Anabolic Androgenic Steroid Testing by Liquid Chromatography Quadrupole Time-Of-Flight Mass Spectrometry in Urine

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Abstract

Sports Doping is one of the hottest topics in sports from high school through the professional levels. In the arena of sports doping, Anabolic Androgenic Steroids (AASs) are used for quick gains, building lean muscle, and increasing strength. AASs have been shown to mimic the actions of male sex hormones. There are several benefits to AASs leading to their approval for treatment of several disorders including chronic wasting conditions, growth disorders, and anemia. The benefits of taking an AAS for many outweigh the risks. In an effort to deter athletes from using an AAS, regulatory organizations like the World Anti-Doping Agency (WADA) has established a list of banned substances for competition in sports. In addition, WADA has suggested instrumentation to use for testing to ensure the most accurate and precise testing possible. Sports doping tests as performed in the United States per WADA are expensive, prohibitively so in many cases. As such, we developed a hyphenated panel that cover the most common WADA banned substances by High Pressure Liquid Chromatography Quadrupole Time-Of-Flight Mass Spectrometry (LC/Q-TOF MS). Using this platform, we are able to duplicate the sensitivity of a single quadrupole instrument while increasing the accuracy due to the exact mass capabilities.

Methods & Materials

Urine samples were spiked with internal standard, cleaned up by solid phase extraction, and run on a 19-analyte LC/Q-TOF MS method.

Sample Collection & Preparation:

Urine samples were collected using standard practices, transferred to a Monovette®, and shipped to the laboratory for testing. 5mL of each sample was added to a 15mL glass centrifuge tube. Samples are hydrolyzed and spiked with internal standard. Post hydrolysis, 8% v/v of Carbonate and Bicarbonate buffers were added to each sample, vortexed, and applied to a column. Sample were eluted, dried, and reconstituted with 100% methanol.

Standards:

All standards were purchased from Cerilliant with the exception of Clostebol, 6β-Hydroxyboldenone, Oxandrolone, and Oxymesterone which were purchased from Alltech.

Instrumentation and Method:

Samples were run on a Micromass Q-TOF-2 coupled to an Alliance 2795 HPLC Autosampler. The mass spectrometer was calibrated according to manufacturer's guidelines and the LTEFF was set using Testosterone as a reference. Mobile phase contained 2.5mM Ammonium Formate and 2.5mM Ammonium Acetate in Water and Methanol for A and B, respectively.

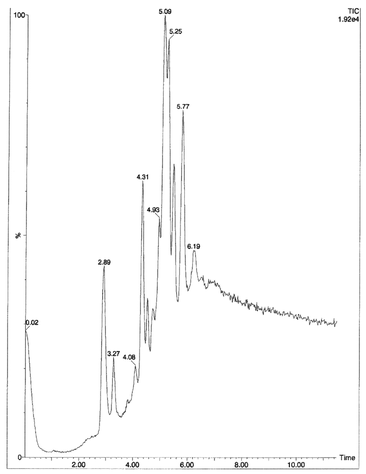
Methods & Materials, Continued

Instrumentation and Method, cont.:

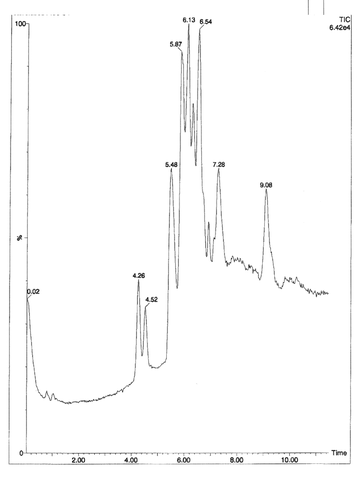
All samples were run on a Pinnacle® DB Biphenyl 5µm 140Å 150 X 2.1mm Column (Restek) except Testosterone, Epitestosterone, and Dehydroepiandrosterone (DHEA) which was tested on an Epic C18 MS 5µm 120Å 150 X 2.1mm HPLC Column (ES Industries). Please note the internal standard was run on both columns.

