Steroids 75 (2010) 710–714

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Analysis of anabolic steroids in human hair using LC–MS/MS

Nawed Deshmukh[a,*], Iltaf Hussain[a], James Barker[a], Andrea Petroczi[b], Declan P. Naughton[b]

[a] School of Pharmacy and Chemistry, Kingston University, London, UK
[b] School of Life Sciences, Kingston University, London, UK

ARTICLE INFO

Article history:
Received 3 March 2010
Accepted 22 April 2010
Available online 18 May 2010

Keywords:
Human hair
Anabolic steroids
Stanozolol
Nandrolone
LC–MS/MS
Doping

ABSTRACT

New highly sensitive, specific, reliable, reproducible and robust LC–MS/MS methods were developed to detect the anabolic steroids, nandrolone and stanozolol, in human hair for the first time. Hair samples from 180 participants (108 males, 72 females, 62% athletes) were screened using ELISA which revealed 16 athletes as positive for stanozolol and 3 for nandrolone. Positive samples were confirmed on LC–MS/MS in selective reaction monitoring (SRM) mode. The assays for stanozolol and nandrolone showed good linearity in the range 1–400 pg/mg and 5–400 pg/mg, respectively. The methods were validated for LLOD, interday precision, intraday precision, specificity, extraction recovery and accuracy. The assays were capable of detecting 0.5 pg stanozolol and 3.0 pg nandrolone per mg of hair, when approximately 20 mg of hair were processed. Analysis using LC–MS/MS confirmed 11 athletes' positive for stanozolol (5.0 pg/mg to 86.3 pg/mg) and 1 for nandrolone (14.0 pg/mg) thus avoiding false results from ELISA screening. The results obtained demonstrate the application of these hair analysis methods to detect both steroids at low concentrations, hence reducing the amount of hair required significantly. The new methods complement urinalysis or blood testing and facilitate improved doping testing regimes. Hair analysis benefits from non-invasiveness, negligible risk of infection and facile sample storage and collection, whilst reducing risks of tampering and cross-contamination. Owing to the wide detection window, this approach may also offer an alternative approach for out-of-competition testing.

1. Introduction

Anabolic androgenic steroids (AASs) are synthetic derivatives of the endogenously produced male sex hormone testosterone which exhibit both anabolic (protein synthesizing) and androgenic (masculinising) effects [1]. AASs are one of the most potent and the most widely used performance-enhancing substances and were thus included in the list of banned substances by the International Olympic Committee (IOC) since the mid-1970s [1–3]. According to 'The 2010 Prohibited List' of the World Anti-Doping Agency (WADA), both exogenous and endogenous AASs belong to Class S1.1 and their use in- and out-of-competition is prohibited [4]. Despite these restrictions, AAAs are still commonly abused by athletes [5–7], in particular nandrolone and stanozolol as revealed by numerous adverse analytical findings during the past few years [8]. As the current official prohibition-based anti-doping approach automatically places the emphasis on detection, there is an ever-increasing need to develop new methods for testing drug abuse in sports for both detection and deterrence [9].

Urinalysis carried out in accredited laboratories is generally considered to be a standard technique for detecting drug doping. The major disadvantage of urinalysis is that it provides only short term information of the individual's drug consumption and does not determine sustained drug abuse [2]. Thus, it is not particularly effective at identifying athletes who partake in long term steroid use during training periods and cease to take them prior to competition, resulting in a sufficient drug-free period to give false negative results. Inter-individual variations in the amount of steroids detected in urine have also been reported owing to differences in the prevalence of UGT2B17, a major steroid metabolizing enzyme [10]. In addition, urinalysis can lead to false positive results due to single accidental intake of steroids [11] leading to debate whether the presence of drug is considered as doping. Therefore for anti-doping purposes, it is necessary to identify a matrix which can prevent such ambiguous results.

Hair analysis has been employed for the past three decades to detect chronic drug use and is gaining attention in controlling drug doping [2]. Hair analysis provides wider surveillance window (weeks to months) depending on hair length which helps in scrutinizing long term histories of drug use [12]. Considering that the average human hair growth rate is 1–1.5 cm per month, hair samples of a few centimetres will provide retrospective information on drug intake well beyond than that obtained from urine or blood

* Corresponding author.
E-mail address: D.Naughton@kingston.ac.uk (N. Deshmukh).

