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Development and validation of a multi-analyte method for the detection of anabolic steroids in bovine urine with liquid chromatography–tandem mass spectrometry

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Abstract

Detection of anabolic steroids in animal urine samples is currently performed with GC–MS in our lab. However we found that the detection of 17α-trenbolone (17α-TbOH), 4-chloroandrost-4-ene-3,17-dion (CLAD), 16-β-OH-stanozolol (16OHstan) and α- and β-boldenone (α-Bol, β-Bol) was very difficult, if not impossible. Therefore a sensitive, specific and selective qualitative multi-analyte LC–MS–MS method was developed. The LC separation was achieved by using a Symmetry® C_{18} column and methanol–water–formic acid (54.7–44.7–0.6) as a mobile phase at a flow-rate of 0.3 ml/min. The mass spectrometer was operated in multiple reaction monitoring mode with positive electrospray interface. Validation of the method was done according to draft SANCO/1805/2000 Rev.1 and a CCβ smaller then 1 ng/ml was obtained for each compound.

Keywords: Steroids, anabolic

1. Introduction

Because of the fact that in the European Union the use of anabolic steroids is prohibited in food-producing animals [1], animal urine is routinely screened in our laboratory for the presence of anabolic steroids. Until now this was done with GC–MS. The steroids are extracted from urine samples on a combination of C_{18} and NH_2 solid-phase extraction (SPE) columns and are, after high-performance liquid chromatography (HPLC) fractionation, derivatised with heptafluorobutyric anhydride (HFBA) prior to GC–MS analysis. Some molecules, however, are not easy to detect in this way.

17β-Trenbolone acetate is administered by subcutaneous implantation in the ear and is rapidly hydrolysed to the active compound 17β-trenbolone. Subsequently 17β-trenbolone undergoes an oxidation followed by a reduction leading to the formation of 17α-trenbolone (17α-TbOH). This is finally excreted in the urine as a glucuronide or sulphate conjugate [2]. Quantitative and reproducible derivatisation of trenbolone with HFBA seemed to be very difficult probably due to the keto function in position 3 (Fig. 1). This results in high detection limits.

4-Chlorotestosterone acetate can be administered intramuscularly or orally. After intramuscular administration 4-chloroandrost-4-ene-3,17-dion (CLAD)

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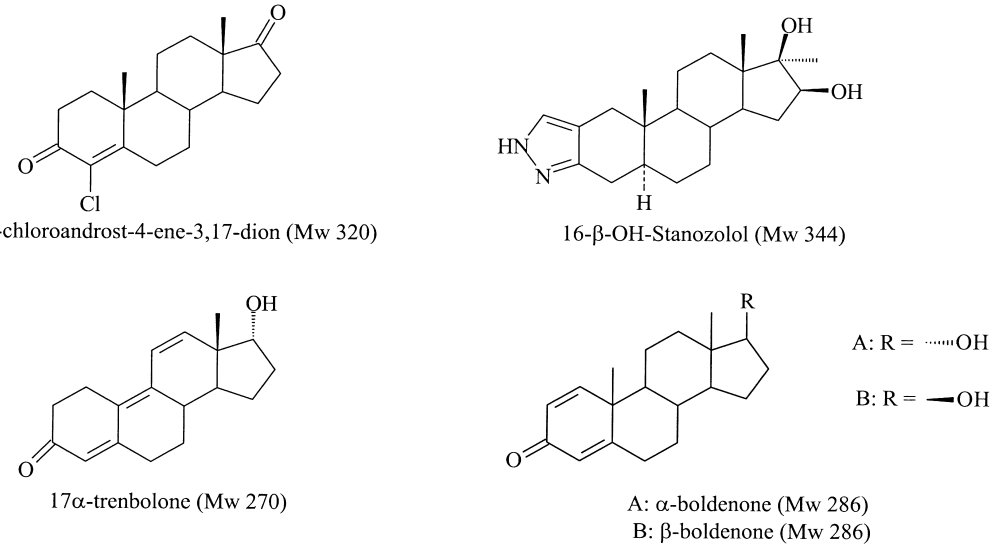

Fig. 1. Structural formulae of CLAD, 16OHstan, α-TbOH and α- and β-Bol.

is identified as one of the major urinary metabolites under the form of a sulphate conjugate [3]. The detection of CLAD with HFBA derivatisation was not possible.

Stanozolol is usually administered through injection. Once injected it is metabolised to 16-β-OH-stanozolol (16OHstan) which is the major metabolite in urine [4,5]. A multi-laboratory study [6] showed that LC–MS–MS is the method of choice for the detection of 16OHstan.

