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Multi-residue screening and confirmatory analysis of anabolic steroids in urine by gas chromatography coupled with tandem mass spectrometry

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Abstract

The diversity of substances used illegally as growth promoters in meat production requires the development of multi-analyte methods of analysis involving a sample pretreatment step that is as rapid and as easy as possible, followed by a specific and sensitive determination of several residues within the same run. A general strategy for the screening and confirmatory analysis of fifteen artificial anabolic compounds in urine samples is described. It is based on solid-phase extraction on C_{18} Empore discs and amino-bonded columns followed, after derivatization (trimethylsilyl or methyloxime-trimethylsilyl derivatives), by gas chromatography coupled with collisionally activated dissociation tandem mass spectrometry.

1. Introduction

All over the world, and particularly in the European Community (EC), the attention of consumers is increasingly focused on chemical compounds generating residues in foodstuffs of animal origin. Public health authorities and the agrofood industries are faced with many difficulties in satisfying consumers and exportation market demands regarding the quality of meat and the safety and control of such residues. The detection of artificial anabolic agents illegally used as growth promoters in industrial farming is considered as a priority in the EC.

The diversity of substances used illegally as growth promoters in meat production has made it essential to develop a multi-residue strategy of analysis, involving a rapid and easy sample pretreatment followed by a specific and sensitive determination of several residues in the same run.

The official techniques used until now for these controls present many drawbacks. On the one hand, the immunological screening methods, radio- [1] and enzyme immunoassays [2], are very sensitive but, in most instances, are mono-residue tests. Moreover, the cross-reactivity properties of the antibodies [3] used in these tests with structural analogues of the controlled residue preclude an unambiguous identification of the compound in the complex matrix of a biological sample. As a consequence, positive results have to be validated by other, more reliable, analytical methods. The confirmatory

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analysis techniques most often used are thin-layer chromatography [4] and low-resolution mass spectrometry coupled with gas chromatography [5]. To reach acceptable detection limits (μg/kg or ppb levels), they require extensive purification of the biosample (HPLC and/or multi-step column chromatography) before the analytical determination itself. Moreover, these efficient purifications are selective and do not allow multi-residue detection within the same chromatographic run.

The technique of determination that appears to be the most powerful for the analysis of anabolic steroid residues is collisionally activated dissociation tandem mass spectrometry (CAD-MS–MS) in the multiple reaction monitoring mode (MRM), coupled with gas chromatography.

The mass spectrometer used in our laboratory is a VG-AutoSpecQ ("EBEQQ" hybrid instrument), which allows the CAD mode of MS–MS to be used. It involves the selection of a precursor ion (formed in the ion source) in the sector part (EBE) of the mass spectrometer, its fragmentation in the quadrupolar collision cell (energetic collision with a gas such as air, helium or argon), followed by the determination of the mass to charge ratio (m/z) of the product ions by the last quadrupolar analyser. In this way, it is possible to monitor transitions from selected precursor ions to particular product ions (MRM mode), the first part of the instrument thus being considered as a physical mass filter that will avoid a major part of the biological matrix interferences to be analysed by the second analyser.

This is a very selective method that can be used with simple pretreatment of purification of the biosample. It is sensitive enough to reach the detection limits in the ppb range and also permits the determination of several residues within the same chromatographic run.

The aim of this study was to develop a general strategy for the screening and confirmatory analysis of artificial anabolic compounds in urine samples of animal origin. This paper describes the development of a rapid procedure for the detection of these residues, involving solid-phase extraction on C_{18} Empore discs and amino (NH_2)-bonded columns for sample pretreatment of large volumes of urine (30 ml), followed by gas chromatography coupled with tandem mass spectrometry for determination. The target compounds selected in this study were artificial and naturally occurring anabolic compounds commonly but illegally used in industrial farming.

2. Experimental

2.1. Material and reagents

All the solvents were of analytical-reagent grade from Merck (Darmstadt, Germany). β-Glucuronidase–arylsulphatase from *Helix pomatia* was obtained from Boehringer (Mannheim, Germany), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), N,N-bistrimethylsilyltrifluoroacetamide (BSTFA) and dry pyridine from Macherey–Nagel (Düren, Germany), iodotrimethylsilane (TMSI) from Aldrich (Brussels, Belgium), dithioerythritol (DTE) from Sigma (Deisenhofen, Germany) and methoxylamine hydrochloride from Supelco (Bellefonte, PA, USA).

