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Detection of Anabolic Steroids in Head Hair

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ABSTRACT: We developed a gas chromatography/mass spectrometry method for detection and quantitation of anabolic steroids in head hair. Following alkaline digestion and solid-phase extraction, the MO-TMS derivatives gave a specific fragmentation pattern with EI ionization. For stanozolol, the TMS-HFBA derivative showed several diagnostic ions. For androstanolone, mestanolone (methylandrostanolone), and oxymetholone two chromatographic peaks for cis and trans isomers of derivatives were seen. Recoveries were 35 to 45% for androstanolone, oxymetholone, chlorotestosterone-acetate, dehydromethyltestosterone, dehydrotestosterone, fluoxymesterone, mestanolone, methyltestosterone, and nandrolone; 52% for mesterolone, trenbolone; 65% for bolasterone; 24% for methenolone and 17% for stanozolol. Limits of detection were 0.002 to 0.05 ng/mg and of quantitation were 0.02 to 0.1 ng/mg. Seven white male steroid abusers provided head hair samples (10 to 63 mg) and urine. In the hair samples, methyltestosterone was detected in two (confirmed in urine); nandrolone in two (also confirmed in urine); dehydromethyltestosterone in four (but not found in urine); and clenbuterol in one (but not in urine). Oxymethalone was found in urine in one, but not in the hair. One abuser had high levels of testosterone: 0.15 ng/mg hair, and 1190 ng/mL urine. We conclude that head hair analysis has considerable potential for the detection and monitoring of steroid abuse.

KEYWORDS: forensic science, hair, anabolic steroids, clenbuterol, substance abuse detection

Anabolic steroids include both natural and synthetic hormonal drugs. Although banned, they continue to be abused by athletes and bodybuilders (1–3). Athletes often take massive doses for months but abstain for weeks before competition and use diuretics to dilute the urine prior to drug testing, making detection more difficult (4).

Hair has been proposed as an alternative to urine for detecting drug abuse, and many drugs can be quantified in human hair (5,6). An important feature of hair analysis is the possibility of detecting drugs in the hair for a long period of time after use. Consequently the method may have particular value in detecting steroids, given their typical pattern of misuse. Detection of the anabolic steroid methyltestosterone has been reported in calf hair using high-performance liguid chromatography (HPLC) and enzyme immunoassay (7), and stanozolol has been detected in rat hair by gas chromatography/mass spectrometry (GC-MS) (8). We have previously reported the quantification of endogenous testosterone, epitestosterone, oestradiol and progesterone in human hair by GC-MS (9). In the present study we have used a similar methodology to detect anabolic steroids in head hair as well as urine from known steroid abusers.

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Methods

Testosterone, epitestosterone, oestradiol, progesterone, androstanolone, bolasterone, clenbuterol, chlorotestosterone acetate, dehydromethyltestosterone, dehydrotestosterone, fluoxymesterone, mestanolone, mesterolone, methenolone, methyltestosterone, nandrolone, oxymetholone, stanozolol, trenbolone, deuterium-labeled testosterone, methoxyamine hydrochloride (MO), methanol, pyridine, trimethylsilylimedazole (TSIM) were purchased from Sigma (Poole, UK). N,O-bis-(trimethyl-silyl)-trifluroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL), N-methyl-N-trimethylsilyl-heptafluorobutyramide (MSHFBA) from Aldrich (Dorset, UK) and N-methyl-bis-heptafluorobutyramide (MBHFBA) from Camlab Ltd (Cambridge, UK). C_{18} Isolute SPE columns (200 mg, 500 mg sorbents) were purchased from International Sorbent Technology Ltd (Tiry-Berth Industrial Estate, Mid-Glamorgan, UK). All other solvents and reagents employed were of analytical grade or higher.

The gas chromatograph was a Fisons GC 8000 series fitted with a WCOT fused silica column (CP-Sil 5 CB, 15 m $\times$ 0.25 mm id, DF 0.25 μm). Samples were injected using a Fisons AS 800 autosampler and a split injector (1 min delay). Helium was the carrier gas (1 mL/min) and 1 μL of reconstituted extract was injected. The mass spectrometer was a Fisons MD 800 with Finnigan Masslab software. The injector temperature was 250°C and the column oven was programmed from 100°C (held for 1.0 min) at 20°C/min to 300°C (held for 5.0 min). Acquisition was performed in selected ion monitoring (SIM) mode using electron impact (EI) ionization at 70 eV. Individual scans of derivatized steroids and standards were performed to obtain the mass fragmentation patterns. The most suitable ions (high ion intensity, high mass and low background) were selected for analysis in SIM mode.

