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Determination of anabolic steroids in bovine urine by liquid chromatography–tandem mass spectrometry☆

George Kaklamanos[a], Georgios Theodoridis[b,*], Themistoklis Dabalis[a]

[a] Veterinary Laboratory of Serres, Terma Omonoias, 62110, Serres, Greece
[b] Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University, 54124, Thessaloniki, Greece

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ABSTRACT

A liquid chromatography–tandem mass spectrometric (LC–MS/MS) multi-method has been developed for the determination of 15 anabolic steroids in bovine urine (diethylstilbestrol, dienestrol, hexestrol, β-estradiol, ethynylestradiol, α/β-boldenone, α-nortestosterone, α/β-zearalenol, α/β-zaeralanol, zearalenone, stanozolol and 16β-OH-stanozolol). The procedure involved enzymatic hydrolysis, extraction with tert-butyl methyl ether, a washing step with hexane and final clean-up with SPE with Oasis HLB and Amino cartridges. The analytes were quantified by liquid chromatography coupled to a tandem mass spectrometer (LC-TSQ Quantum AM) operating in both positive and negative atmospheric pressure chemical ionisation (APCI). Data acquisition was performed in multiple reaction monitoring (MRM) mode quantifying two diagnostic product ions from a chosen precursor. The method was validated according to the Commission Decision 2002/657/EC, for the detection and confirmation of residues in products of animal origin. The method specificity, sensitivity, accuracy and precision were evaluated. The decision limits CCα ranged from 0.06 to 0.26 ng/ml and the detection capabilities CCβ ranged from 0.11 to 0.49 ng/ml. The developed method is sensitive and useful for detection, quantification and confirmation of these anabolic steroids in bovine urine and can be used for residue control programs.

1. Introduction

The use of anabolic steroids for growth promotion purposes in meat producing animals results in an improvement in muscle growth, more lean meat and a higher feed efficiency. However, toxicological/epidemiological studies show that there are harmful effects to consumers; as a result the public health is placed in risk. As a consequence, the use of anabolic steroids for fattening purposes has been banned in the European Union since 1986 [1]. Therefore, National Plans of the individual Member States were developed to monitor the use of anabolic steroids. The development of sensitive, specific and multi-residue analytical methods is therefore required for a successful control of the illegal use of growth promoters in meat production, which must be in compliance with the criteria of the Commission Decision 2002/657/EC [2].

Several analytical procedures have been developed for the efficient clean-up of biological matrices, such as liquid–liquid extraction (LLE), solid phase extraction (SPE) and liquid chromatography (LC) fractionation. In the protocols reported in the literature combinations of the above-mentioned pre-treatment procedures are used for the successful determination of anabolic steroids.

Also, different techniques have been developed for the determination of anabolic steroids in biological samples. As such high-performance liquid chromatography (HPLC) [3–6], gas chromatography (GC), GC coupled with mass spectrometry (GC–MS) [7–15] GC-high resolution MS [16], GC–MS/MS (mostly on ion traps see [17–23]) and LC coupled with mass spectrometry (LC–MS) have all been utilized [24–36].

Gas chromatography coupled to mass spectrometry (GC–MS) is a sensitive, robust and suitable technique for the assay of hormones, but it is time-consuming because it requires derivatization due to the analytes polarity and thermal instability. The combination of liquid chromatography coupled to mass spectrometry (LC–MS/MS) offers a rapid, simplified, specific and sensitive alternative to GC–MS methods involving simple extraction procedures and removing the need for derivatization reactions. In most of the reported LC–MS/MS works, electrospray ionisation (ESI) mode is applied.

Both GC–MS and LC–MS have found use in the analysis of steroids in serum, urine meat and hair. At farm level, misuse of anabolic steroids in living animals is being monitored by analyses of the animal's urine. Therefore, the development of analytical procedures for the determination of anabolic steroids in urine has always been a challenge.

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* Corresponding author. Tel.: +30 2310 997718; fax: +30 2310 997719.
E-mail address: gtheodor@chem.auth.gr (G. Theodoridis).

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The present study describes a relatively simple methodology for the detection of 15 anabolic steroids of wide interest in food control programs (due to the potential usage in livestock farming). Prior to the APCI-LC–MS/MS analysis urine underwent enzymatic hydrolysis, liquid–liquid extraction and solid phase extraction. Analysis with APCI provides an attractive alternative to ESI as the two modes differ on the ionisation mechanism and thus the ionisation efficiency for a given compound. The presently developed method provides satisfactory analytical figures of merit and is thus useful for detection, quantification and confirmation of these anabolic steroids in bovine urine and can be used for residue control programs.

2. Experimental

2.1. Chemicals and reagents

α-Zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, zearalenone, diethylstilbestrol, dienestrol, hexestrol, β-estradiol, ethynylestradiol, β-boldenone, α-boldenone, α-nortestosterone, stanozolol, 16β-OH-stanozolol, 17β-estradiol-d3, testosterone-d3, α/β-zearalanol-d4, diethylstilbestrol-d6 and 16β-OH-stanozolol-d3 were purchased from Cerilliant (Promochem, Wesel, Germany), NARL (Pymble, NSW, Australia), RIVM (Bilthoven, The Netherlands) and Sigma (Sigma–Aldrich, Steinhem, Germany).

Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany), tetr-butylmethyl ether (TBME), hexane, acetone, acetic acid and potassium acetate were from Sigma (Steinhem, Germany), ammonia (25%) was from Panreac (Barcelona, Spain) and Helix Pomatia Juice from BioSepra (Cergy, France).