17β-Boldenone-undecanoate is hydrolysed to 17β-boldenone (β-Bol) following intramuscular injection. One of the main urinary metabolites of β-Bol is 17α-boldenone (α-Bol), but both are found in the urine of cattle. α-Bol is believed to be endogenous in cattle [7] although this is doubted by Van Puymbroeck et al. [8]. α- and β-Bol are detectable with GC–MS using HFBA derivatisation but we were not able to separate them chromatographically.

It was clear that there was a need for (a) supplemental method(s) to be able to detect the steroids mentioned above. Because we wanted to cover all components with as few methods as possible, and because LC–MS–MS was the method of choice for 16OHstan, we decided to focus on liquid chromatography coupled to mass spectrometry (LC–MS) for all molecules and/or metabolites which are difficult to detect by GC–MS. It was our aim to develop a qualitative LC–MS–MS method for the confirmation of 16OHstan, 17α-TbOH, CLAD, α- and β-Bol in animal urine in order to check for the illegal use of stanozolol, trenbolone acetate, chlorotestosterone acetate and β-boldenone (ester) in animal breeding.

2. Experimental

2.1. Chemicals and reagents

Sodium acetate, glacial acetic acid, formic acid (pro analyse) and methanol for HPLC were purchased from Merck (Overijse, Belgium), ethyl acetate was supplied by Acros (Geel, Belgium) and Helix pomatia digestive juice (Cat. No. 127 698; β-glucuronidase activity: 4.5 standard units; arylsulfatase activity: 14 standard units) by Boehringer Mannheim (Germany). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

17α-Trenbolone, 17β-19-nortestosterone-D_3 (NT-D_3) and 17α-boldenone were obtained from RIVM (Bilthoven, the Netherlands), 16-β-OH-stanozolol from WIV-LP (Brussels, Belgium), 4-chloroandrost-

4-ene-3,17-dion from Dr. Willems Instituut (Diepenbeek, Belgium) and 17β-boldenone from Sigma (Bornem, Belgium).

2.2. Preparation of standard solutions

The stock standard solutions (1 mg/ml) of CLAD and β-Bol were prepared by dissolving 5.0 mg in 5.00 ml of methanol. Stock standard solutions (0.1 mg/ml) of α-TbOH, NT-D_3 and α-Bol were prepared from ampoules containing 0.1 mg of lyophilised powder. These standards were reconstituted by adding 1 ml of methanol in the ampoules, vortexing, and transferring the methanol into a glass tube. These manipulations were repeated three times. Finally the methanol was evaporated to dryness and the residue was dissolved in 1 ml of methanol giving a standard solution of 0.1 mg/ml. 16OHstan was available in an ampoule containing a solution of 0.1 mg/ml. Stock solutions are stable for at least 1 year. Working standard solutions, which are stable for 3 months, were made by dilution with methanol to the appropriate concentration (10 ng/μl and 1 ng/μl).

2.3. Materials and apparatus

C_{18} (6 ml/500 mg) and NH_2 (3 ml/200 mg) SPE columns were purchased from Baker (Deventer, The Netherlands). The C_{18} columns were conditioned by passing through 2×5 ml of methanol followed by 2×5 ml of water. The NH_2 columns were conditioned with 6 ml of ethyl acetate.

The steroids were separated on a Symmetry® C_{18} (5 μm; 2.1×150 mm) column from Waters (Brussels, Belgium). A Symmetry® guard column (3.5 μm; 2.1×10 mm) was used prior to the analytical column.

Analyses were performed on a Waters Alliance 2690 HPLC instrument coupled to a Quattro LCZ mass spectrometer (Micromass, Manchester, UK) utilising an electrospray interface. The mass spectrometer was operated in the electrospray positive (ESI+) mode. Capillary voltage was set at 3.5 kV, extractor at 3 V, source block temperature at 100 °C and desolvation temperature at 300 °C. High-purity nitrogen was used as the drying gas and ESI nebulising gas. For collision-induced dissociation, argon was used as the collision gas. Data were collected in the multiple reaction monitoring (MRM) mode.