Urine for calibration curves was from a child; hair samples for calibration curves and recovery studies were from a four-year-old boy; steroid abusers' urine and full-length hair samples, collected from the posterior vertex, were provided by a local harm reduction clinic. No self-reported drug use data were available from the sample donors, who were all adult white males. For urine extraction, to 5 mL of sample an equivalent volume of acetate buffer (0.1 M, pH 5.6) was added, and hydrolyzed at 50°C for 2 h with 150 μL of β-glucuronidase (98 700 units/mL) from Helix pomatia, type HP-2. After addition of 50 ng of deuterium-labeled testosterone internal standard, the hydrolyzed urine was passed through C_{18} Isolute SPE cartridges (500 mg sorbent) that had been pre-conditioned by sequentially adding 5 mL of methanol and then 5 mL of distilled water. After loading the samples, the cartridges were washed with 5 mL of 10% methanol and eluted with 5 mL of methanol. The extract was dried under nitrogen at 50°C. After cooling to room temperature, the residue was dissolved in 2 mL

of acetate buffer (0.1 M, pH 5.6), and then about 50 mg of 10:1 (w/w) mixture of sodium bicarbonate-sodium carbonate was added (pH 8.5). The extraction was performed twice with 3 mL of diethyl ether, the organic layer dried over anhydrous sodium sulfate and evaporated to dryness under nitrogen at 50°C.

The hair samples were not segmented for analysis. The full-length hair samples were washed three times with 5 mL phosphate buffer (pH 7.4) and once with 5 mL dichloromethane, then cut into small pieces, mixed thoroughly and accurately weighed. For hair sample digestion, 2 mL of 1 N NaOH (sodium hydroxide) was added and heated at 60°C for 30 min. After digestion the sample pH was adjusted to 5.6 with 6 N HCl (hydrogen chloride). The sample was mixed and then centrifuged at 2500 rpm for 5 min. Deuterium-labeled testosterone internal standard (50 ng) was added at this time (there was no significant difference in recovery whether the internal standard was added before or after digestion). The sample was loaded onto C_{18} Isolute SPE cartridges (200 mg sorbent) which had been preconditioned by sequentially adding 2 mL of methanol and 2 mL of distilled water. After loading the samples, the cartridges were washed with 2 mL of 10% methanol and eluted with 2 mL of methanol. The extract was dried under nitrogen at 50°C.

For derivatization (10), a solution of methoxyamine hydrochloride (2%, w/v in pyridine, 50 μL) was added to each extract and heated at 60°C for 30 min. The residue was dried. Triethylamine (0.05 M in pyridine, 100 μL) and BSTFA with 1% TMCS (50 μL) were added and the resulting mixture was heated at 100°C for 15 min. Following incubation, the solvent was evaporated at 50°C under nitrogen and the residue was reconstituted in toluene (50 μL) and analyzed by GC-MS. For stanozolol derivatization (8), MSHFBA-TSIM (40 μL, 1000:20, v/v) was added; the vials were vortex mixed and then heated at 80°C for 5 min. After cooling to room temperature, 10 μL of MBHFBA was added. The mixture was vortex mixed and heated at 80°C for 30 min. The vials were allowed to cool to room temperature, and the liquid was analyzed by GC-MS.

Extraction recovery was determined by preparing two sets of samples. Set A ($n = 10$) consisting of androstanolone, bolasterone, clenbuterol, chlorotestosterone, dehydromethyltestosterone, dehydrotestosterone, fluoxymesterone, mestanolone, mesterolone, methenolone, methyltestosterone, nandrolone, oxymetholone, stanozolol, and trenbolone (25 ng of each). This set was added to blank hair, digested, extracted and derivatized as described above, except that the deuterium-labeled testosterone (50 ng) was added to the organic solvent immediately prior to the first evaporation. For set B ($n = 10$), the same compounds (25 ng of each), and deuterium-labeled testosterone (50 ng) were directly derivatized and analyzed. The compound:internal standard ratio for set A was expressed as a percentage of the ratio for set B to obtain the percent recovery.

Repeatability of the chromatographic analysis was determined by ten replicate 1-μL injections of a mixture of derivatized standards. Reproducibility for hair samples was evaluated by ten injections of 1 μL of hair samples at 25 ng/mg. Hair aliquots (50 mg each) spiked with increasing amounts of standards (0.05, 0.1, 0.5, 1 and 5 ng/mL, corresponding to 0.001, 0.002, 0.01, 0.02, and 0.1 ng/mg) were analyzed to determine the detection limit. A hair blank without added anabolics was analyzed at the same time in duplicate. The limit of detection was determined as the lowest concentration producing a chromatographic peak with a signal to noise ratio greater than or equal to 3. The limit of quantitation was determined as the lowest concentration at which the calibration graph was linear. All samples were run in duplicate and within one run.

Urine and hair from seven male steroid abusers were analyzed using the above procedure.