Standards of steroids (see Fig. 1) were as follows. β-nortestosterone (nandrolone, 17β-hydroxy-4-estren-3-one), methylboldenone (17-methyl-1,4-androstadien-17β-ol-3-one), methyltestosterone (17-hydroxy-17-methylandrost-4-en-3-one), boldenone (1,4-androstadien-17β-ol-3-one), α-testosterone (17α-hydroxyandrost-4-en-3-one), β-testosterone (17β-hydroxyandrost-4-en-3-one), chlorotestosterone (clostebol, 4-chloro-17β-hydroxyandrost-4-en-3-one), chlorotestosterone acetate, *trans*-diethylstilbestrol [*trans*-DES; 4,4'-(1,2-diethyl-1,2-ethenediyl)bisphenol], ethinylestradiol [17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol), α-estradiol [estra-1,3,5(10)-treine-3,17α-diol] and β-estradiol [estra-1,3,5(10)-treine-3,17β-diol] were obtained from Sigma (Deisenhofen, Germany). α-Trenbolone (17α-hydroxy-19-norandrosta-4,9,11-trien-3-one) and β-trenbolone (17β-hydroxy-19-norandrosta-4,9,11-trien-3-one) were

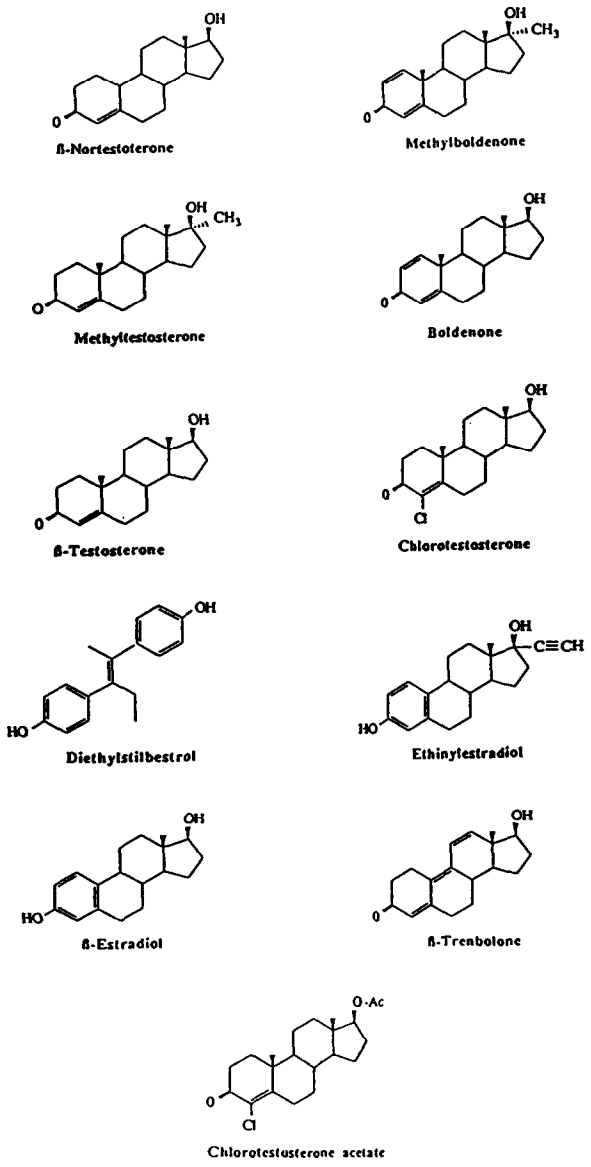

Fig. 1. Structures of the target anabolic compounds.

gifts from Roussel Uclaf (Romainville, France). α-nortestosterone (17α-hydroxy-4-estren-3-one) was a gift from CER (Marloie, Belgium).