2.4. Sample clean-up

Sample clean-up was performed according to a method developed in our lab [9]. The pH of 20 ml urine was adjusted to 4.6 with 3 *M* acetate buffer (pH 4.6) and then passed through a C_{18} column. After washing with 2×5 ml of water the column was eluted with 2 ml of methanol. The eluate was evaporated at 40 °C under nitrogen and the residue dissolved in 100 μl of methanol. Enzymatic hydrolysis was done by adding 5 ml of a 0.2 *M* acetate buffer (pH 4.6) and 50 μl of Helix pomatia juice. The samples were then kept for 2 h at 60 °C and subsequently centrifuged for 10 min at 1100 *g*. The supernatant phase was passed through a C_{18} column, which was washed with 2×5 ml of water. After drying the C_{18} column an NH_2 column was coupled underneath it. The columns were eluted in tandem with 2 ml of ethyl acetate, and the eluent was evaporated to dryness at 40 °C under nitrogen. The residue was dissolved in 150 μl of mobile phase (methanol–water–formic acid (64.7–34.7–0.6)).

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry conditions

The liquid chromatographic method was based on earlier work done in the lab [10]. The injection volume used was 50 μl and separation of the different compounds was obtained by using a flow of 0.3 ml/min, column at room temperature and a gradient program (Table 1) with a run time of 40 min.

In Table 2 the precursor ion, daughter ions, cone voltage and collision energy of each compound are presented. Analyses were done in multiple reaction monitoring (MRM), because of its higher sensitivity, using the conditions and ions mentioned in Table 2.

Table 1
Gradient timetable

Time (min)	Solvent A	Solvent B	Flow (ml/min)	Curve
0	55	45	0.3	–
15	55	45	0.3	–
17	65	35	0.3	Linear
30	65	35	0.3	–
32	55	45	0.3	Linear
40	55	45	0.3	–

Solvent A, 0.3% formic acid in methanol; solvent B, 0.3% formic acid in water.

3.2. Validation

The method validation was done according to the draft SANCO/1805/2000 Rev.1 of July 18th 2000 [11]. According to this revision, validation includes the determination of detection capability (CCβ), decision limit (CCα) and specificity for a qualitative confirmation method.

Specificity was checked in two different manners. Firstly, 20 different blank bovine urine samples were analysed to look for possible matrix interferences. No interfering peaks were detected (Fig. 2). Secondly, blank bovine urine samples were spiked with the following compounds: dienestrol, diethylstilbestrol, ethinylestradiol, fluoxymesterone, methylboldenone, 17α-methyltestosterone, norethandrolone, norgestrel, α-zeranol, β-zeranol, hexestrol, ethylestraandiol, methylandrostanediol, progesterone and methandriol. This was done to look for compounds that possibly could interfere with the detection of the compounds under investigation. The chromatograms of this experiment were very similar to the ones obtained by analysing blank urine samples. From these two experiments it could be concluded that the method was specific.

The decision limit (CCα) is defined by the draft SANCO/1805/2000 Rev.1 as: 'the limit from which it can be decided that a sample is truly violative with an error probability of α'. With a qualitative method CCα is determined by analysing at least 20 blank samples and calculating the signal at the time window in which the analyte is expected. Three times the S/N can be used as the decision limit. The results of this analysis are presented in Table 3.

The detection capability (CCβ) is 'the smallest content of the analyte that may be detected and or identified with an error probability of β'. This β-error should be less than or equal to 5%. Twenty fortified bovine urine samples were used to determine the CCβ of each of the components. Only one sample per analyte is allowed to give a false

Table 2
Precursor and most abundant daughter ions and their optimal ESI (+) MS–MS conditions

Compound	Precursor ion (m/z)	Daughter ions (m/z)	Cone voltage (V)	Collision energy (eV)
16-β-OH-Stanozolol	345	81[a]	40	45
		95	40	45
		107	40	45
17α-Trenbolone	271	107	45	25
		199	45	25
		253[a]	45	25
4-Chloroandrost-4-ene-3,17-dion	321	131	45	20
		143[a]	45	20
		173	45	20
α-Boldenone	287	121[a]	25	15
		135	25	15
		269	25	15
β-Boldenone	287	121[a]	25	15
		135	25	15
		149	25	15
17β-19-Nortestosterone-D3	278	83	45	28
		109[a]	45	28

[a] The most abundant ion.