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doi:10.1016/j.steroids.2010.04.007

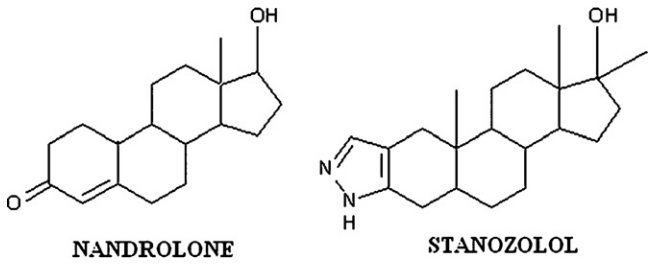

Fig. 1. Structures of nandrolone and stanozolol [1].

specimens [13]. Thus, hair can be an ideal matrix to detect out-of-competition doping. Anabolic steroids are found in the picograms per milligram range in hair and generally get incorporated into the hair matrix by a number of ways: (i) endogenous pathway: passive diffusion of the drug molecule from systemic circulation into growing hair, and (ii) endogenous–exogenous pathway: absorption or transfer of drug molecules into hair shaft from transdermal excretion, sweat and sebum [14–16].

The process of drug incorporation into the hair matrix depends on several factors such as pharmacokinetics, bioavailability, dosage and physico-chemical properties of the drug molecule. Individual variations like metabolic pathways, hair pigmentation and cosmetic treatments may also influence the concentrations of drug detectable in hair. Hair has an isoelectric pH close to 6 and thus favours incorporation of undissociated basic drugs. Amongst the anabolic steroids, stanozolol gets preferentially distributed into pigmented hairs due to the basic nature of the pyrazole ring present in its chemical structure (Fig. 1).

Researchers have observed that hair analysis favours the detection of parent drug in comparison to their metabolites. This owes to the fact that parent drugs are less polar and hence their incorporation into the keratin matrix is favoured. Conversely, drug metabolites are mainly detected in urine in the form of conjugates and relatively low concentrations of parent drugs are excreted in urine. For example, detection of stanozolol in hair is more facile than in urine, whereas the opposite is the case for 3'OH stanozolol (major metabolite of stanozolol) [17,18].

Hair testing is non-invasive, tamper resistant and the most convenient technique for the detection and control of drug doping in comparison to urinalysis and blood tests. Although hair analysis may not completely replace urinalysis it can be used in special cases to provide information complementary to that obtained from urine and blood tests. It can be used to confirm repetitive intake of drugs by directly detecting the parent drugs. The aim of this work was to develop sensitive, specific and reproducible methods for the detection and quantification of stanozolol and nandrolone in human hair using ELISA and tandem liquid chromatography-mass spectrometry. The hair samples were first screened by ELISA. All positive samples were then confirmed using the robust, quantitative technique of tandem liquid chromatography-mass spectrometry.

2. Materials and methods

2.1. Specimens

Hair samples were obtained from 180 European participants (108 males, 72 females, 62% athletes) of age ranging from 18 to 53 years. A positive hair control sample for stanozolol was obtained from a male body builder who reported use of stanozolol for the past 30 years by oral and intramuscular route. All hair samples consisted of approximately 50 untreated hair strands minimum 3 cm in length (circa 100 mg in weight), cut directly at the skin surface at the vertex posterior of the head. This area was preferred as it shows less variability in hair growth rate than compared to other areas. The hair samples were stored individually in labelled, sealable paper envelopes, according to the protocols established and approved by the Kingston University Faculty Research Ethics Committee. Blank hair was obtained from healthy, non-athlete volunteers in a similar manner.

2.2. Standards and reagents

ELISA kits for nandrolone and stanozolol were obtained from Neogen Corporation (Lexington, KY 40511, USA). Each kit comprised of a specific enzyme immunoassay (EIA) buffer, wash buffer concentrate, drug–enzyme conjugate, 3,3′,5,5′-tetramethylbenzidine substrate (TMB), stop solution and antibody coated plate containing 96 wells. Nandrolone, stanozolol and stanozolol D3 (internal standard) were obtained from LGC standards (Teddington, UK). Dichloromethane, pentane, sodium hydrogen phosphate heptahydrate, sodium phosphate monobasic dihydrate, sodium hydroxide, hydrochloric acid, deionised water, formic acid and acetonitrile were obtained from Sigma Aldrich (Poole, Dorset, UK). All chemicals and reagents were of HPLC grade.