Results and Discussion

The ions chosen for analysis are listed in Table 1. Under the chromatographic conditions used, there was no interference with the anabolic agents or deuterated internal standard by any extractable materials in the control human urine and hair. With EI ionization, the MO-TMS derivatives gave a specific fragmentation pattern: M^+, $[M-15]^+$(-CH3), $[M-31]^+$(-OCH3), $[M-90]^+$(-TMSOH) or $[M-105]^+$(-CH3-TMSOH). For chlorotestosterone acetate, only the MO derivative was formed because the 17-position is occupied by acetate, which hinders reaction with TMS. Consequently the fragmentation pattern for chlorotestosterone acetate is M^+, $[M-15]^+$(-CH3), $[M-31]^+$(-OCH3), $[M-35]^+$(-Cl).

TABLE 1—*Ions of MO-TMS derivatives of steroids.*

Compound	M[a]	M[b]	Diagnostic Ions, m/z	Retention Time, min
Androstanolone	290.22	***391.29***	286.22, 360.27, 376.27, 391.29	10.12
Bolasterone	316.24	***417.31***	327.21, 386.29, 402.28, 417.31	10.65
Clenbuterol*	277.09	420.16[c]	***86.10,*** 243.00, 262.02, 333.05	6.87
Chlorotestosterone-Acetate	364.18	***393.21***[d]	358.24, 362.19, 378.18, 393.21	11.45
Dehydromethyl-testosterone	300.21	***401.28***	298.22, 370.26, 386.25, 401.28	10.38
Dehydrotestosterone	286.19	***387.26***	340.21, 356.24, 372.24, 387.26	10.20
Epitestosterone	288.21	***389.27***	268.21, 358.26, 374.25, 389.27	9.77
Fluoxymesterone	336.21	***437.28***	347.23, 404.24, 422.25, 437.28	11.48
Mestanolone	304.24	405.31	360.28, 375.30, ***390.28,*** 405.31	10.40
Mesterolone	304.24	***405.31***	315.26, 374.29, 390.28, 405.31	10.17
Methenolone	302.46	***403.29***	360.28, 372.27, 388.27, 403.29	10.53
Methyltestosterone	302.23	***403.29***	300.23, 372.27, 388.27, 403.29	10.40
Nandrolone	274.19	***375.26***	328.21, 344.24, 360.24, 375.26	9.93
Oxymetholone	332.24	462.33	399.26, ***431.29,*** 447.30, 462.33	11.53
Testosterone	288.21	***389.27***	268.21, 358.26, 374.25, 389.27	10.05
$[^2H_3]$-Testosterone (I.S.)	291.41	***392.29***	271.23, 361.28, 377.27, 392.29	10.00
Trenbolone	270.16	***371.23***	266.15, 340.21, 356.20, 371.23	10.32
Stanozolol**	328.25	596.27	491.19, 506.22, ***581.24,*** 596.24	11.43

[a] Molecular mass; [b] Molecular mass of MO-TMS; [c] Molecular mass of clenbuterol-*bis*-TMS (cannot be seen in the spectrum); [d] Molecular mass of MO-chlorotestosterone-acetate; * β-agonist; ** TMS-HFBA form; I. S. = internal standard; ions in bold italics were used for quantitation.

Clenbuterol is a β-agonist used as a growth promoter, and is the best known β-agonist having an anabolic effect (11). The mass spectrum of its bis-TMS derivative showed mainly a base peak formed by α-cleavage fragment ions. No molecular ion (M^+ 420) was detected and confirmation relies on its characteristic ions, which are of suitable intensity. With MO-TMS derivatization, stanozolol-TMS was formed [M^+ 400] but its chromatographic behavior was poor because the active hydrogen of the NH group in the pyrazole ring, which is largely responsible for the poor chromatographic behavior, was not converted. With MSHFBA-TSIM and MBHFBA derivatization, both active hydrogens of the NH and OH groups were derivatized and stanozolol TMS-HFBA was formed. The mass spectrum of stanozolol TMS-HFBA showed several diagnostic ions: 596 (M^+), 581 (-CH3), 506 (-TMSOH), and 491 (-CH3-TMSOH) among others.

Each of the steroids studied gave a single peak on the total-ion chromatogram except for androstanolone, mestanolone (methylandrostanolone) and oxymetholone, for each of which two peaks were seen, representing the cis and trans isomers of the derivatized compounds. These isomers were well separated and the ion abundance of the trans isomer was usually higher than that of the cis isomer. However, since the cis:trans ion intensity ratio was not constant, the sum of the peak areas of both isomers was used for quantitation.