Solid-phase Empore (3M) octadecyl-bonded Bakerbone discs (47 mm) and a Bakerbond Speamino (NH_2-bonded column (200 mg, 3 ml) were obtained from J.T. Baker (Deventer, Netherlands).

A DB-5 fused-silica capillary GC column (Ultra-2; 20 m × 0.2 mm I.D., 0.33-μm film thickness) was supplied by Hewlett-Packard (Avondale, PA, USA).

2.2. Apparatus

The extraction apparatus was composed of a Vac Elut SPS24 vacuum manifold from Varian (Brussels, Belgium) for Empore discs and a Baker Van Elut for disposable columns. An HP-5890 Series II gas chromatograph and an HP-7673 automatic injector were supplied by Hewlett-Packard. The tandem mass spectrometer used was a VG AutoSpecQ (VG Analytical, Manchester, UK).

2.3. Hydrolysis of conjugated steroids

Blank, fortified (2,5 and 10 ng/ml) and incurred urine samples (30 ml) were adjusted to pH 4.6 with 3 *M* sodium acetate buffer. They were hydrolysed at 60°C for 3 h with 75 μl of β-glucuronidase–arylsulphatase digestive juice from *Helix pomatia*.

2.4. Solid-phase extraction of steroids

The hydrolysed urine samples were centrifuged at 2000 *g* for 10 min at room temperature. The supernatant was extracted on a C_{18} Empore disc (47 mm) under a depression of 0.4 bar. The discs were preconditioned with ethyl acetate (10 ml), methanol (10 ml) and water purified with a Milli-Q system (2 × 10 ml). The sample was then applied to the extraction disc. The washing sequence consisted of 5 ml of methanol–water (55:45, v/v) and 5 ml of hexane. The disc was dried under vacuum (0.15 bar depression) for 10 min and the steroids were eluted with 3 × 5 ml of ethyl acetate. This extract was dried under a stream of nitrogen in a water-bath at 40°C. The dry residue was dissolved in 1 ml of ethyl acetate and applied to a Bakerbond column (200 mg) under a depression of 0.2 bar. The columns were preconditioned with 3 × 2 ml of ethyl acetate. The steroids were collected and further eluted with 500 μl of ethyl acetate.

2.5. Derivatization procedures

Trimethylsilylation

The extracts were evaporated to dryness at 40°C under a stream of nitrogen and derivatized with 50 μl of MSTFA–TMSI–DTE (100:10:5) at 60°C for 30 mins [7].

Methyloxime/trimethylsilyl ether (MO/TMS) derivatization

The extracts were evaporated to dryness at 40°C under a stream of nitrogen and derivatized with 100 μl of methoxylamine hydrochloride in dry pyridine solution (20 mg/ml) at 60°C for 1 h. Pyridine was evaporated to dryness under a stream of nitrogen (40°C) and trimethylsilylation was performed with 50 μl of BSTFA (60°C, 30 min) [7–9].

2.6. Gas chromatography

The temperature of the GC column oven was initially 120°C for 1 min, then increased at 15°C/min to 240°C and subsequently at 5°C/min to 300°C, the final temperature being maintained for 9 min. The carrier gas has helium (grade N_{60}) with a column head pressure of 100 mbar and a flow-rate of 1 ml/min. A 1-μl volume of the derivatization mixture was injected in the splitless mode; the injector and transfer line temperatures were 300°C.

2.7. Tandem mass spectrometry

The positive electron impact mode (EI^+) was applied with an electron energy of 70 eV and a trap current of 200 μA. The source temperature was 190°C. The collision gas used for CAD was air at a pressure of 10^{-6} mbar and with a collision energy of 30 eV. The resolution (at 10% of valley) of the sector part of the hybrid instrument (VG-AutoSpecQ) for the MRM mode was 100 and the quadrupole span was 0.5 mass unit. The dwell time for each transition was 80 ms.

