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應用手冊

Screening for Anabolic Steroids and Their Esters Using Hair Analyzed by GC Tandem Quadrupole MS

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For forensic toxicology use only.

Abstract

A sensitive, specific, and reproducible method has been developed to detect and quantify 10 anabolic steroids and 11 anabolic steroids esters in hair by GC-MS/MS. This approach, not yet validated by the world anti-doping agency (WADA), allows scientist to dramatically increase the window of detection after steroids misuse.

Introduction

Use of anabolic steroids was officially banned in the mid-1970s by sports authorities. The first control of anabolic steroids (particularly metandienone found in Dianabol) was achieved in Montreal in 1976 during the Olympic games.[1]

The official detection of anabolic steroid misuse in sports is based on the analysis of urine samples. However, some athletes take long-term treatment of anabolic steroids during the winter months and stop before the competition,[2] or will take them for periods ranging from four to 18 weeks, alternating with drug-free periods of one month to one year.[3] This is the reason why abusers can be found drug-free. Anabolic steroids are detectable in urine only two to four days after exposure, except for the ester forms.

Hair specimens have been used for 20 years in toxicology to document long-term exposure in various forensic, occupational, and clinical situations.[3]

Hair analysis allows an increase in the detection window (from weeks to months) depending on the hair length. It allows a distinction to be made between a single or a repetitive use, and documents an estimation of consumed quantities.[4-7] For this reason, doping during training and abstinence during competition can be detected by hair analysis.

Anabolic steroids are prohibited. Their presence in a urine sample leads to a positive result except for endogenous steroids (testosterone, epitestosterone, DHEA, and nandrolone, which have a threshold of positivity). In hair, the parent compound is the target analyte, that is the opposite to urine where the metabolites are of interest. Due to low amount to be detected, a sensitive and specific method is necessary.[8-9]

A sensitive, specific, and reproducible method has been developed to detect and quantify 10 anabolic steroids and 11 anabolic steroids esters (Figures 1 and 2) in hair by GC-MS/MS. For practical reasons,