2.3. Sample preparation and analysis

Hair samples were initially decontaminated by rinsing them with 2 mL dichloromethane. This ensures that hair is free from contaminants like sweat, sebum, shampoo, etc., which may interfere with analysis. After decontamination, hair samples were cut into 1 mm segments which were then used to carry out both ELISA screening as well as LC–MS/MS confirmatory analysis.

2.3.1. Enzyme linked immunosorbent assay (ELISA)

For ELISA screening, 50 mg of hair segments were incubated with 1 mL of 1 M sodium hydroxide at 95 °C for 15 min. Prior to screening, the homogenate was neutralized with hydrochloric acid and then diluted with EIA buffer (1:1, v/v). The ELISA screening system consisted of a Cary 50 MPR microplate reader (Varian, UK). The manufacturer's protocol was followed for analysis. The ELISA kit employed operated on the basis of competition between the drug in the hair matrix and the drug–enzyme conjugate for a limited number of antibody binding sites on the microplate. Initially, the samples were added to the wells followed by addition of the drug–enzyme conjugate. After incubation, the wells were washed using the wash buffer supplied with the kit. Washing was essential to remove any unbound drug from each well. The presence of bound drug–enzyme conjugate was recognized using 3,3′,5,5′-tetramethylbenzidine (TMB). The enzymatic reaction was stopped after 30 min using a stop solution. The extent of colour development is inversely proportional to the amount of drug present in the hair. The absorbance of each well was measured at a wavelength of 450 nm. A calibration curve and quality controls were prepared in EIA buffer to ensure the kit was working properly. Controls were made by spiking blank hair preparations with known concentrations of selected drug. Hair samples were first screened by ELISA and all positive samples were then analysed on LC–MS/MS for confirmation.

2.3.2. LC–MS/MS procedure

The LC–MS/MS system consisted of an Accela UPLC system (Thermo Scientific, UK) coupled to a Triple Quadrupole TSQTM mass spectrometer (Thermo Electron Corp, UK). All positive samples were quantified using LC–MS/MS. For confirmation, 20 mg of hair segments were incubated with 1 mL 1 M sodium hydroxide at 95 °C for 15 min in the presence of stanozolol D3, which was used as an internal standard. After cooling the homogenate was neutralized with 1 M hydrochloric acid followed by addition of 2 mL

Table 1
LC mobile phase gradient composition.

LC run time (min)	Acetonitrile (%)	0.1% formic acid (%)
0	50	50
10	80	20
11	100	0
12	50	50
15	50	50

Table 2
Retention times, SRM transitions and conditions of each steroid.

Analytes	Retention time (min)	Transition (m/z)	Collision energy (eV)
Nandrolone	3.53	275.2 → 109.2	27
Stanozolol	4.74	329.2 → 81.1	50
Stanozolol D3 (I.S)	4.79	332.2 → 81.2	42

of 0.2 M phosphate buffer (pH 7.0). The homogenate was purified by liquid–liquid extraction (LLE) using pentane. After vortex mixing and centrifugation (4 min at 1257 × g), the organic layer was transferred into a fresh glass tube after filtering through a PTFE membrane (0.45 μm). The organic layer was then dried under a gentle stream of nitrogen gas at 50 °C. The extracted residue was reconstituted with 100 μL acetonitrile. A 4 μL aliquot of the solution was injected into the LC–MS/MS system. An Agilent ZORBAX SB-C18 column (2.1 mm × 50 mm, 1.8 μm) was used for analysis. The column oven temperature was maintained at 60 °C. The two mobile phases used composed of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The gradient flow composition is shown in Table 1. The total flow rate through the column was 100 μL/min.