3. Results

3.1. Solid-phase extraction recoveries

The recoveries of the extraction procedure (Empore disc and amino-bonded column) for several anabolic steroids were determined for 30-ml urine samples, using tritiated standards. The samples were spiked with tritiated steroids before hydrolysis and the complete extraction procedure was performed for each compound separately. The final extract (in ethyl acetate) was evaporated to dryness under a stream of nitrogen and the dry residue was dissolved in 200 μl of methanol. A 4-mol volume of scintillation liquid (EcoScint A from National Diagnostics) was added and the radioactivity of the extract was measured using a Beckman LS5000CE liquid scintillation counter. The extraction recoveries obtained are given in Table 1.

3.2. Derivatization procedures

The fastest and most sensitive derivatization technique was trimethylsilylation, using MSTFA–TMSI–DTE. This method allows the derivatization of the alcohol functions and, moreover, the enolization and trimethylsilylation of the ketone function of the 3-keto-steroids [6,7].

Unfortunately, trenbolone, one of the androgenic steroids tested, generates numerous isomers using this derivatization mode [7–9], owing to its three conjugated double bonds. One solution to this problem was to block the A-ring of trenbolone to form a 3-methyloxime group, and further derivatize the 17-alcohol function with a trimethylsilylating agent (BSTFA). In this way, only one peak was observed under the GC conditions used. The other 3-keto-steroids could also be MO-TMS derivatized (see Table 2), but for methyltestosterone we could not reach a detection limit below 10 ppb (in the MRM mode), owing to its poor MO-TMS derivatization efficiency under the conditions used.

Fig. 2 presents, as examples, the EI^+ full-scan ion spectra of the enol–TMS–TMS ether derivative of nortestosterone and of the MO-TMS derivative of trenbolone.

Table 1
Recoveries of tritiated anabolic steroids in spiked urine samples (30 ml) by the solid-phase extraction procedure

Compound	Mean recovery (%) ($n = 5$)	Standard deviation (%)
β-Nortestosterone	96	3
Methyltestosterone	95	4
β-Trenbolone	89	5
Ethinylestradiol	98	3
Diethylstilbestrol	87	6
β-Estradiol	98	2
Testosterone	94	5
α-Trenbolone	91	4

3.3. Tandem mass spectrometry

Different tandem mass spectrometric modes of analysis were tested (daughter and parent ion spectra, constant neutral loss scanning and multiple reaction monitoring) in both the first field-free region and collision quadrupole, using these derivatives. Only the MRM mode using the quadrupole collision cell air pressure 10^{-6} mbar;

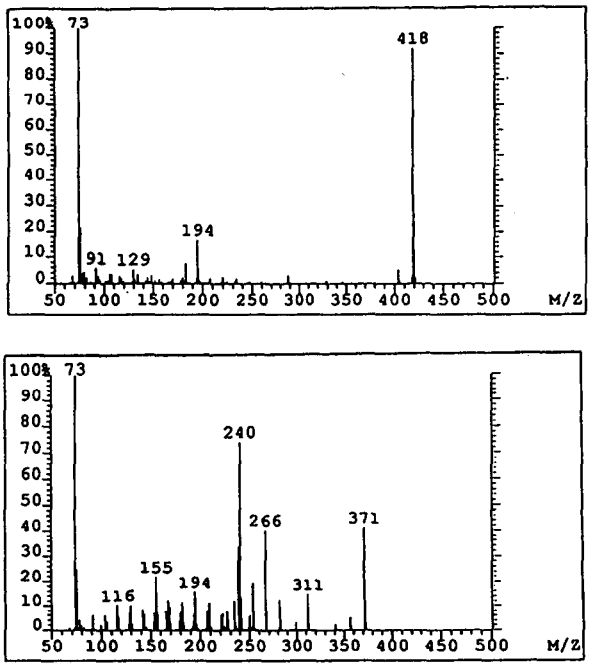

Fig. 2. Electron impact full-scan spectra of the enol–TMS–TMS ether derivative of 19-nortestosterone (m/z 418) and of the MO-TMS derivative of trenbolone (m/z 371).

