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Short communication

Detection of testosterone propionate administration in horse hair samples

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Abstract

A sensitive and specific method has been developed to detect semi-quantitatively testosterone in horse hair samples. The method involved a washing step with sodium dodecylsulfate aqueous solution. The mane and tail hair samples (100 mg) were dissolved in 1 mL of sodium hydroxide for 15 min at 95 °C in the presence of d3-boldenone used as internal standard. The next three steps involved diethyl ether extraction and a solid phase extraction on Isolute C18 (EC) cartridges eluted with methanol. The residue was derivatized by adding 100 μL of acetonitrile and 30 μL of PFPA then incubating for 15 min at 60 °C. After evaporation, 30 μL of hexane was added and 2.5 μL was injected into the column (a bonded phase fused silica capillary column DB5MS, 30 m × 0.25 mm i.d. × 0.25 μm film thickness) of a Trace GC chromatograph. In order to improve the sensitivity of the method, damping gas flow has been optimized. Testosterone was identified in MS^2 full scan mode on the Polaris Q instrument. The assay was capable of detecting less than 1 pg mg^{-1}. The recovery was close to 90%. The analysis of tail and mane samples collected from a gelding horse having received a single dose of testosterone propionate (1 mg kg^{-1}) showed the presence of testosterone in the range of 1–6 pg mg^{-1} in hair collected during 5 months after administration.

Keywords: Testosterone; Testosterone propionate; Horse hair; Gas chromatography–mass spectrometry; Ion trap mass spectrometry; Triple quadrupole mass spectrometer

1. Introduction

In forensic science, hair has been used for more than 25 years to document chronic drug exposure. Since the early 1980s, the development of highly sensitive and specific assay methods such as gas chromatography mass spectrometry (GC/MS or GC/MS–MS) [1,2] and more recently liquid chromatography mass spectrometry (LC/MS) has made possible the analysis of various drugs incorporated in hair. The main advantage of hair testing compared to urine and blood testing is its larger detection window, which is at least weeks to months, depending on the length of hair shaft analysed, against a few days or weeks in urine depending of the drug. As a result, the value of hair analysis for the identification of drug users is continuously gaining recognition [3,4]. Hair analysis is also an important tool for veterinary drug monitoring in livestock production [5]. The detection of veterinary drugs in animal hair was begun by the detection of clenbuterol. Since then several drugs have been detected in livestock and horse hair. In horse hair, the large detection window also allowed for the detection of several anti-microbial drugs such as trimethoprim and procaine benzylpenicillin [6,7], clenbuterol [8,9]. The detection in hair samples is able to discriminate between natural and administered hormones. Since 1999, several informative reports on the detection of esters, free testosterone [10], and nandrolone [11,12] have been released. According to Segura et al. [11], it is difficult to detect testosterone esters in hair after a single administration. However, an increase of free testosterone was observed; Concentrations of endogenous testosterone in hair in male volunteers has been determined in tests on a large sample population by at least two different groups [10,13]. The same authors have reported the detection of the esters in human and bovine hair samples.

More recently, Gratacos-Cubarsi et al. [5] Nielen et al. [14], have shown the great possibilities of bovine hair analysis in detecting intact testosterone esters and boldenone undecylenate by GC/MS or LC/MS.

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Regarding steroid analysis in horse hair samples, boldenone was detected in the mane and tail retrospectively for up to 12 months after boldenone undecylenate administration to two different horses [15]. As recently showed by Anielski et al. [16] and Popot et al. [17], testosterone has been detected in mane and tail samples from a stallion but was not detected in hair of a gelding before administration of testosterone enanthate. Testosterone was detected in a hair sample taken 1 month after administration.

Taking advantage of previous work, the goal of the present study is to check the possibility of detecting an acute administration of testosterone propionate using sensitive and specific assays for the determination of free testosterone and testosterone propionate in post-administration hair samples.

2. Experimental

The bay thoroughbred horse selected for this experiment was a 4-year-old gelding. A testosterone propionate single dose of 1 mg kg^{-1} was given orally. Before starting the experiment, specific areas on the mane and tail were selected for sampling. Hair samples were taken each month over a period of 6 months. This experiment lasted from November 2004 to April 2005.

Testosterone analysis was conducted on these samples or sections (3–4 cm) of samples. Testosterone propionate analysis was conducted only on some samples in which testosterone was detected (i.e. tail samples taken 1 month and 2 months after administration, a mane sample taken 1 month after administration and Section 1 of a mane sample collected 3 months after administration).