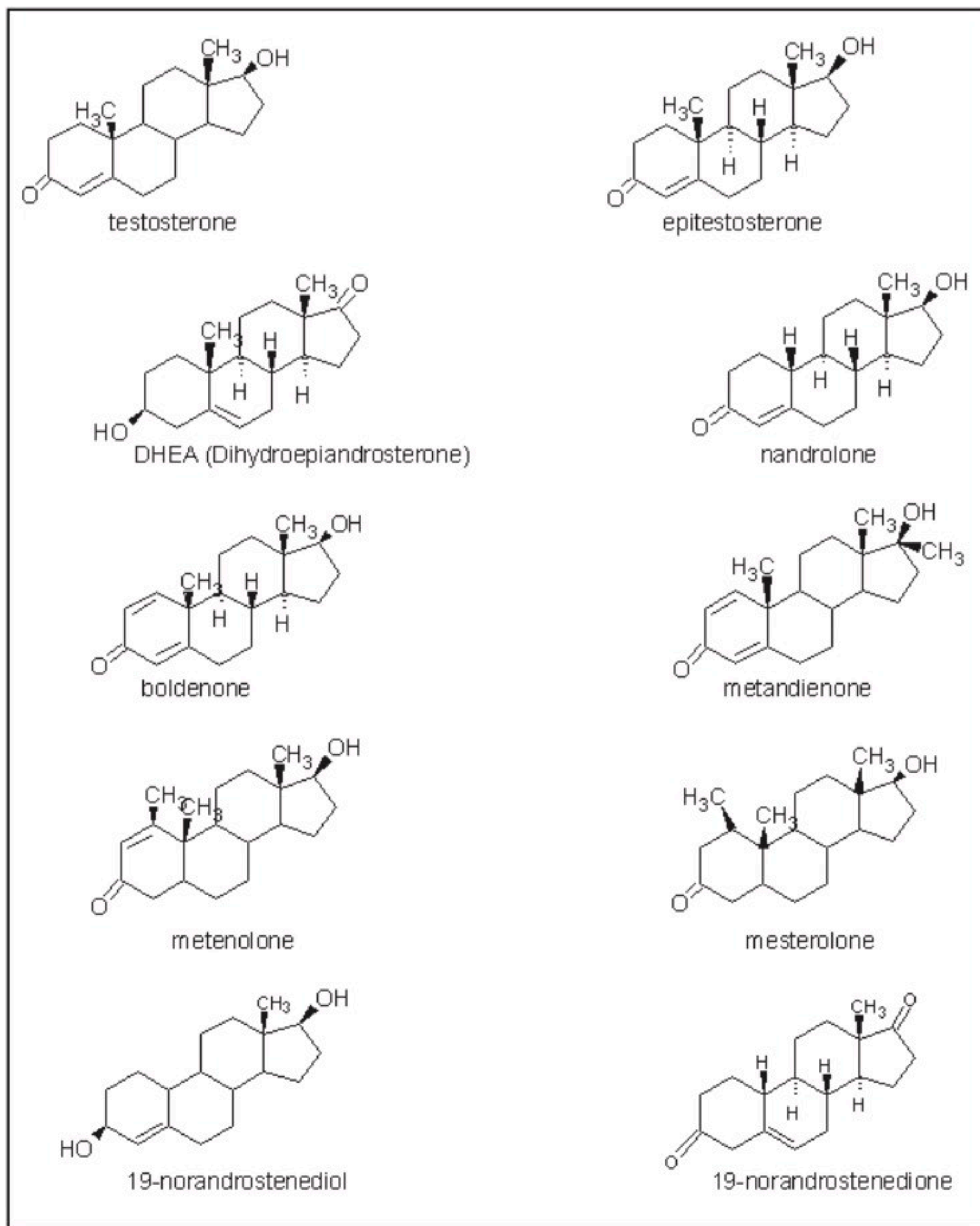


Figure 1. Structures of anabolic steroids detected by GC-MS/MS.