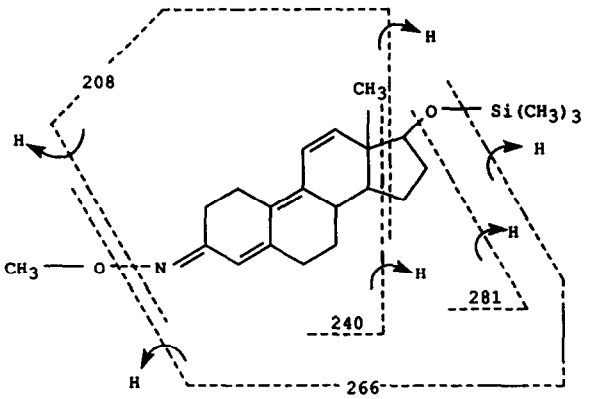

Fig. 3. Tandem mass spectrometric fragmentation (CAD) scheme for the molecular ion (m/z 371) of the MO-TMS derivative of trenbolone. Air pressure, 10^{-6} mbar; collision energy, 30 eV.

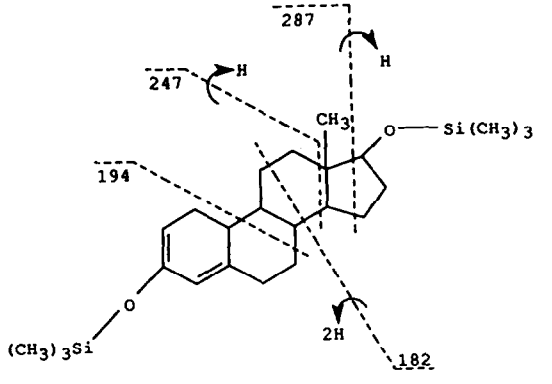

Fig. 4. Tandem mass spectrometric fragmentation (CAD) scheme for the molecular ion (m/z 418) of the enol–TMS–TMS ether derivative of nortestosterone. Air pressure, 10^{-6} mbar; collision energy, 30 eV.

Table 2
Most sensitive transitions monitored [CAD with air (10^{-6} mbar), collision energy 30 eV] and detection limits for each target compound, relative to their derivatization mode

Steroid	Derivatization mode	Retention time (min) (min:s)	Transition monitored (m/z)	Relative intensity (%)	Approximate detection limit (ppb)
α/β-Nortestosterone	TMS	13:10/13:40	418–287	60	0.5
			418–259	25	1
			418–247	20	1
			418–194	100	0.2
			418–182	25	1
	MO-TMS	13:20/13:50	375–344	45	1
			375–295	40	1
			375–254	100	0.5
Boldenone	TMS	14:00	430–415	50	0.5
			430–325	25	1
			430–299	10	2
			430–206	100	0.2
	MO-TMS	14:30	387–356	100	0.5
			387–266	40	1
			387–120	50	1
Methylboldenone	TMS	15:00	444–339	30	1.5
			444–299	25	1.5
			444–206	100	0.5
	MO-TMS	15:30	401–370	100	0.5
			401–280	85	1
α/β-Testosterone	TMS	13:30/14:10	432–417	50	0.5
			432–327	100	0.2
			432–301	60	0.5
			432–208	75	0.5
	MO-TMS	13:40/14:20	389–268	70	1
			389–153	80	1
			389–125	100	0.5
Methyltestosterone	TMS	15:10	446–356	10	0.5
			446–301	100	0.1
α/β-Trenbolone	MO-TMS	14:00/14:50	371–281	65	1
			371–266	90	0.5
			371–240	100	0.5
Chlorotestosterone	TMS	16:50	466–431	70	1
			466–335	100	0.5
			466–230	25	2
	MO-TMS	17:10	423–266	100	0.5
			423–240	25	2
			423–208	20	2.5
Chlorotestosterone acetate	TMS	17:50	436–401	75	1
			436–432	50	1
			436–230	100	0.5
	MO-TMS	18:20	393–302	20	2.5
			393–266	25	2
			393–171	100	0.5
α/β-Estradiol	TMS	13:40/14:10	416–326	20	1
			416–285	100	0.2
			416–232	25	1
Ethinylestradiol	TMS	15:30	440–425	100	0.5
			440–285	60	1
			440–196	30	1.5
trans-Diethylstilbestrol	TMS	11:00	412–397	70	1
			412–383	100	0.5
			412–217	55	1

collision energy 30 eV), allowed to reach ppb (μg/kg) detection limits in multi-residue analysis, as other modes required residue levels over 10 ppb.

