"Development of a two-dimensional HPLC method for the GC/C/IRMS analysis of corticosteroids and low concentrated urinary metabolites"

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Project overview

The project that we propose implies the development of a novel two-dimensional HPLC purification method for the carbon isotopic analysis of two groups of molecules, corticoids and low concentrated steroids. Prednisolone is a direct metabolite of the synthetic corticoid prednisone but it can also be derived from the dehydrogenase bacterial enzymatic activity of cortisol. In the same manner cortisone, the parent molecule of cortisol, could produce considerable quantities of prednisone as well, yielding false positive cases. Additionally, numerous methods for the purification and ^{13}C analysis of boldenone and nandrolone metabolites have been developed in the past years; however, to our knowledge, none yet has proven to produce measurements as precise and accurate as the testosterone metabolites, being unquestionably more concentrated.

Furthermore two HPLC purifications are commonly required for their purification, from which testosterone metabolites are left aside due to the complexity of the separation even if the latter come necessary in the detection of multi-positives cases. To ascertain the exogenous origin of the corticoids, difficult to chromatographically purify due to their higher polarity, and to efficiently purify boldenone or nandrolone metabolites including testosterone metabolites, the development of a novel two dimensional HPLC method through the heart-cutting technique is sought for a rapid purification of those compounds from the urine matrix without any derivatization. The rationale behind this method development is (i) to save instrumental time for the purification of the compounds, (ii) obtain the same purification power as with two subsequent HPLC cleansing runs and (iii) to gain intensity, precision and accuracy owed to fewer manipulations and chemical reactions. Finally, we wish to validate the methods by confirming the absence of isotopic fractionation through the analysis of all the tested metabolites using the developed HPLC technique and to publish the methods used for routine $\delta^{13}C$ measurements.

Results and Conclusions:

The use of compound specific isotope analysis (CSIA) to investigate stable isotopic abundances of is still considered a niche discipline for well-trained specialists (Brand 2012) and indeed, the implementation of reliable and robust IRMS methods has proven to be quite a challenge for anti-doping labs. Possibly the most critical aspect of CSIA is achieving adequate purification of compounds prior to IRMS analysis since unlike most other mass

spectrometric methods, the specificity of CSIA analysis hinges on achieving baseline separation of target GC peaks from all other carbon-containing molecules in the urinary matrix. Insufficient purification results in peak contamination and inconsistent or irreproducible $\delta^{13}C$ values. The world anti-doping agency (WADA) recommends the use of high performance liquid chromatography (HPLC) for sample cleanup, however many WADA-accredited labs suggest more than just one purification step (Piper et al. 2008) or their methods require long HPLC columns and extended purification time (75 minutes, Ouellet, LeBerre and Ayotte, 2012).

This WADA project provides a description of an automated two dimensional HPLC purification (2D-HPLC) method for urine extracts that has made possible the highest throughput CSIA purification of urine extracts described thus far by WADA-accredited labs, requiring only 35 minutes per sample or approximately 20-25 samples/day. In contrast to previously established CSIA methods, no sample manipulation is required between purification steps (e.g. Piper 2008). Six urinary steroids including testosterone/DHEA and 4 metabolites (5α-androstanediol, 5β-androstanediol, androsterone and etiocholanolone) as well as two endogenous reference compounds (pregnanediol and 3α-hydroxy-5α-androst-16-ene) were eluted and collected during HPLC purification.

Comparative GC chromatograms are used to contrast the efficiency of 2D purification to a previously established 1D HPLC method (Ouellet et al 2014). While each sample requires less than half the time of 1D purification, sample purity is actually improved. Precision of $\delta^{13}C$ for all analyzed compounds in negative and positive controls was 0.3 ‰ or better, which is comparable to the precision of pure compounds at similar concentrations. The appeal of 2D-HPLC is indeed that a properly configured method can easily surpass the efficiency of the highest performing 1D system – producing clean peaks in much shorter run times. No extra expenditure is incurred by the use of 2D-HPLC, aside from the initial instrument cost. The potential for a dramatic increase in the IRMS sample load offered by 2D-HPLC would be especially useful during international sporting events, during which a large number of tests are required over very short time delays. Increased sample throughput makes possible a larger number of IRMS tests, necessarily improves the likelihood of detection of the abuse of testosterone or testosterone-related steroids.