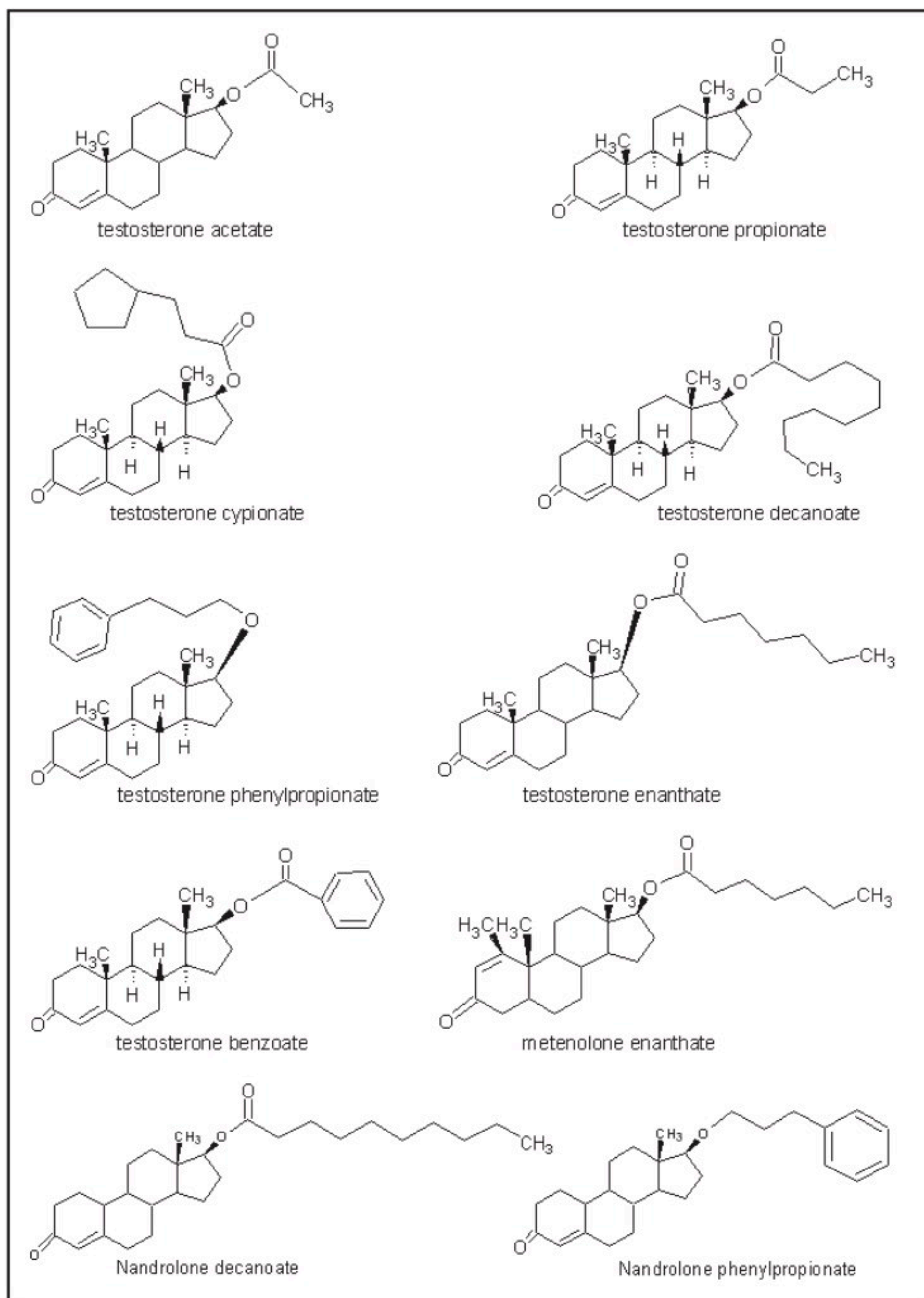


Figure 2.

Structures of anabolic steroids esters detected by GC-MS/MS.

Experimental

Specimens

The method has been validated with controlled drug-free specimens from laboratory personnel spiked with different concentration of anabolic steroids and their esters. The samples were analysed on an Agilent 6890 GC coupled to a Waters Quattro micro GC tandem quadrupole mass spectrometer operated in EI+ mode (Figure 3).

Figure 3. Waters Quattro micro GC MS System.

Anabolic Steroids

Extraction

The hair was decontaminated twice using 15 mL of dichloromethane, for 2 min at room temperature.

The segmentation of hair in doping cases allows differentiation of a single from use from repetitive use. Each segment measures 1 cm to estimate the pattern of anabolic steroids or their esters on a one month basis (at the vertex posterior, hair growth is about 1 cm/month).[7]

A 100 mg of decontamined hair was incubated in 1 mL 1 M NaOH, 10 min at 95 °C, in the presence of 2 ng of testosterone-d_3 and nandrolone-d_3 (final concentration 20 pg/mg) used as internal standard. After cooling, the homogenate was neutralized with 1 mL 1 M HCl and 2 mL of 1 M phosphate buffer (pH 7.0) were added.

C18 based SPE cartridges (such as Waters Sep-Pak C_{18}) were conditioned with 3 mL of methanol, followed by 3 mL of deionized water. After sample addition, the columns were washed twice with 1 mL of deionized water. After column drying, analyte elution occurred with the addition of three 0.5 mL aliquots of methanol. The eluent was evaporated to dryness, and the residue reconstituted in 1 mL of 1 M phosphate buffer (pH 7.0). A further purification step was achieved by addition of 100 mg of Na_2CO_3 - $NaHCO_3$ (1:10, w/w) and 2 mL of pentane. After

agitation and centrifugation, the organic phase was removed and evaporated to dryness. The residue was derivatized by adding 5 µL MSTFA-NH_4I-2-mercaptoethanol (250 µL, 5 mg, 15 µL respectively) and 45 µL MSTFA, then incubated for 20 min at 60 °C.

GC method

The samples were injected by splitless injection (1 µL, 270 °C, purged at 50 mL/min after 1 min) into a carrier gas of helium at a constant flow rate of 1.0 mL/min delivered from an Agilent 6890 GC. The column was an Ohio Valley OV 30 m x 0.25 mm i.d., 0.25 µm. The following temperature ramp rate was used: 150 to 295 °C (10 min) at 15 °C/min. The total run time was 19.67 min.

