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Automated and sensitive determination of four anabolic androgenic steroids in urine by online turbulent flow solid-phase extraction coupled with liquid chromatography–tandem mass spectrometry: A novel approach for clinical monitoring and doping control

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ABSTRACT

A novel method for automated and sensitive analysis of testosterone, androstenedione, methyltestosterone and methenolone in urine samples by online turbulent flow solid-phase extraction coupled with high performance liquid chromatography–tandem mass spectrometry was developed. The optimization and validation of the method were discussed in detail. The Turboflow C18-P SPE column showed the best extraction efficiency for all the analytes. Nanogram per liter (ng/L) level of AAS could be determined directly and the limits of quantification (LOQs) were 0.01 ng/mL, which were much lower than normally concerned concentrations for these typical anabolic androgenic steroids (AAS) (0.1 ng/mL). The linearity range was from the LOQ to 100 ng/mL for each compound, with the coefficients of determination (r^2) ranging from 0.9990 to 0.9999. The intraday and interday relative standard deviations (RSDs) ranged from 1.1% to 14.5% ($n=5$). The proposed method was successfully applied to the analysis of urine samples collected from 24 male athletes and 15 patients of prostate cancer. The proposed method provides an alternative practical way to rapidly determine AAS in urine samples, especially for clinical monitoring and doping control.

1. Introduction

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone originally designed for therapeutic uses to provide enhanced anabolic potency with negligible androgenic effect [1,2]. These substances promote the growth of skeletal muscle and the development of male sexual characteristics. They are naturally produced in the testicles and adrenal glands and can also be synthetically created. Testosterone is one of the predominant endogenous anabolic androgenic steroids [3]. It has anabolic effects, causing muscle and bone growth and maturation. Androstenedione is the most common precursor of sex hormones [4]. It works by increasing the amount of androstenedione which in turn increases the amount of testosterone in the body [4,5]. Exogenous anabolic androgenic steroids which have more anabolic and less androgenic activity than testosterone are synthesized and used therapeutically in medicine. Methyltestosterone is the first synthetic anabolic steroids and its importance of therapeutic use was recognized in the 1950s [6]. Since then, a large amount of anabolic steroids are synthesized and tested.

Both endogenous and exogenous AAS continue to be used clinically today to treat conditions resulting from steroid hormone deficiency, such as delayed puberty, as well as diseases that result in loss of lean muscle mass, such as cancer and AIDS [7]. However, long term usage of anabolic steroids can cause many adverse effects such as gynecomastia, elevated blood pressure, liver damage, cardiovascular disease or coronary artery disease. Serum concentrations of AAS are of particular importance and often monitored for diagnosis, treatment or research on the relationship between their concentrations and diseases [8–11]. Urinary concentrations are also received great attention because of its non-invasive detection [12–14]. Furthermore, AAS are even misused by athletes to improve their sports performance [6,15]. Although the use of AAS has been banned in sports and governed by the World Anti-Doping Agency since 1976, some of the priority controlled substances such as methenolone were still detected in urine at the

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London Olympic Games in 2012 [16] and the National Basketball Association Championship (USA) in 2013 [17], which are regulated as “Zero detection” in the games.

Some analytical methods have been used to analyze anabolic androgenic steroids, including Immunoassay [18], GC–MS/MS [19,20] and LC–MS/MS [21–23]. Immunoassay is a feasible method in the clinical laboratory but it is susceptible to matrix interference especially for determination of low concentration. The specificity also limits its application for simultaneous detection of a number of analytes. GC–MS/MS is widely used due to its high sensitivity and selectivity, but the derivatization procedure is complicated and sometimes the derivative efficiency is low. LC–MS/MS with relative simpler pretreatment, good sensitivity and selectivity receives analysts' favor especially in the analysis of low levels of AAS in biological samples [24]. The traditional pretreatment procedures, such as liquid–liquid extraction and off-line solid phase extraction, are not only time-consuming but also solvent and labor cost. Meanwhile, the operative error is hard to be avoided. In order to simplify the pretreatment procedure and improve the method sensitivity, some new techniques such as molecular imprinted polymer solid phase extraction (SPE) [25,26], solid phase microextraction (SPME) [27], stir bar microextraction (SBME) [28] and online SPE [29,30], have also been applied to determine AAS in diverse biological samples.

In this work, an automated and sensitive method for detecting testosterone, androstenedione, methyltestosterone and methenolone in urine by online turbulent flow solid-phase extraction coupled with liquid chromatography–tandem mass spectrometry (TF-SPE–HPLC–MS/MS) was developed. The development, validation and implementation of the method were discussed in detail. The LOQs were 10–100 times lower than the reported method, and the nanogram per liter (ng/L) level of AAS could be determined directly with large volume injection of 1.0 mL urine samples. The proposed method was successfully used to analyze 39 urine samples including 24 ones from male athletes and 15 ones from patients of prostate cancer.

2. Experimental

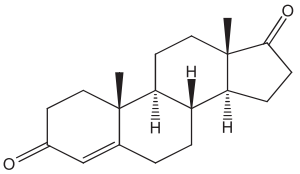
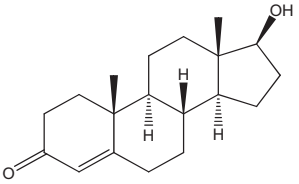
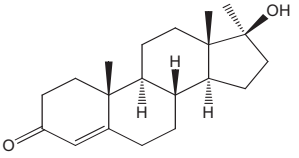
2.1. Chemicals and materials

Standards for testosterone (T), androstenedione (ADD), methyltestosterone (MTT), were purchased from Dr. Ehrenstorfer GmbH, Germany. Methenolone (MET) was purchased from Sigma-Aldrich, USA. Their molecular structures are shown in Table 1. Stock solutions (1 mg/mL) were prepared by dissolving 10 mg of each standard in 10 mL of methanol and stored at 4 °C. Working solutions were prepared by diluting the stock solutions with water. HPLC grade Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ). HPLC grade formic acid was purchased from Dikma Technology Inc., USA. Ultrapure water was produced with a Milli-Q system (Millipore, Billerica, MA). All reagents were of analytical grade unless otherwise noted.

2.2. Instrumentation

The UltiMate™ 3000 system (Thermo, USA) consisted of a WPS-3000TSL autosampler with large-volume loop (2.5 mL) for injection. A TCC-3200 thermostated column compartment with a two-position, six-port (2P–6P) valve, a DGP 3600M dual-gradient pump, and a SRD 3600 solvent rack with integrated vacuum degasser were included. The whole system was controlled by Chromeleon® Chromatography Management Software (v. 6.80, Dionex, USA). A Quattro Ultima triple quadrupole mass spectrometer (Premier XE, Waters, USA) equipped with an electron spray ionization (ESI) source (Waters, USA) was used for measuring target compounds. The data were recorded by Masslynx 4.1 software.

Table 1
The molecular structures of the four AAS and their MRM parameters for tandem mass spectrometry.

Compounds	MRM transitions (m/z)	Dwell time (s)	Cone (V)	Collision (V)	Ion mode	Molecular structures
ADD	287 > 97 287 > 109	0.1	30 30	20 20	PI	
T	289 > 97 289 > 109	0.1	40 40	25 25	PI	
MTT	303 > 97 303 > 109	0.1	40 40	35 35	PI	
MET	303.4 > 82.9 303.4 > 187	0.1	35 35	20 20	PI