The LC was interfaced with the MS/MS system without a flow split. The mass spectrometer was operated in the positive electrospray ionisation mode at a spray voltage of 4500 V and capillary temperature of 350 °C. The protonated molecules of nandrolone, stanozolol, and stanozolol D3 generated, act as precursor ions for collision induced dissociation (CID) for MS–MS analysis. Selective reaction monitoring (SRM) was used for the confirmation of steroids. The most abundant SRM ion transitions for each analyte were acquired using the conditions given in Table 2.

Calibration curves and quality controls at low, medium and high concentration levels were prepared by spiking negative hair control with known amounts of steroids and internal standard. The assays were validated for specificity, lower limit of detections (LLOD), intraday precision, interday precision, accuracy and recovery for each analyte. LLOD for both analytes were determined by decreasing their concentrations until a response equivalent to 3 times the background noise was observed. The relative extraction recoveries were determined by comparing the representative peak areas of nandrolone and stanozolol extracted from blank hair samples (N = 6) spiked at final concentration of 50 pg/mg with peak areas of standard solutions prepared in acetonitrile of the same final concentration. Validation results are summarised in Table 3.

3. Results and discussion

Steroids were identified and quantified on the basis of their retention times and relative abundance of their respective product ions. Assays were linear in the range 1–400 pg/mg for stanozolol and 5–400 pg/mg for nandrolone. The assays were capable of detecting 0.5 pg stanozolol and 3.0 pg nandrolone per mg of hair.

Amongst the 180 hair samples screened by ELISA, 16 were positive for stanozolol and 3 for nandrolone. In order to avoid any false positive results due to cross reactivity in ELISA, these 19 samples were analysed on LC-ESI–MS/MS for confirmation (Figs. 2 and 3). Twelve athletes were successfully confirmed positive for stanozolol and 1 for nandrolone. An athlete who admitted use of stanozolol for the past 30 years by oral and intramuscular routes was consid-

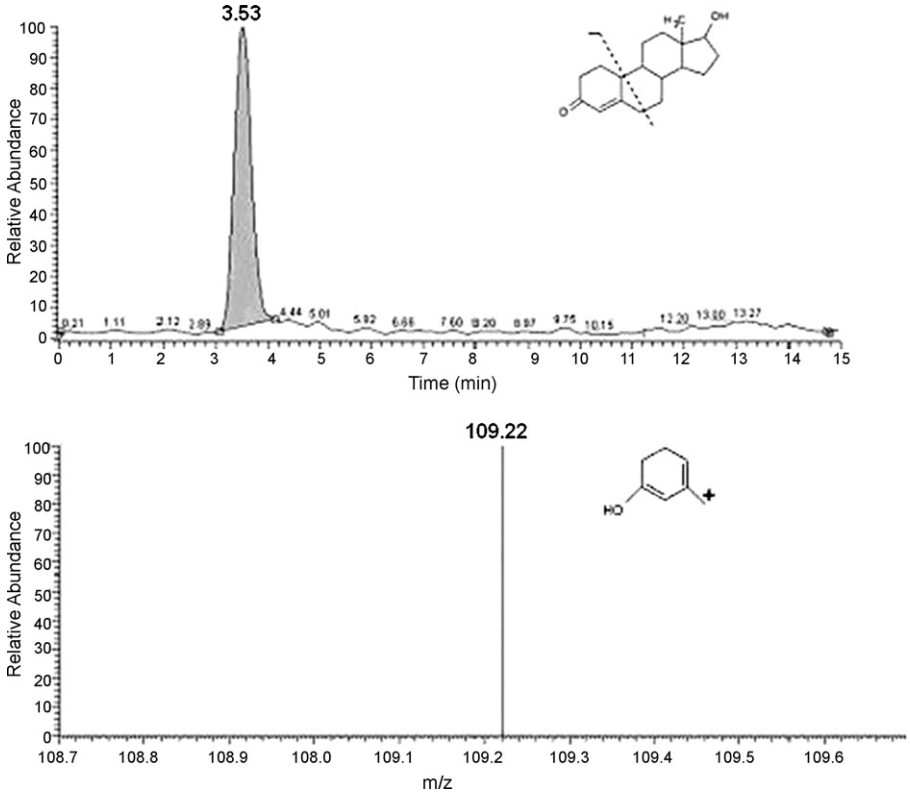

Fig. 2. LC–MS/MS chromatogram and CID spectra of nandrolone.