Anabolic Steroid Esters

Extraction

A 100 mg of decontamined hair was incubated overnight at 50 °C in 1 mL MeOH in presence of 2 ng testosterone-d_3 and nandrolone-d_3. After incubation, methanol was evaporated to dryness. The residue was reconstituted in 3 mL of 1 M phosphate buffer (pH 7.0). As anabolic steroids, extraction was made with C_{18} based SPE cartridges. The eluent was evaporated to dryness, and the residue reconstituted in 0.5 mL of 1 M phosphate buffer (pH 7.0). A further purification step was achieved by addition of 4 mL of hexane-ethyl acetate (70:30, v/v). After agitation and centrifugation, the organic phase was removed and evaporated to dryness. The residue was derivatized by adding 5 µL MSTFA-NH_4I- 2-mercaptoethanol (250 µL, 5 mg, 15 µL respectively) and 45 µL MSTFA, then incubated for 20 min at 60 °C.

GC method

The samples were injected by splitless injection (1 µL, 270 °C, purge at 50 mL/min after 1 min) into a carrier gas of helium at a constant flow rate of 1.2 mL/min delivered from an Agilent 6890 GC. The column was an Ohio Valley OV 30m x 0.25 mm i.d., 0.25µm. The following temperature ramp rate was used: 60 to 325 °C (5 min) at 20 °C/min. The total run time was 18.25 min.

MS method

The Quattro micro GC Mass Spectrometer was used in electron impact (EI+) mode. The ion source was operated at 180 °C with an electron energy of 70 eV and a trap current of 200 µA. The mode of acquisition was multiple reaction monitoring (MRM) at an argon collision gas pressure of 3.00×10^{-3} Bar.

The list of anabolic steroids, ester forms, MRM transitions, collision energies and retention time for the method are listed in Table 1.

Anabolic steroids	Retention time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Collision energy (eV)
Testosterone	9.68	432.4	209.2	10
Epitestosterone	9.42	432.4	209.2	10
DHEA	9.30	432.4	327.3	10
Testosterone-d_3	9.67	435.4	420.4	10
Nandrolone	9.45	418.4	194.2	10
Nandrolone-d_3	9.44	421.4	194.2	15
Metenolone	9.84	195.1	179.0	10
Mesterolone	9.62	448.3	141.1	15
19-Norandrostenedione	9.33	416.3	234.2	10
19-Norandrostenediol	9.13	420.3	340.3	5
Boldenone	9.60	416.3	326.3	5
Metandienone	10.04	444.2	206.1	15
Esters				
Testosterone acetate	12.44	402.4	209.1	10
Testosterone propionate	12.82	416.3	209.1	10
Testosterone enanthate	14.38	472.4	209.2	10
Testosterone benzoate	15.29	464.3	209.1	20
Testosterone cypionate	15.49	484.4	209.2	15
Testosterone phenylpropionate	16.30	492.3	477.3	10
Testosterone decanoate	16.06	514.5	209.2	10
Metenolone enanthate	14.57	486.3	208.1	10
Nandrolone decanoate	15.76	500.4	194.2	15
Nandrolone phenylpropionate	15.97	478.3	182.2	15

Table 1. Anabolic steroids, their esters and MRM method parameters.

Method Validation

A standard calibration curve was obtained by adding 0.1 ng (1 pg/mg), 0.2 ng (2 pg/mg), 0.5 ng (5 pg/mg), 1 ng (10 pg/mg), 2.5 ng (25 pg/mg), 5 ng (50 pg/mg), and 10 ng (100 pg/mg) of anabolic steroids or steroid esters to 100 mg of control hair, previously decontamined. From this standard calibration, the precision had been evaluated. The accuracy has not been determined because there is no qualified matrix.

Intra-day and between-day precisions for anabolic steroids or steroid esters were determined using negative control hair spiked with the drugs at final concentration of 25 pg/mg (n=8).

Relative extraction recovery was determined by comparing the representative peak of anabolic steroids or steroid esters extracted from negative control hair spiked at the final concentration of 25 pg/mg with the peak area of a methanolic standard at the same concentration.

The limit of detection (LOD) was evaluated with decreasing concentrations of anabolic steroids or steroid esters until a response equivalent to three times the background noise was observed for all the transitions. For the limit of quantification (LOQ), a response superior to ten times the background noise is necessary.