The CAD-MS–MS fragmentation schemes proposed, as examples, for the MO-TMS derivative of trenbolone and for the enol–TMS–TMS ether derivative of nortestosterone, under the described conditions, are presented in Figs. 3 and 4, respectively [9,10].

The detection limits reached under these conditions are summarized in Table 2 for each compound and relative to the derivative used. All of them range from 0.1 to 2 ppb, allowing the monitoring of such residues in urine samples (decision level of 2 ppb for artificial anabolic compounds). Another advantageous point is that, for all the target residues tested, one of the three mentioned transitions is more sensitive with detection limits below 0.5 ppb. The monitoring of this unique transition per target residue allows the screening of up to fifteen anabolic compounds during the same chromatographic run.

As an example, Fig. 5 shows the GC–MS–MS traces obtained for a 2 ppb spiked urine sample, for two transitions of the enol–TMS–TMS ether derivative of chlorotestosterone (m/z 466–431/466–335) and two transitions of the MO-TMS derivative of trenbolone (m/z 371–240/371–266). The chemical background observed in these low-resolution MS–MS analyses is much lower than in the chromatograms obtained in the low- or medium-resolution single-ion monitoring mode (SIR), in which it is impossible to interpret data in the ppb range without further purification of the urine sample.

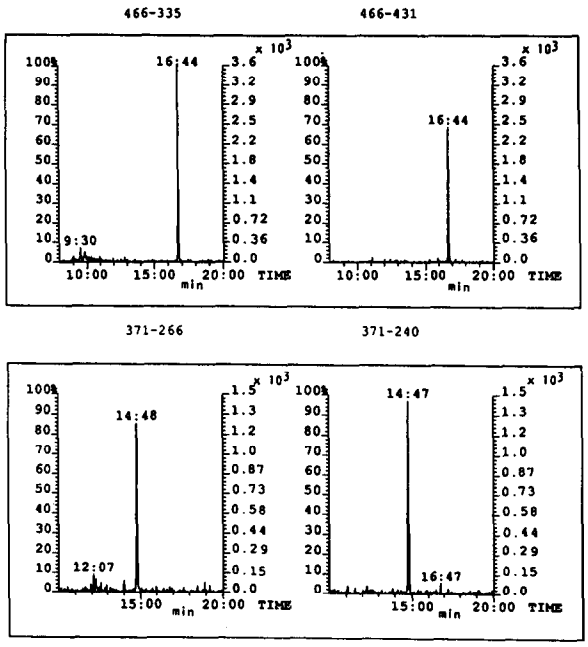

Fig. 5. GC–MS–MS for a 2 ppb spiked urine sample, for two transitions of the enol–TMS–TMS ether derivative of chlorotestosterone (m/z 466–431/466–335) and two transitions of the MO-TMS derivative of trenbolone (m/z 371–266/371–240). Collision cell air pressure, 10^{-6} mbar; collision energy, 30 eV.

4. Discussion

The method developed here, requiring minimal sample preparation, allows, on the same extract, both screening and/or confirmation for multi-residue analysis of anabolic compounds. If ambiguities still remain, the switch, using the same protocol, from TMS to MO-TMS derivatives brings a further confirmation step for 3-keto-steroids. The MO-TMS derivatization mode is necessary for the detection of trenbolone owing to its isomerization during TMS derivatization. Such an MS–MS method could be implemented on cheaper tandem mass spectrometric quadrupolar instruments.

A parallel approach using high-resolution mass spectrometry (HR-MS with resolution >5000) in the single-ion recording (SIR) mode gives the same detection limit for individual compounds. The simultaneous detection of several anabolic steroid residues would imply an acquisition based on voltage SIR. In this instance, the available mass range is only 10% of the nominal mass fixed at the magnet. The narrowness of this mass range does not allow a multi-residue strategy of analysis.

Work is in progress on the direct determination (without derivatization) of extracts using HPLC coupled with electrospray ionization at the inlet to the MS instrument.

Acknowledgements

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