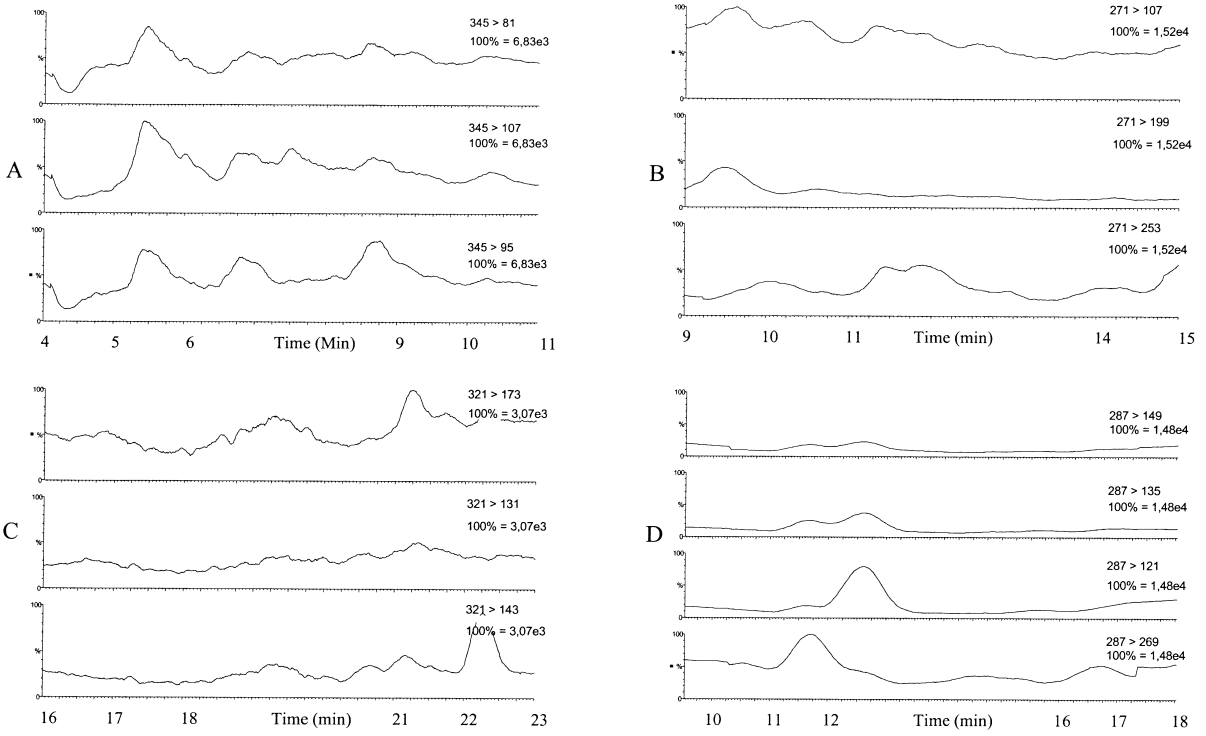

Fig. 2. Chromatogram of blank urine samples. Ions monitored for (A) 16-β-OH-stanozolol; (B) 17α-trenbolone; (C) 4-chloroandrost-4-ene-3,17-dion and (D) α- and β-boldenone. Conditions: recorded in MRM; injection of 50 μl; flow-rate 0.3 ml/min; mobile phase start condition methanol–water–formic acid (54.7–44.7–0.6) linear increasing at 15 min to (64.7–34.7–0.6).

Table 3
Calculated CCα (according to draft SANCO/1805/2000 Rev.1)

Compound	Ion (m/z)	CCα
16-β-OH-Stanozolol	95	2.12
	81	1.44
	107	2.94
17α-Trenbolone	253	4.17
	107	4.42
	199	3.77
4-Chloroandrost-4-ene-3,17-dion	143	2.08
	173	2.01
	131	1.73
α-Boldenone	121	1.22
	135	0.90
	269	1.33
β-Boldenone	121	2.87
	135	5.45
	149	1.88

negative result in order to have a CCβ of the concentration at which it was spiked. In this case blank urine samples were spiked with each of the components at a concentration of 1 ng/ml. In Fig. 3 a chromatogram is presented of each of the analytes spiked at 1 ng/ml. Out of 20 urine samples no false negative results were obtained, for either one of the analytes.

In accordance with the draft SANCO/1805/2000 Rev.1 a sample can only be determined positive when the following criteria are met. The S/N of two diagnostic ions have to be greater then three. The relative retention time of the analyte should correspond to that of the standard analyte, from a spiked sample, with a tolerance of 2.5%. And the relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense transition, must correspond to those of the standard analyte, from a spiked sample, with the tolerances given in Table 4. All these criteria were fulfilled with

