Identification of anabolic agent abuse in sport: determination of androgen glucuro- and sulfoconjugates in urine by LC-MS/MS.

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AIMS: Anabolic androgenic steroids are an important class of abused drugs in sport. Most steroids are excreted in urine with their hydroxyl function being substituted by either sulphate or -glucuronide. The aim of this study was to develop a specific and sensitive method based on liquid chromatography-multiple mass spectrometry (LC-MS/MS) for the determination of 16 androgen conjugates (9 glucuronide – 5a-Androstane-3b,17b-diol-3-glucuronide; Androsterone glucuronide; 5a-Dihydrotestosterone glucuronide; Epitestosterone glucuronide; Etiocholanolone glucuronide; Testosterone glucuronide; 19-Noretiocholanolone glucuronide; 19-Norandrosterone glucuronide; DHEA glucuronide and 7 sulphates – Androsterone sulphate; 5a-Dihydrotestosterone sulphate; Epitestosterone sulphate; Etiocholanolone sulphate; 19-Norandrosterone sulphate; Testosterone sulphate; DHEA sulphate) and 8 deuterated analogs – d4- Androsterone sulphate; d3-Testosterone sulphate; d3-5a-Dihydrotestosterone sulphate; d3-Epitestosterone glucuronide; d3-Epitestosterone sulphate; d5-Etiocholanolone sulphate; d3-Testosterone glucuronide; d3-5a-Dihydrotestosterone glucuronide.

METHODS: Method development consisted of: optimisation of ionisation conditions on an ion trap LC-MS/MS instrument (positive ion electrospray, negative ion electrospray, positive ion atmospheric pressure chemical ionisation, negative ion atmospheric pressure chemical ionisation); optimisation of multiple mass spectrometry experiments [collision voltage amplitude, collision time, multiple reaction monitoring (MRM)]; optimisation of LC separation (type of column, elution system, buffer molarity, isocratic or gradient elution,); optimisation of extraction from urine samples (solid-phase extraction using a C18 cartridge with two different elution programmes, liquid/liquid extraction with dichloromethane, liquid/liquid extraction with ether); ion suppression testing under different ionization conditions was performed comparing the areas obtained by injection of standard solutions with those of spike-after-extraction samples. After the best conditions for each parameter were identified, full method validation was performed in terms of linearity, limits of detection and quantification (LOD and LOQ), precision, accuracy.

RESULTS: The optimised method consisted of: C18 SPE extraction, LC separation on a Luna C18 column with gradient elution (solvent A, 5 mM ammonium acetate-formic acid buffer pH 4,2 and solvent B, acetonitrile); electrospray ionisation and multiple reaction monitoring on selected ion species specific for each analyte. The whole chromatographic run was divided in six segments differing for ionisation conditions (positive ion or negative ion), tuning conditions, and MRM setting. The developed method was applied to the analysis of urine from healthy subjects which do not use any anabolic drugs and subjects that declared taking oral preparations containing different dosages of the pro-hormones (such as DHEA, DHT, androstenedione). Differences between the urinary sulphate and glucuronate steroid profile were elucidated.

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CONCLUSIONS: Intra-day and inter-day precision was always better than 12 and 15% respectively; LOD (with the criterion of signal to noise ratio of 3) were in the range 0,5 - 3 ng/ml while LOQ (the concentration giving deviation from nominal value within 20%) were in the range 2 - 10 ng/ml. Accuracy was assessed by means of fortified samples and expressed by percent bias; it was always better than 17 %. The LC-MS/MS method developed and validated is sensitive, selective and precise and has the potential for routine determination of anabolic agent conjugates in urine.

KEYWORDS: *Androgens, Glucuroconjugates, Sulfoconjugates, LC-MS/MS, Ion trap*

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