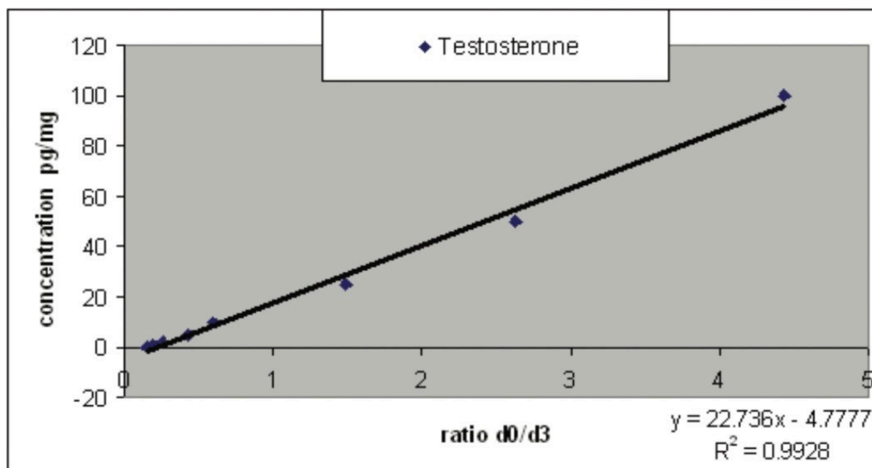
Results and Discussion

Validation Results

It is desirable to produce an intense ion signal that is characteristic for the target compound. Selectivity and sensitivity are increased by almost suppressing the noise level. The parent ion of anabolic steroids or steroid esters corresponds to the molecular ion or was chosen based upon criterion of specificity and abundance.

The calibration curve corresponds to the linear regression between the peak area ratio of anabolic steroids or steroids esters to I.S. and the final concentration of the drug in spiked hair.

Response for anabolic steroids was linear in the range 1 to 100 pg/mg; except for 19-norandrostenedione, mesterolone, metenolone, metandienon, boldenone and steroid esters for which, the response was linear in the range 2 to 100 pg/mg. For 19-norandrostenediol, testosterone enanthate and metenolone enanthate, the response was linear in the range 5 to 100 pg/mg. Representatives curves for testosterone and testosterone acetate are illustrated in Figure 4.



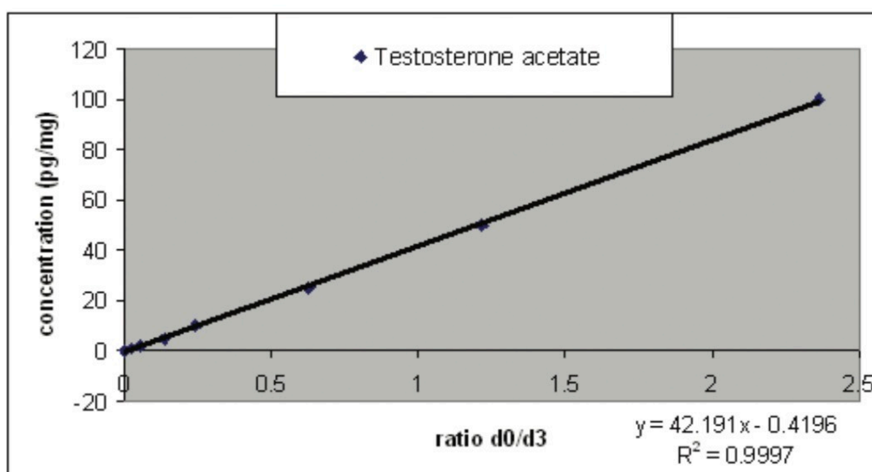


Figure 4. Representative calibration curve for testosterone (top) and testosterone acetate (bottom).

The extraction recovery (n=8), the correlation coefficient, LOD and LOQ were determined and are given in Table 2. The chromatogram which corresponds to the LOD of nandrolone is illustrated in Figure 5 and the one from testosterone acetate in Figure 6. Figure 7 is the chromatogram obtained for DHEA in hair of laboratory personnel.