Table 3
Summary of assay validation results.

Compounds	Linear range (pg/mg)	LLOD (pg/mg)	Recovery (%) at 50 pg/mg ($N=6$)	Concentration (pg/mg)	Precision RSD (%)		Accuracy (%)
					Intraday $N=6+6+6$	Interday $N=18+18+18$	
Stanozolol	1–400	0.5	82.5	2.5	6.3	9.6	103.9
				20	11.4	6.7	92.9
				100	7.6	7.3	92.2
Nandrolone	5–400	3.0	43.1	10	9.1	8.8	99.4
				20	8.6	7.4	97.5
				100	6.3	5.5	106.7

ered as a positive control. Table 4 shows the quantity of steroids detected in hair.

The results indicate that the methods are efficient in detecting steroids in hair even at very low levels when only circa 20 mg hair was processed (Figs. 2 and 3). Less hair required for analysis makes the method more convenient for drug testing. Shen et al. quantified anabolic steroids in guinea pig hair at a concentration as low as 10 pg/mg using LC–MS/MS [19]. These newly developed methods for human hair analysis now provide extended LLOQs and LLODs and hence can be employed more efficiently for doping testing using circa 20 mg human hair.

Under the chromatographic conditions employed, there were no detectable interferences by extractable endogenous materials present in hair. Also, owing to the efficient hair decontamination step employed, no external interferences with hair analyses were observed. Since anabolic steroids get incorporated into the hair shaft in low quantities—alkali digestion of hair followed by purification using LLE combined with injection of 4 μL aliquot through the

Table 4
Hair analysis results of athletes using LC–MS/MS.

	Sex	Age	Stanozolol (pg/mg)	Nandrolone (pg/mg)
Control[a]	M	53	47.4	–
Athlete 1	M	22	5.0	–
Athlete 2	M	19	11.2	–
Athlete 3	M	21	33.0	–
Athlete 4	F	18	–	14.0
Athlete 5	F	19	9.8	–
Athlete 6	F	22	10.0	–
Athlete 7	F	20	12.7	–
Athlete 8	F	20	26.9	–
Athlete 9	F	18	40.2	–
Athlete 10	F	20	56.1	–
Athlete 11	F	22	63.3	–
Athlete 12	F	20	86.3	–

[a] Male bodybuilder who admitted use of stanozolol for the past 30 years by intramuscular and oral route.