3. Standards and reagents

Analytical grade solvents were obtained from Prolabo (Paris, France) and PFPA (pentafluoropropionic anhydride) from Pierce (Rockford, Illinois, USA). Drug standards were purchased from Sigma (St. Louis, MO, USA) and d3-boldenone from NARL (Pymble, Australia). Isolute C18 (EC) cartridges (3 mL, 500 mg) were obtained from Interchim (Montluçon, France).

4. Sample preparation

Except those taken 1 or 2 months after administration, samples were cut into 3–4 cm sections. These samples were washed using an aqueous solution of sodium dodecylsulfate at 1 g L^{-1} before being pulverised in a Retsch MM200 instrument from Labo Modern (Paris, France).

4.1. Testosterone

One hundred milligrams of hair samples (entire or sections) supplemented with 1 ng of d3-testosterone as internal standard were treated with 1 mL of 1 M sodium hydroxide and then incubated for 15 min at 95 °C. The solution was neutralised by adding 1 mL of 1 M hydrochloric acid and adjusted to pH 7.6 by the addition of 2 mL of Soerensen buffer. Solvent extraction was carried out three times using 4 mL of diethyl ether and the residue was diluted in 5 mL of phosphate buffer (pH 7). A solid phase extraction was performed using 3 mL C18 (EC) cartridges and testosterone was eluted with methanol. Derivatization was performed by adding 100 μL of acetonitrile and 30 μL of PFPA and heating 15 min at 60 °C. After evaporation, 30 μL of hexane was added and 2.5 μL was injected into the GC/MS system.

4.2. Testosterone propionate

One hundred milligrams of entire hair samples or hair sections supplemented with 1 ng of d3-boldenone as internal standard were treated with 3 mL of methanol and then incubated one night at 50 °C. The hair solution was adjusted to pH 7.6 by addition of 50 μL of 3 M potassium hydroxide and 0.5 mL of Soerensen buffer before being extracted three times using 4 mL of a mixture hexane/ethylacetate (70:30). The residue was diluted in 5 mL of phosphate buffer (pH 7) before solid phase extraction using 3 mL C18 (EC) cartridges. Testosterone propionate was eluted with methanol. After PFP-derivatization, 30 μL of hexane was added and 2.5 μL was injected into the GC/MS system.

5. Instrumental

5.1. Testosterone

GC–EI–MS–MS analyses were performed in positive ion electron impact (EI) mode on PolarisQ spectrometer, coupled to a TraceGC gas chromatograph equipped with a bonded phase fused silica capillary column DB5MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness). Operating conditions were as follows: time for the splitless injection was set to 1 min, helium inlet flow was 1 mL min^{-1}, initial temperature was set to 150 °C for 1 min and ramped at 18 °C min^{-1} up to 305 °C min^{-1},which was held for 3 min. The injector temperature was set at 250 °C, the transfer line temperature was 300 °C and the source temperature was 220 °C. The damping gas was set to 2.5 mL min^{-1}. Detection was performed by MS–MS of the $M^{+\bullet}$ ion of the PFP testosterone and PFP d3-testosterone derivatives at *m/z* 580 (Ec = 3.90 V) and *m/z* 583 (Ec = 3.65 V), respectively. A quantitative approach was made by summing the area of the three daughter ions at *m/z* 401, *m/z* 416, *m/z* 565 for testosterone and *m/z* 404, *m/z* 419, *m/z* 568 for d3-testosterone in the CID mass spectra.

5.1.1. Testosterone propionate

Two different spectrometers were tested.

5.1.1.1. Varian 1200L triple quadrupole mass spectrometer. The samples were injected by splitless injection (2.5 μL at 250 °C) into a carrier gas of helium at a constant flow rate of 1 mL min^{-1} delivered from a Varian CP-3800 gas chromatograph with a CTC Combi PAL sampler attached (injection speed = 100 μL s^{-1}). The column employed was a DB1MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The following temperature ramp was used: 150 °C (1 min) to 320 °C at 18 °C/min. The temperature of the source was 280 °C and the transfer line was set to 300 °C. The sample were analysed on a

Table 1a
Transitions selected for analysis in the SRM mode on the Varian 1200L triple quadrupole mass spectrometer