Anabolic steroids	Relative extraction recovery (%)	Correlation coefficient (%)	LOD (pg/mg)	LOQ (pg/mg)
Testosterone	93.5	8.6	1	5
Epitestosterone	74.8	7.9	1	5
DHEA	23.2	14.4	1	5
Nandrolone	113.5	11.9	1	5
19-Norandrostenedione	118.1	6.2	2	5
19-Norandrostenediol	39.8	14.5	5	10
Mesterolone	89.4	14.9	2	5
Metenolone	91.8	14.0	2	5
Metandienone	99.7	9.9	2	5
Boldenone	74.9	16.0	2	5
Esters				
Testosterone acetate	118.3	12.0	2	5
Testosterone propionate	120.7	17.4	2	5
Testosterone enanthate	122.4	13.4	5	10
Testosterone cypionate	133.9	16.9	2	5
Testosterone benzoate	121.9	14.9	2	5
Testosterone phenylpropionate	119.8	15.9	2	5
Testosterone decanoate	141.4	19.1	2	5
Metenolone enanthate	96	14.1	5	10
Nandrolone decanoate	92.4	13.1	2	5
Nandrolone phenylpropionate	75.9	14.9	2	5

Table 2. Mean recovery (n=3) and correlation coefficient (n=8) established at 25 pg/mg and LOD and LOQ.

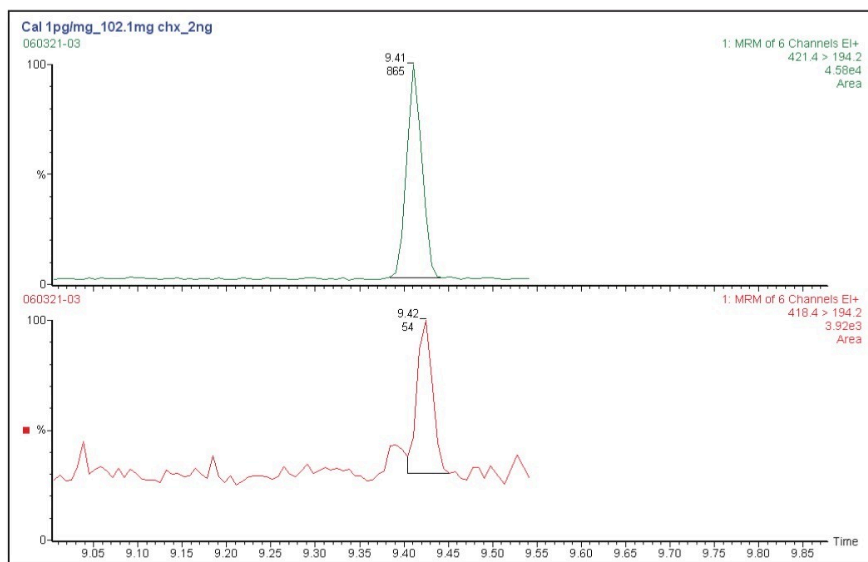


Figure 5. Chromatogram obtained after extraction of nandrolone by the established procedure of a 102.1 mg hair specimen spiked for a final concentration at 1 pg/mg. Top: nandrolone-d3 with its daughter ion at m/z 194.2. Bottom: quantification transition m/z 418.4 to 194.2.

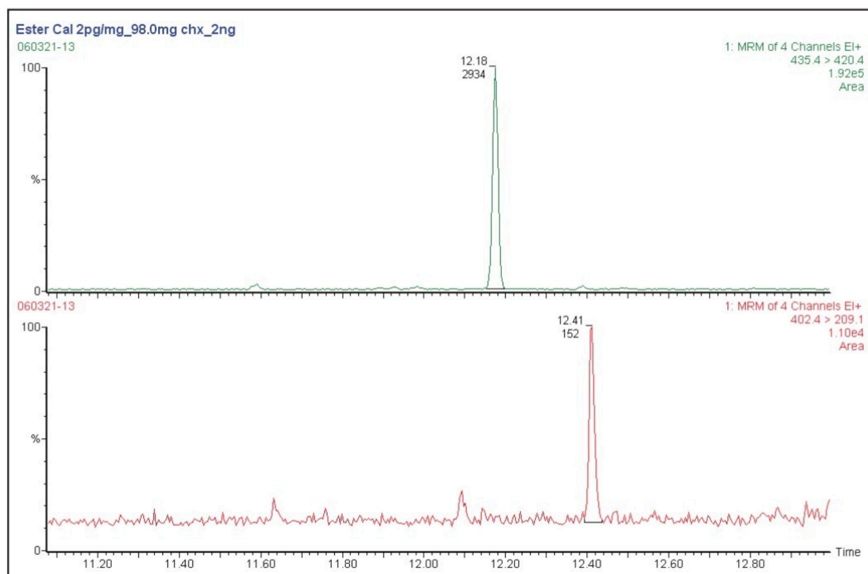


Figure 6. Chromatogram obtained after extraction of testosterone acetate by the established procedure of a 98.0 mg hair specimen spiked for a final concentration at 2 pg/mg. Top: testosterone-d_3 with its daughter ion at m/z 420.4. Bottom: quantification transition m/z 402.4 to 209.1.