Acetate buffer 2 M (pH 5.2) was prepared by dissolving 25.2 g of acetic acid and 129.5 g of potassium acetate in 1000 ml of water. Ultrapure water was produced with a Pure Lab system (Sation 9000, Spain). 2% ammonium/water solution was prepared by adding 8 ml ammonium 25% in 92 ml of water. Oasis HLB (60 mg, 3 ml) cartridges were obtained from Waters (Milford, MA, USA) and Amino Supelclean NH_2 cartridges from Supelco (Bellenfonte, USA).

Stock standard solutions (1 mg/ml) were prepared in methanol and stored at −20 °C in the absence of light. Working solutions were prepared by appropriate dilution of the stock standard solutions with methanol and were stored at 4 °C in the dark for a maximum period of 6 months.

2.2. Samples

Urine samples collected from untreated bovine animals at slaughterhouses were used as blank and, after fortification with the different steroids, as quality control samples. Urine samples from bovine animals were collected as part of the national program for residue control in Greece, were assayed for the presence of the steroids. The samples were received in frozen condition and were kept frozen (−20 °C) until analysis.

2.3. Instrumentation

LC–MS/MS analysis was performed on a ThermoElectron TSQ Quantum AM mass spectrometer equipped with a Finnigan Surveyor MS pump Plus, a Finnigan Surveyor Autosampler plus and a Dell computer system with Xcalibur data acquisition software (ThermoElectron, San Jose, CA, USA).

2.4. LC–MS/MS analyses

A reversed phase Hypersil ODS column (150 mm × 4.6 mm i.d., 5 μm; ThermoElectron) was used for the analyses. The mobile phase was composed of deionised water as solvent A and methanol as solvent B. The gradient program used was as follows: 40% methanol as solvent B at the start (t = 0 min), increased linear to 70% (t = 12 min, held for 6 min), increased to 85% (t = 18.10 min, held for 1 min) and equilibrated for 3.5 min at the initial conditions. The flow rate was kept at 0.7 ml/min. Injection volume was 15 μl throughout the study. The ionisation of each compound was tested in APCI positive and negative multiple reaction monitoring (MRM) mode. The source conditions were optimized to obtain four identification points (two product ions) for each compound, according to the criteria of the Commission Decision 2002/657/EC. Capillary temperature was tested in the range 300–360 °C and the highest value (360 °C) was employed in the study. The nitrogen sheath and auxiliary gas flow rates were set at 10–50 and 0–5 arbitrary units, respectively. Vaporizer temperature was set at 450 °C. The discharge current was studied in the range 4–8 μA and the value of 6 μA was applied in the study. The peak width for quadrupoles Q1 and Q3 was set at 0.70. The collision energy (CE) and tube lens were optimized for each compound (see Section 3.1 and Table 1).

2.5. Sample preparation

5 ml of urine was spiked with a mixture of internal standards (17β-estradiol-d3, testosterone-d3, α/β-zearalanol-d4, diethylstilbestrol-d6 and 16β-OH-stanozolol-d3) at the concentration of 4 ng/ml and 2 ml of 2 M acetate buffer was added. The pH was controlled for being 5.2 and 25 μl of Helix Pomatia was added. The mixture was hydrolysed for 2 h at 50 °C. After cooling down to room temperature, the mixture was extracted with 10 ml TBME (10 min rotating and centrifuged at 3327 × g). The extract was evaporated in a water bath (55 °C) under a stream of nitrogen. After addition of 4 ml methanol/water (4/1, v/v) the mixture was washed twice with 2 ml of Hexane. The tube was vortexed for 30 s and was subsequently centrifuged for 3 min at 1872 × g. The hexane layers were decanted. The resulting solution was next evaporated in a water bath (at 55 °C) and under a mild nitrogen stream to reduce its volume to a final volume of 0.5 ml. After the addition of 3 ml methanol/water (1/9, v/v), the mixture was loaded on an Oasis HLB cartridge, which was previously conditioned with 3 ml of methanol and 3 ml of water. After three washing steps with (i) 3 ml of 5% (v:v) methanol in 2% ammonium in water, (ii) 3 ml of 40% (v:v) methanol in 2% ammonium in water and (iii) 3 ml water, the analytes were eluted with 3 ml of acetone:methanol (80/20, v/v). The extract was then loaded on an Amino cartridge, which was previously conditioned with 3 ml of acetone:methanol (80/20, v/v) and was directly collected. This final extract was evaporated to dryness in a water bath at 55 °C under a gentle stream of nitrogen. The residue was dissolved in 600 μl methanol, transferred to an injection vial, evaporated under a stream of nitrogen at 55 °C to dryness, redissolved in 100 μl of methanol and analysed on the LC–MS/MS system.

The developed procedure for the extraction–purification of the anabolic steroids in urine samples is shown in Fig. 1.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry conditions

Acquisition parameters of the mass spectrometer were optimized in ion spray mode by direct continuous pump infusion of standard working solutions of the analytes (10 ng/μl) at a flow rate of 10 μl/min in the mass spectrometer. Data acquisition was performed preliminary on the standard compounds in full scan, to choose an abundant precursor $[M+H]^+$/$[M-H]^-$. Although ESI is applied in the majority of the published works in preliminary studies we investigated both ESI and APCI and observed higher detection