	Transition 1	Transition 2	Transition 3	Transition 4	Transition 5	Transition 6
Testosterone propionate	490 → 131 (−25 V)	490 → 133 (20 V)	490 → 146 (−10 V)	490 → 237 (−10 V)	490 → 252 (−10 V)	490 → 311 (−10 V)
D3-boldenone	581 → 417 (−10 V)	581 → 433 (−10 V)	581 → 444 (−10 V)	–	–	–

Table 1b
Transitions selected for analysis in the SRM mode on the Waters Micromass Quattro micro-GC tandem quadrupole mass spectrometer

	Transition 1	Transition 2	Transition 3	Transition 4	Transition 5	Transition 6
Testosterone propionate	490 → 131 (−25 V)	490 → 133 (15 V)	490 → 146 (−10 V)	490 → 237 (−10 V)	490 → 252 (−10 V)	490 → 311 (−10 V)
D3-boldenone	581 → 417 (−5 V)	581 → 433 (−10 V)	581 → 254 (−20 V)	–	–	–

Varian 1200L triple quadrupole mass spectrometer operated in EI+ mode (70 eV). For the MRM mode, the transitions for each analyte and their collision energy are listed in Table 1a. Argon pressure for the collision gas was 1.5 mTorr.

5.1.1.2. Waters Micromass Quattro micro-GC tandem quadrupole mass spectrometer. The samples were injected by splitless injection (2 μL at 250 °C) into a carrier gas of helium at a constant flow rate of 1 mL min^{-1} delivered from an Agilent 6890 gas chromatograph with a 7683 autosampler attached (injection speed = 100 μL s^{-1}). The column employed was a RTX1MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The following temperature ramp was used: 150 °C (1 min) to 320 °C (3 min) at 18 °C/min. The temperature of the interface was 275 °C. The samples were analysed on a Waters Micromass Quattro micro-GC tandem quadrupole mass spectrometer operated in EI+ mode (70 eV). For the MRM mode, the transitions for each analyte and their collision energy are listed in Table 1b. Argon pressure for the collision gas was 2 mTorr.

6. Results and discussion

6.1. Testosterone

6.1.1. Assay development

In a previous study dedicated to the detection of 17β-boldenone [18] and testosterone [17] in hair, PFP derivatisation was chosen in order to obtain the best specificity and sensitivity. As shown in Fig. 1, the diagnostic ions selected for identification and quantitative approach were fragment ions at *m/z* 565, *m/z* 416 and *m/z* 401. A coefficient of variation higher to 15% was obtained by measuring the sum of the area corresponding to the three fragment ions to those corresponding to the internal standard. The repeatability was not improved by measuring the ratio of the area of one of the three diagnostic ions separately. Consequently, a semi-quantitative approach was considered in which the range of concentrations was determined.

In order to improve the sensitivity of the technique, the effect of the damping gas flow variation was studied. The damping gas is an option available on the Polaris Q instrument which allows one to optimize the helium gas flow. The default value of 0.3 mL min^{-1}, was used at the beginning of the study. In order to improve the detection limit and the stability of the response, several values of damping gas were tested (0.3, 2, 2.5 and 3 mL min^{-1}). The best result (i.e. an improvement of a factor 5 of the signal intensity on the three fragment ions) was obtained by choosing a damping gas at a flow rate of 2.5 mL min^{-1} associated with an optimization of three other parameters (Ec, *q*, width). There was no improvement at a flow rate value higher than 2.5 mL min^{-1}.

6.1.2. Validation

As previously observed [17], under the chromatographic conditions described above there was no detectable interference with