The recoveries of the steroids from hair were: androstanolone 35%, bolasterone 65%, chlorotestosterone-acetate 40%, dehydromethyltestosterone 40%, dehydrotestosterone 41%, fluoxymesterone 40%, mestanolone 45%, mesterolone 52%, methenolone 24%, methyltestosterone 39%, nandrolone 42%, oxymetholone 36%, trenbolone 53%, and stanozolol 17%. The recovery of anabolic steroids from urine has been reported to be similarly low (12). The recovery of stanozolol, at 17%, is comparable with the 14 to 33% reported for animal hair, using alkaline digestion (8). We previously reported that recoveries of oestradiol and progesterone from hair were low (13%) also, while the recoveries of testosterone and epitestosterone were better, at about 40% (9). Coefficients of variation (CV) for repeatability were 3 to 6%, and for reproducibility were 5 to 12.5%. Limits of detection, limits of quantitation, and ranges of linearity for the steroids analyzed are set out in Table 2.

TABLE 2—*Limits of detection (LOD), limits of quantitation (LOQ), and ranges of linearity for the steroids analyzed.*

Compound	LOD ng/mg	LOQ ng/mg	Range* ng/mg
Testosterone	0.002	0.02	0.02–5
Androstanolone	0.01	0.05	0.05–1
Bolasterone	0.002	0.02	0.02–1
Clenbuterol	0.02	0.1	0.1–5
Chlorotestosterone-acetate	0.05	0.1	0.1–5
Dehydromethyl-testosterone	0.005	0.02	0.02–5
Dehydrotestosterone	0.002	0.02	0.02–5
Fluoxymesterone	0.01	0.1	0.1–5
Mestanolone	0.05	0.1	0.1–5
Masterolone	0.005	0.05	0.05–1
Methenolone	0.02	0.1	0.1–5
Methyltestosterone	0.005	0.05	0.05–1
Nandrolone	0.002	0.02	0.02–5
Oxymetholone	0.01	0.1	0.1–5
Trenbolone	0.005	0.05	0.05–5
Stanozolone	0.05	0.01	0.01–1

* Regression coefficients all >0.990.

TABLE 3—*Steroids detected in urine and hair from known abusers* (all male).*

Case	Urine (ng/mL)		Hair (ng/mg)		Hair Wt. (mg)
1	Nandrolone	21	Dehydromethyl-testosterone	0.02	40
			Nandrolone	0.02	
			Testosterone	0.06	
			Oestradiol	0.11	
			Progesterone	0.04	
			Epitestosterone	0.05	
2	Testosterone	88	Dehydromethyl-testosterone	TR	30
	Nandrolone	20			
			Nandrolone	TR	
			Testosterone	0.02	
			Oestradiol	TR	
			Progesterone	TR	
3	Oxymetholone	21	Clenbuterol	TR	28
			Testosterone	TR	
			Oestradiol	TR	
4	Methyltestosterone	15	Methyltestos-terone	0.17	20
5	Testosterone	1190	Testosterone	0.15	12
6	Methyltestosterone	35	Methyltestos-terone	TR	63
			Dehydromethyl-testosterone	TR	
			Testosterone	0.07	
			Oestradiol	TR	
			Progesterone	TR	
7	(sample not provided)		Methyltestos-terone	TR	10
			Dehydromethyl-testosterone	TR	
			Progesterone	TR	

TR = Trace, i.e., above level of detection but below level of quantitation (see Table 2).

* No self-reported drug use data were available from the sample donors.

Despite the relatively low recoveries, abused steroids and endogenous steroids were detectable in human hair from seven male steroid abusers (Table 3). Methyltestosterone was detected in both urine and hair samples from two steroid abusers, and nandrolone was detected in both urine and hair samples from two others.

Dehydromethyltestosterone was found in hair from four of the seven subjects, but not in urine. Trace amounts of clenbuterol were detected in one hair sample but not in the matching urine, which contained oxymetholone. High concentrations of testosterone in both urine and hair (at 0.15 ng/mg in hair) from one individual (case 5) clearly reflects abuse. The maximum testosterone level in 20 male hair samples collected at autopsy from nonabusers (age range 17 to 70 years) was reported as 0.026 ng/mg with a median of 0.004 ng/mg (9), casting suspicion on two other cases (cases 1 and 6, Table 3). However, it is not possible presently, given the limited available data, to offer guidance on the maximum normal testosterone concentration in male or female head hair. At least one of the four endogenous steroids (testosterone, oestradiol, progesterone, and epitestosterone) could be detected in all seven hair samples.

We have previously reported the detection of endogenous steroids in human hair by GC-MS using a NaOH digestion technique (9). In the present study we have applied the same basic methodology to the detection of abused steroids and detected methyltestosterone, dehydromethyltestosterone, nandrolone, and clenbuterol, as well as the endogenous steroids, in the head hair of known

steroid abusers. We conclude that head hair analysis has considerable potential for the detection and monitoring of steroid abuse.

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