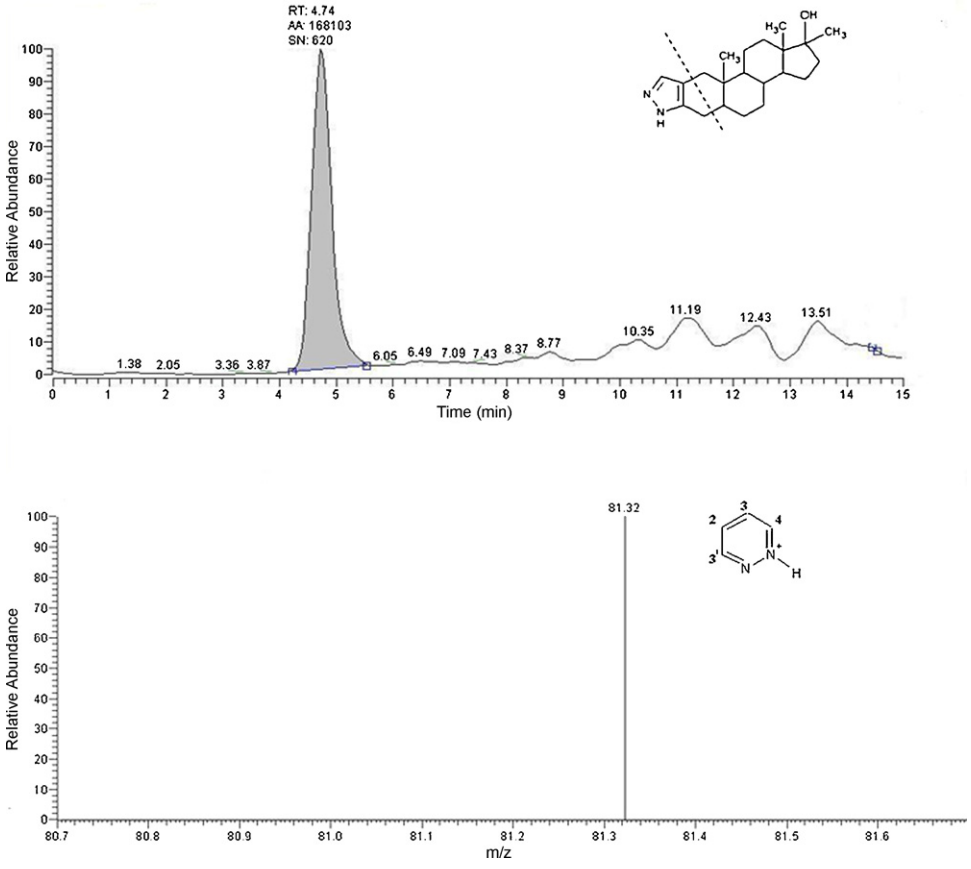

Fig. 3. LC–MS/MS chromatogram and CID spectra of stanozolol.

column were analytical prerequisites for proficient identification of target steroids in the hair matrix.

3.1. Application

To ensure doping-free sport, out-of-competition doping testing regimes are carried out by sport organizations to ensure that athletes are not using substances (steroids) [20]. Such doping control regimes require top elite athletes to be available for doping tests on a very regular basis. However, on a regular basis this can be cumbersome and time consuming for athletes. Hence, hair specimens that are obtained with less intrusion than urinalysis can be used to determine retrospective information on an individual's drug use. These new hair testing methods can also identify athletes who attempt to evade urinalysis or blood tests by deliberately stopping steroid intake before doping testing or by diluting urine, as these attempts will not affect drug concentration in hair [2,3].

Urinalysis can give false positive results due to single accidental intake of steroids or metabolites through contaminated food or water [21]. It has been found that nutritional supplements that are generally taken by athletes are either accidentally contaminated (during manufacturing, transportation or packaging) or deliberately adulterated with anabolic steroids in an attempt to improve the effectiveness of supplement products. De Cock et al. carried out a study and found that urinalysis reported false positive results for healthy volunteers who were administered with a nutritional supplement that was found to contain 19-nor-4-androstene-3, 17-dione as a non-labeled ingredient [11]. Urinalysis confirmed the presence of norandrosterone (nandrolone metabolite) at a concentration above 2 ng/mL. According to WADA, urine concentrations of nandrolone or its metabolite, exceeding 2 ng/mL indicates offense [22]. These findings reveal the possibilities of positive urinalysis results due to unintentional consumption of steroids. Stanozolol has been reported to be one of the most important synthetic anabolic steroids used to promote weight gain in food-producing animals and as a result its use is prohibited since 1981 by the European Union (Directive 81/602/EEC) [17,18]. Inline with De Cock et al. [11], it can be assumed that urinalysis of individuals who consume meat from animals that may be administered with steroids may also lead to false doping results. However, this will depend on several factors like time between steroid administration and animal slaughter, metabolic pathway, dosage and route of administration of steroids in animals and individuals diet. This can be unjust for those athletes who do not practice doping but unknowingly consume such contaminated nutritional supplements, meat and water.

4. Conclusions

The new methods that have been developed are sensitive, specific, reliable and reproducible for the determination of stanozolol and nandrolone when circa 20 mg human hair is processed. Hair specimens can be collected without invasion of privacy and are easy to store. The extended lower limit of detection and lower limit of quantification are capable of detecting these steroids even at very low concentrations, hence reducing the amount of hair required to 14–15 hairs affording sample collection without leaving noticeable bald patch. These methods facilitate improved doping testing regimes that are non-invasive, tamper resistant and benefit from facile storage and negligible risk of infection. Owing to the wide detection window, this approach may also offer an alternative approach for out-of-competition testing. This methodology can be used in combination with urinalysis or blood test in special cases to prevent false results so that no athlete can evade doping testing.

Acknowledgements

This research was partially supported by the World Anti-Doping Agency Social Science Research Grant 2008-09/Petroczi. The authors thank the athletes who participated in this study.

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