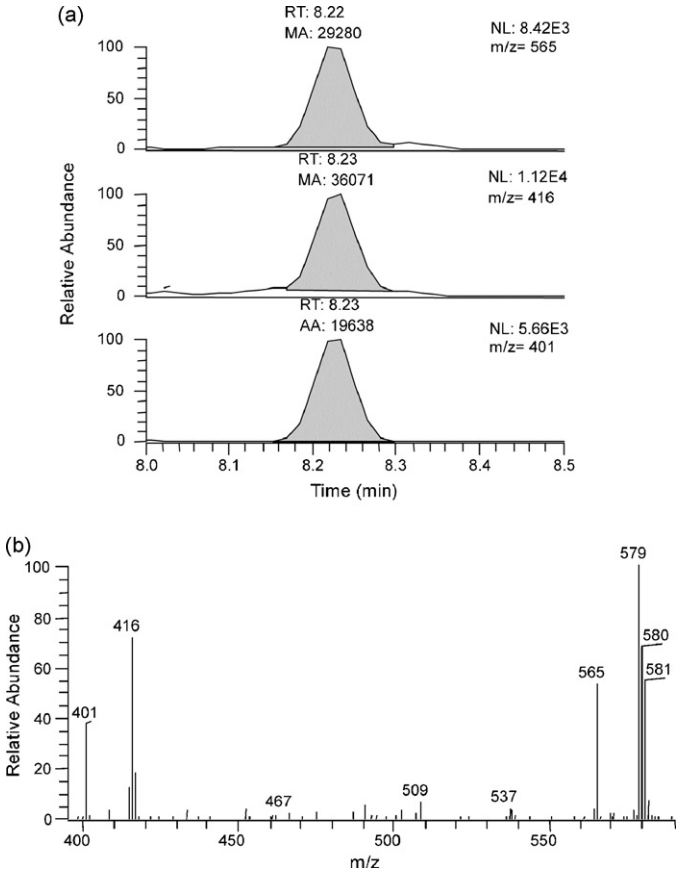

Fig. 1. (a) Extracted ion mass chromatograms at *m/z* 565, *m/z* 416 and *m/z* 401 corresponding to the PFP derivate of testosterone obtained from a tail sample spiked at 10 pg mg^{-1}, (b) CID spectrum of *m/z* 580 prepared under EI conditions.

testosterone and d3-testosterone by any extractable endogenous material present in horse hair (i.e. the mane and the tail). Extraction recovery determined in a previous study was higher than 90% [17]. The limit of detection was less than 1 pg mg^{-1} of testosterone in a 100 mg sample size. Compared to our previous work [17], in which the limit of detection was estimated at 2 pg mg^{-1}, this detection limit was better. This is due to the use of damping gas at the optimized flow rate. As reported before [16,17] testosterone is not detectable in hair samples collected from non-treated geldings. In the present study, testosterone was not detectable in the mane and tail samples obtained before administration.

6.1.3. Detection of testosterone in post-administration hair samples

As compared to a spiked tail sample at the concentration of 10 pg mg^{-1} (Fig. 1a and b), testosterone was detected in a 1 month post-administration tail sample (Fig. 2a and b) collected from a gelding treated with testosterone propionate (1 mg kg^{-1}). The mass spectrum and the chromatograms were in accordance with the Association Official Racing Chemist criteria [19] generally used for the detection of drugs in urine or blood. The testosterone concentration was estimated at 5.5 pg mg^{-1}.

As shown in Table 2, analyses were carried out on mane and tail samples n1–n6 collected from November to April; testosterone was detected in the entire mane and tail hair collected 1 month and 2 months after testosterone propionate administration.

Testosterone was also detected in one section of mane and tail samples collected 3, 4 and 5 months after testosterone propionate administration. Testosterone was better detected in mane than in tail samples. The same observation was seen before for the detection of boldenone [15].