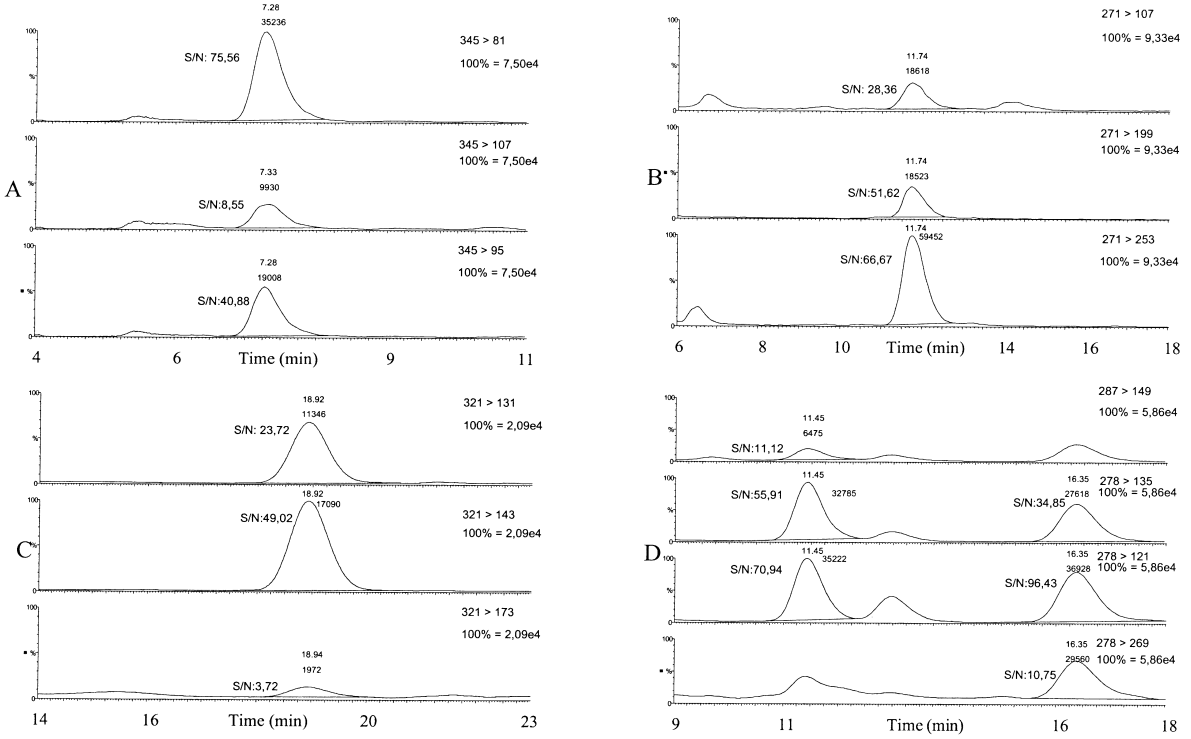

Fig. 3. Chromatogram of blank urine samples spiked at 1 ng/ml showing the diagnostic ions of (A) 16-β-OH-stanozolol; (B) 17α-trenbolone; (C) 4-chloroandrost-4-ene-3,17-dion and (D) α- (RT: 16.35) and β-boldenone (RT: 11.45). Conditions: recorded in MRM; injection of 50 μl; flow 0.3 ml/min; mobile phase start condition methanol–water–formic acid (54.7–44.7–0.6) linear increasing at 15 min to (64.7–34.7–0.6).

the samples spiked at 1 ng/μl. This led for each analyte to a CCβ smaller than 1 ng/μl.

4. Conclusion

It was our aim to develop a multi-analyte method that could confirm the illegal use of stanozolol, trenbolone acetate, chlorotestosterone acetate and β-boldenone (ester) in animal breeding in agreement with the draft SANCO/1805/2000 Rev.1 of July 18th 2000. This was achieved by searching for their main urinary metabolites (16OHstan, α-TbOH, CLAD, α-Bol and β-Bol) in urine. We were able to develop an LC–MS–MS method that separated the different components in retention time and in mass, thus leading to a specific method. Furthermore, detection up to at least 1 ng/μl of each analyte was possible, due to the sample clean-up procedure. This resulted in a sensitive and specific qualitative confirmation method for the detection of the aforementioned compounds in bovine urine. This method was validated in accordance with draft SANCO/1805/2000 Rev.1 and is used in routine analyses in our laboratory. Because of the ease of the method and the high sensitivity this method showed for each compound, we have decided to expand the method to other steroids.

Table 4
Maximum permitted tolerances for relative ion intensities (according to the draft SANCO/1805/2000 Rev.1)

Relative intensity (% of the most intense peak)	Relative tolerance
>50%	±20%
>20–50%	±25%
>10–20%	±30%
≤10%	±50%

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