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Review

Revisiting the metabolism of 19-nortestosterone using isotope ratio and high resolution/high accuracy mass spectrometry

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ABSTRACT

The synthetic anabolic androgenic steroid 19-nortestosterone is prohibited in sports according to the regulations of the World Anti-Doping Agency (WADA) due to its performance-enhancing effects. Today, doping controls focus predominantly on one main urinary metabolite, 19-norandrosterone glucuronide, which offers the required detection windows for an appropriate retrospectivity of sports drug testing programs. As 19-norandrosterone can also be found in urine at low concentrations originating from in situ demethylation of other abundant steroids or from endogenous production, the exogenous source of 19-norandrosterone needs to be verified, which is commonly accomplished by carbon isotope ratio analyses.

The aim of this study was to re-investigate the metabolism of 19-nortestosterone in order to probe for additional diagnostic long-term metabolites, which might support the unambiguous attribution of an endo- or exogenous source of detected 19-nortestosterone metabolites. Employing a recently introduced strategy for metabolite identification, threefold deuterated 19-nortestosterone (16,16,17-$^{2}H_3$-NT) was administered to one healthy male volunteer and urine samples were collected for 20 days. Samples were prepared with established methods separating unconjugated, glucuronidated and sulfated steroids, and analytes were further purified by means of high-performance liquid chromatography before trimethylsilylation. Deuterated metabolites were identified using gas chromatograph/thermal conversion/isotope ratio mass spectrometer comprising an additional single quadrupole mass spectrometer. Additional structural information was obtained by gas chromatography/time-of-flight mass spectrometry and liquid chromatography/high resolution mass spectrometry.

In general, sulfo-conjugated metabolites were excreted for a longer time period than the corresponding glucuronides. Several unexpected losses of the arguably stable isotope labels were observed and characterized, attributed to metabolic reactions and sample preparation procedures. The detection window of one of the newly detected metabolites was higher than currently used metabolites. The suitability of this metabolite to differentiate between endo- or exogenous sources could however not be verified conclusively.

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1. Introduction

Most substances relevant to doping controls undergo considerable metabolism following administration. The generated metabolites are commonly less toxic and more polar which allows for renal clearance. Consequently, in urine the analysis of metabolites is more promising than attempts to detect the ingested or injected compound, particularly when these metabolites are excreted for a longer time period than the drug. These so-called long-term metabolites are of great interest especially for those doping agents that are predominantly used during out-of-competition periods as for instance in case of anabolic androgenic steroids [1–6].

In order to identify drug metabolites in biological matrices, stable isotope labeling of compounds has proven to be a helpful tool over the past decades. Commonly, hydrogen is replaced by its heavier isotope deuterium at 3 to 5 locations within the steroid backbone and the resulting mass shift is detected by means of conventional mass spectrometers. The sensitivity of this approach has recently been considerably improved by using hydrogen isotope ratio (HIR) mass spectrometry instead of conventional mass spectrometers [7]. These HIR-dedicated systems are built to determine the deuterium/hydrogen ratio at natural abundance, i.e. with approximately 1 out of 10,000 hydrogen atoms representing a deuterium atom. For deuterium-labeled compounds commonly up to 10% of hydrogen is substituted and thus enables a highly sensitive detection of any metabolite of the administered compound as long as it comprises the deuterium-label.

In a pilot study performed with deuterated metandienone, the potential of combining isotope ratio mass spectrometry (IRMS) with high and low resolution mass spectrometry (MS) to identify minor and long-term metabolites of a compound was demonstrated [7]. A similar approach was to be exploited to investigate the metabolism of nortestosterone (17β-hydroxyestra-4-en-3-one, NT). Since numerous years NT is within the scope of sports drug testing programs and an extended systematic study of its metabolism appears warranted to support both its long-term detection and the determination of its source (i.e. endogenous or artificial). Currently, doping control analyses concerning NT rely on the detection of one main metabolite referred to as norandrosterone (NA) excreted conjugated as glucuronide. Findings beyond the established threshold of 2 ng/mL trigger carbon isotope ratio investigations due to the aforementioned possibilities of endogenous or artificial production of the analyte [8]. Besides other known and well described urinary metabolites such as noretiocholanolone (NE) or norepiandrosterone (NEpiA) [9,10], a substantial number of additional hydroxylated or reduced metabolites is expected to exist that could support and facilitate the separation of an endogenous NA production from NT administration. During phase-II-metabolism and prior to renal elimination, many metabolites are glucuronidated (GLUC) or sulfated (SULF) to increase water solubility. Therefore the sample preparation procedure was adopted to address both conjugates separately and to also include possible unconjugated metabolites. Additionally, due to the expected large number of metabolites and their presumed large variance in urinary concentrations, all samples were further purified and separated into different fractions using a high-performance liquid chromatography (HPLC) system. As it has recently been reported that testosterone metabolites may also be excreted as cysteine bound metabolites, samples were further analyzed for potential metabolites cleaved after alkaline hydrolysis of urine specimens [11].

2. Experimental

2.1. Reagents and chemicals

Steroid reference material including NA (3α-hydroxy-5α-estran-17-one), NE (3α-hydroxy-5β-estran-17-one), NEpiA (3β-hydroxy-5α-estran-17-one) and NT (17β-hydroxyestra-4-en-3-one) was purchased from Steraloids (Newport, RI, USA). Threefold deuterated NT (16,16,17-$^{2}H_3$-NT) was synthesized in house [12,13]. All solvents and reagents were of analytical grade.

2.2. Excretion study

The study was carried out by one male volunteer (39 year, 80 kg, Caucasian). The deuterated NT (40 mg) was dissolved in 20 mL of ethanol/water (50/50, v/v) and administered orally. One urine sample was collected as blank urine prior to the administration followed by the collection of all morning urine samples for the next 20 days. All samples were stored frozen at −20 °C until preparation. The local ethic committee approved the study and written consent was given by the volunteer.

2.3. Sample preparation for gas chromatography-based determinations

The sample preparation strategies for analyzing glucuronidated and sulfated steroids has been delineated in extenso before [14,15] and is described only in brief in the following.

In order to enable the sensitive detection of metabolites present at lowest concentrations, up to 30 mL of urine were applied to Chromabond® C18 cartridges (500 mg, 6 mL) from Macherey & Nagel (Düren, Germany), conditioned with 2 mL of methanol (MeOH) and 2 mL of water. After washing with 2 mL of water, columns were eluted 3 times with 1 mL of MeOH. The combined eluate was dried under a stream of nitrogen and reconstituted in 2 mL of aqueous phosphate buffer (pH 7). A first

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