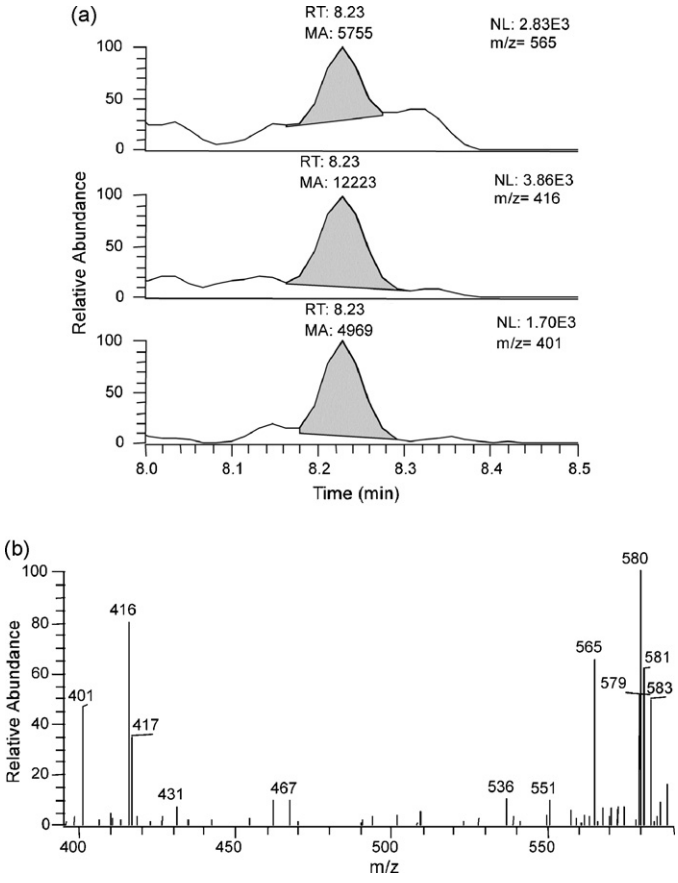

Fig. 2. (a) Extracted ion mass chromatograms at *m/z* 565, *m/z* 416 and *m/z* 401 corresponding to the PFP derivate of testosterone, from a post-administration tail sample collected 1 month after testosterone propionate administration from a gelding, (b) CID spectrum of *m/z* 580 prepared under EI conditions.

Table 2
Testosterone detection in 6 mane sections and 6 tail sections collected from a gelding horse

	Month of sampling	Testosterone detection in mane sections			Testosterone detection in tail sections		
		Section number	Length of the section (mm)	Concentration (pg mg^{-1})	Section number	Length of the section (mm)	Concentration (pg mg^{-1})
n0	November	Entire		ND	Entire		ND
n1	December	Entire		3	Entire		5.5
n2	January	Entire		3	Entire		2
n3	February	S1	0–40	3	Entire		<LQ
		S2	40–80	<LQ			
n4	March	S1	0–30	ND	S1	0–40	ND
		S2	30–60	ND	S2	40–80	1
		S3	60–100	1.7			
n5	April	S1	0–40	ND	S1	0–40	ND
		S2	40–80	ND	S2	40–80	ND
		S3	80–110	1	S3	80–110	1
n6	May	S1	0–40	ND	S1	0–40	ND
		S2	40–80	ND	S2	40–80	ND
		S3	80–120	ND	S3	80–120	ND

Sample n0 was collected before the administration. Detection range was 1–5.5 pg mg^{-1}.

Testosterone was not detected in mane and tail samples n6 collected 6 months after administration. It was suspected that this sample had not been segmented at the right place.

The present data on detection of free testosterone after testosterone propionate administration are in agreement with those obtained after a single dose of testosterone enanthate administration (3 mg kg^{-1}) [17].

6.2. Testosterone propionate

On the Polaris Q instrument, it was not possible to detect testosterone propionate at concentrations less than 10 pg mg^{-1}. This is why we have investigated the possibility offered by two triple quadrupole instruments.

One important point of the assay development is the injection speed optimisation conducted on the Varian 1200 L TQ mass spectrometer. This test was performed using a spiked horse tail sample at 10 pg mg^{-1} (diluted to 1 pg mg^{-1} with a blank hair sample extract), 100 μL s^{-1} was proved to be the most appropriate one. The decrease of the injection speed to 50 μL s^{-1} had reduced the response to 50%. Using a flow of 10 μL s^{-1}, testosterone propionate was not detected.

The detection limit for testosterone propionate achieved on the two TQ mass spectrometers defined as 3 S/N was about 0.5 pg mg^{-1}, which is appropriate [5,14,16] for the analysis of anabolic esters in hair.

The presence of testosterone propionate was investigated in tail and mane samples in which free testosterone was found. In the tail and in the mane samples tested on two TQ mass spectrometers, testosterone propionate was suspected. In order to detect it non-ambiguously, further investigations are necessary.

Our results indicate that testosterone was detected at pg mg^{-1} level in mane and tail samples after testosterone propionate administration. In bovine hair, Nielen et al. [14] did not detect any testosterone propionate after administration of two mixtures containing testosterone propionate (0.1–0.2 and 0.2–0.4 mg kg^{-1}) due to either low concentrations incorporated in hair or low doses administered.

7. Conclusion

The GC–MS/MS method developed on an ion trap mass spectrometer has been suitable for the detection of testosterone in hair after testosterone ester administration to a gelding. Compared to its detection in human and other animal species, the testosterone concentrations were very low. It has been not possible to detect testosterone propionate in these samples on the ion trap instrument but testosterone propionate was highly suspected during the tests carried out on two triple quadrupole mass spectrometers.

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