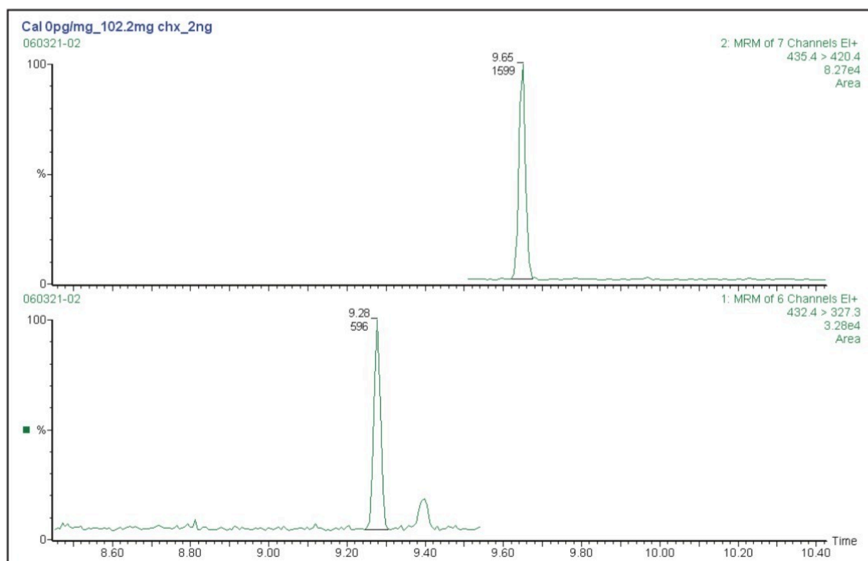


Figure 7. Chromatogram obtained after extraction of DHEA by the established procedure of a 102.2 mg hair specimen. DHEA was quantified at the concentration of 10.7 pg/mg. Top: testosterone-d_3 with its daughter ion at m/z 420.4. Bottom: quantification transition m/z 432.4 to 327.3.

Extensive chromatographic procedures (two purification steps by solid-phase and liquid-liquid extractions, MS-MS detection) were analytical prerequisites for successful identification of anabolic steroids and esters in hair

due to the low target concentrations.

Previously, anabolic steroids were analyzed by GC-MS, but it is appears that GC-MS/MS is of a greater interest. Actually, this method allows us to obtain greater sensitivity and specificity, which is important when trace amounts of analytes are tested for.

The comparison between MRM mode and SIR (selected ion recording) is shown in Figure 8. The GC-MS/MS detection in the MRM mode allows us to obtain better chromatograms which are cleaner (lower background noise) and with less interferences than those obtained with GC-MS.

LOD are less than or equal to those found with the GC-MS.[10]

This method allows us to identify the presence of anabolic steroids or esters in hair. In the case of a positive result, the presence of drug is confirmed using a specific method for each molecule including, in general, two more transitions. A list of anabolic steroids, esters and confirmatory MRM transitions is presented in Table 3.

