenone, and Formestane in urine by on-line-coupling of LC-GC-MS and LC-GC-IRMS*"

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Project Summary

When metabolites of nandrolone, boldenone or formestane are found at low concentrations in doping control tests, a further CIRMS-based analysis is compulsory in order to be sure that steroid misuse has occurred. For each analyte, the factors that could generate such findings are diffrent, but in all cases the urinary concentrations always range between 1 and 20 ng/mL. Due to the low concentration of the analytes and the small contribution of 13C to the analyte molecule, an important level of selectivity and sensitivity is necessary to characterize a ^{13}C difference. Therefore, the sample must be submitted to a long time-consuming sample preparation method.

Nevertheless, to improve detection capabilities, some laboratories include a standalone clean-up step based on the use of high performance liquid chromatography (HPLC). The present project attempts to attain these objectives by means of on-line coupling of liquid chromatography and gas chromatography (LC-GC) using the TOTAD interface, developed and patented by the UCLM research group. This analytical methodology allows the complete LC fraction containing the analytes to be transferred to the GC and, moreover, provides a better signal/noise ratio because it provides cleaner extract. These aspects, will presumably improve sensitivity by one or two orders of magnitude. The project will focus on developing an analytical method in which the hydrolysed extract is derivatised (off-line) and analysed by LC-GC. Two different detectors will be used, leading to LC-GC-MS and/or LC-GC-C-IRMS coupling. Automation of the system by using an autosampler will also be attempted. The objective of the present project is to develop a sensitive, automatic, robust and reliable analytical method to be used for the analysis of the mentioned steroids in urine. The method should allow the detection limits required to be reached, thereby providing a powerful tool for the efficient control of the misuse of anabolic steroids.

Results and Conclusions

It has been developed a sensitive, automatic, robust and reliable method for analyzing steroids in urine using on-line coupled LC-GC-C-IRMS (Liquid Chromatography-Gas Chromatography-Combustion Isotope Ratio Mass Spectrometry) with the TOTAD (Through Oven Transfer Adsorption Desorption)

interface to discriminate between the endogenous or exogenous origin of the same, and by on-line coupled LC-GC-MS to confirm the purity and the identity of the steroid peaks. Urine samples spiked at 5 ng/mL or 10 ng/mL with the steroid to be analysed were used. Of each urine sample 20 mL was hydrolyzed, extracted, purified by a first RPLC step of un-derivatized steroids, derivatized to acetyl-steroids and, finally, submitted to on-line LC-GC with CIRMS or MS detection. In the coupled system a second RPLC cleaning of derivatized steroids is applied, and the LC fractions containing the analytes are completely transferred to GC-CIRMS or GC-MS.

LC-GC-CIRMS methods were developed for the analysis of 19-Norandrosterone (19-NA) and for Bodenone (Bo) and its main metabolite (BoM). The method failed in the case of 19-NA due to an interfering compound. The derivatization of Formestane (F) did not work properly, so that the developed method is not applicable to F analysis. In the case of Bo and BoM the method was developed successfully. The volumes of the fractions transferred from LC to GC through a fraction collector were 1000 and 900 µL, respectively. $^{13}\delta C_{VPDB}$ corrected values for Bo and BoM, as well as the $\Delta\delta$ in relation to the reference compound Pregnandiol (PD), clearly indicated the exogenous origin of the steroids. The Standard Deviations of the $^{13}\delta C_{VPDB}$ values ranged from 0.85 to 1.11 for the entire method. A confirmatory on-line LC-GC-MS analytical method was developed for Bo and BoM. Relative Standard Deviations of the absolute peak areas were below 8%, except when Bo was spiked at 10 ng/L. Detection Limits were 0.5 ng/mL for Bo and 0.05 ng/mL for BoM in full scan. When 3 ions were selected, detection limits were 0.07 and 0.008 ng/mL respectively.

For the first time, an analytical method involving the coupling of LC-GC with C-IRMS has been developed. The presented methods permit the origin of urinary Bo and BoM to be identified as endogenous or exogenous. The sensitivity is substantially improved compared with currently used methods, allowing the detection limits set by WADA to be attained.

The advantages of LC-GC coupling open up the possibility of extending the methodology to other steroids and to other compounds currently analysed by GC. The high sensitivity achieved should permit the amount of urine necessary for such analyses to be decreased.

The LC-GC-C-IRMS method developed for the analysis of Boldenone has been published (J. Chromatog. A 1320 (2014) 171-178). Presumably, the LC-GC-MS method for the analysis of Boldenone will be published shortly.