Steroids in Panel
Boldenone
Clenbuterol
Clostebol
DHEA
Epitestosterone
Fluoxymesterone
Formestane
6β-Hydroxyboldenone
6β-Hydroxyfluoxymesterone
3'-Hydroxystanozolol
Methandrostenolone
Nandrolone
19-Norandrosterone
Oxandrolone
Oxymesterone
Stanzolol
Testosterone
Tetrahydrogestrinone

Table 1: Steroids in the Panel



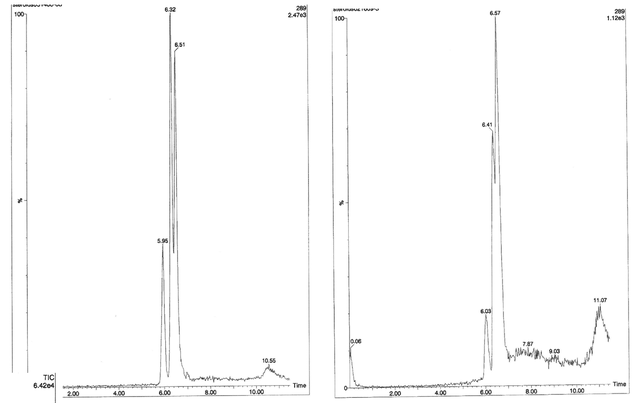
Graph 1: Total Ion Count for Biphenyl Run



Graph 2: Total Ion Count for C18 Run

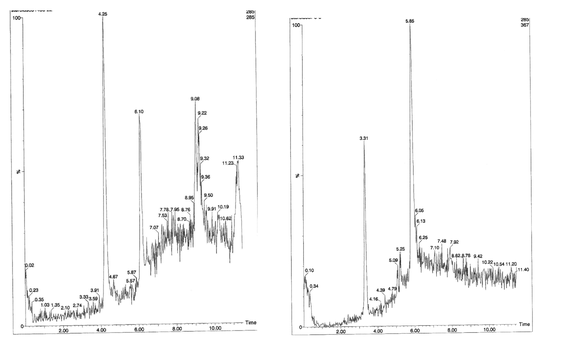
Results

We were able to run all 20 compounds, including the internal standard, in a single run on the C18 column achieving great separation for Testosterone and its epimer, Epitestosterone (Graph Three). Though, the AASs performed well on the C18 column we were able to get better resolution of the metabolites on the Biphenyl column. See comparison of 6β-Hydroxyboldenone in Graph Four; the resolution and the mass contribution in the spectrum (not shown) of the compound are remarkably better on the Biphenyl column. The Biphenyl column does not separate Testosterone and Epitestosterone well. As such, to optimize the chromatography for each compound, all the exogenous AASs were tested on the Biphenyl column while the endogenous compounds remained on the C18 column.



C18 Column: Testosterone and Epitestosterone **Biphenyl Column: Testosterone and Epitestosterone**

Graph 3: Testosterone and Epitestosterone on the C18 and Biphenyl Column Respectively



C18 Column: 6β-Hydroxyboldenone **Biphenyl Column: 6β-Hydroxyboldenone**

Graph 4: 6β-Hydroxyboldenone on the C18 and Biphenyl Column Respectively

Summary & Conclusion

It has been shown that AASs has detrimental effects on humans even when taken at low doses. Despite the negative consequences, AASs are used by several who are seeking the lean muscle, increase in strength, and the quick gains. Regulations of AASs by the Food and Drug Administration, WADA, and similar organizations has increased significantly over the past few decades to ensure the health and safety of individuals. When examining the AASs, most laboratories utilize Enzyme-Linked Immunosorbent Assay (ELISA), High Pressure Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS), or Gas Chromatography Mass Spectrometry (GC/MS). Several have demonstrated the efficacy of using LC/MS/MS urine and blood for AAS testing[1,2,3]. By taking advantage of the benefits of liquid chromatography (no derivation) and mass spectrometry (sensitivity), more accurate testing could be developed. This testing is called LC/Q-TOF MS which offers sensitivity with limits of detection in the low ppb range while being more accurate, less than ten ppm, than LC/MS/MS. Touber *et. al.* (2007) demonstrated that AASs and other compounds can be detected in a single run in urine without interference[4]. We were able to demonstrate consistent results for common AASs not previously tested for by Touber *et.* al. with great separation for the endogenous epimers at low concentrations. Therefore, the LC/Q-TOF MS method as described can be used to test for AASs in urine at WADA cutoff levels. Moreover, this method appears robust and adaptable to lower volumes which allows for universal use.

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