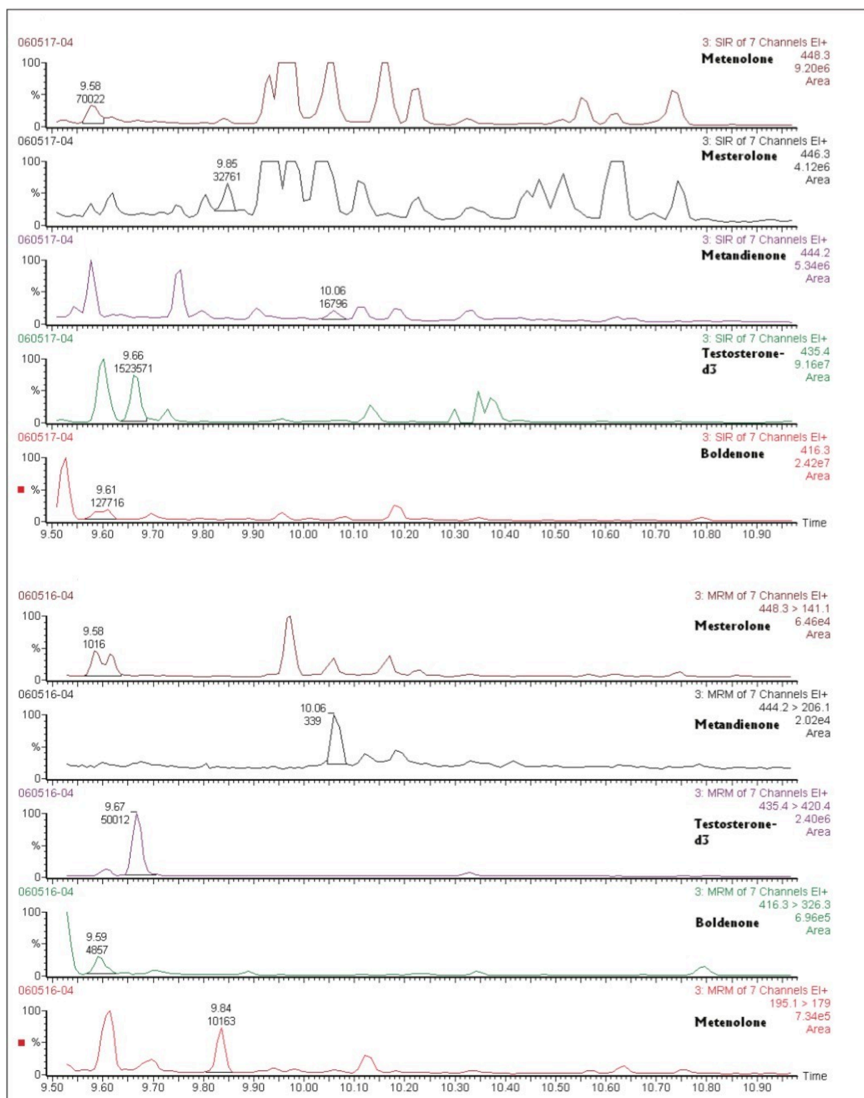


Figure 8. Chromatograms obtained in SIR (top) and MRM (bottom) mode of detection after extraction of anabolic steroids by the established procedure.

Anabolic steroids	Precursor Ion (m/z)	Product Ion (m/z)	Collision energy (eV)
Testosterone Epitestosterone DHEA	432.4	417.4	10
Nandrolone	418.4	182.2	10
Metenolone	446.3	208.1	5
	208.2	193.0	5
Mesterolone	433.3	187.1	10
	349.2	192.1	10
19-Norandrostenedione	416.3	401.3	10
	416.3	220.2	15
19-Norandrostenediol	240.2	225.2	5
	420.3	330.3	5
Boldenone	325.2	229.2	5
	191.0	175.1	10
Metandienone	444.2	339.2	10
	206.1	191.1	10
Esters			
Testosterone acetate	402.4	387.3	10
	387.3	233.2	10
Testosterone propionate	416.3	401.3	10
	416.3	196.2	10
Testosterone enanthate	472.4	457.4	10
	472.4	196.1	10
Testosterone benzoate	464.3	449.3	10
	449.3	327.3	10
Testosterone cypionate	484.4	469.5	10
	469.4	327.3	10
Testosterone phenylpropionate	492.3	209.2	10
	492.3	191.1	10
Testosterone decanoate	514.5	499.5	10
	514.5	191.1	15
Metenolone enanthate	208.2	193.0	10
	195.0	179.0	10
Nandrolone decanoate	500.4	182.2	15
	500.4	246.3	20
Nandrolone phenylpropionate	478.3	194.2	15
	194.1	73.0	15

Table 3. Confirmatory MRM transitions of anabolic steroids and esters in case of requested confirmation.

Conclusion

This sensitive, specific, and reproducible method developed is suitable for the detection and quantification of anabolic steroids and their esters in human hair. This approach, not yet validated by the world anti-doping agency (WADA), allows scientist to dramatically increase the window of detection after steroids misuse.

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