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A downscaled multi-residue strategy for detection of anabolic steroids in bovine urine using gas chromatography tandem mass spectrometry (GC–MS^3)

S. Impens [a,*], J. Van Loco [a], J.M. Degroodt [a], H. De Brabander [b]

[a] *Scientific Institute of Public Health, Department of Pharmacology-Bromatology, Section of Food, J. Wytsmanstraat 14-16, B-1050 Brussels, Belgium*
[b] *University of Ghent, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Salisburylaan 133, B-9820 Merelbeke, Belgium*

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Abstract

Within the scope of the European Community member states' residue monitoring plan, illicit administration of anabolic steroids is monitored at slaughterhouse level as well as on living animals. At farm level, urine is one of the target matrices to detect possible abuse of anabolic steroid growth promoters. Optimisation of the routinely applied analysis method resulted in a procedure for which high performance liquid chromatographic (HPLC) fractionation prior to GC–MS^n analysis was no longer required. Analytical results could be obtained within 1 day and only 5 mL urine was needed tot carry out the screening procedure. Using the downscaled methodology, all validation criteria described in the European Commission document 2002/657/EC could be fulfilled, and the minimum required performance limits (MRPLs) established for anabolic steroids in urine, could be achieved.

A higher GC–MS technique's specificity was achieved by detecting the steroids using GC–MS^3. Nevertheless, it was decided to screen routinely sampled urine with GC–MS^2 whereas GC–MS^3 was applied to confirm the presence of anabolic steroid residues in suspected sample extracts.

Keywords: Gas chromatography; Tandem mass spectrometry; Bovine urine; Sex steroid hormones; Anabolic steroid residues; Minimum required performance limits

1. Introduction

By nature, steroidal hormones are produced by the male and female sex organs (testes, ovaries), the adrenal cortex and the placenta. As they are involved in the development of reproductive structures and secondary sexual characteristics, sex hormones are generally applied in veterinary medicine to regulate rut and improve fertility [1]. Next to endogenous steroids, many semi-synthetic and synthetic analogues have been produced and administered to animals.

Based upon pharmacological effects steroids can be divided into three principal groups: estrogens, gestagens and androgens (EGAs) [2]. Because of their anabolic effects, EGAs have been used in animal husbandry to increase the weight of meat-producing animals. Enhanced nitrogen retention and build-up of proteins result in improved muscle growth, a higher carcass quality (lean meat) [3–5] and a higher feed efficiency. However, based upon results of pharmacological/toxicological studies, the use of steroidal hormones for cattle fattening purposes has been forbidden in the European Community (EC) since 1988 [6–8]. Since then, analytical laboratories are involved to analyse the samples taken by the inspection services. For that reason, many analytical procedures have been developed to screen and confirm the presence of EGAs in several matrices.

In 2002, the EC has proposed to establish minimum required performance limits (MRPLs) which all EC member state accredited analytical laboratories must achieve in order to ensure the quality of analysis carried out on official governmental inspection services' order (2002/657/EC) [9]. In Belgium, the Federal Agency for the Safety of the Food Chain (FAVV-AFSCA) has setup a residue monitoring plan and national MRPLs have been established for substances for which no maximum residue level (MRL) has been imposed (Group A substances).

* Corresponding author. Tel.: +32 2 642 51 75; fax: +32 2 642 56 91.
E-mail address: sandra.impens@iph.fgov.be (S. Impens).

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Table 1
HPLC fractionation of anabolic steroids

Component	Fraction 3–4[a]	Fraction 5–6	Fraction 7–8[a]
Hexestrol	x		
Diethylstilbestrol	x		
Dienestrol	x		
α/β-Nortestosterone	x (β)	x (α)	
α/β-Boldenone	x		
Methylandrostanediol			x
Methandriol		x	
Ethylestranediol			x
Methylboldenone	x		
Methyltestosterone		x	
Ethinylestradiol	x		
α/β-Zeranol	x		
α-Trenbolone	x		
Norgestrel		x	
Norethandrolone			x
Chloroandrostenedione		x	
Fluoxymesterone	x		

[a] Fractions 1, 2 and 9 were of no interest (matrix and hydrolysis enzyme residual components).

Illegal steroid administration is being monitored at various stages in the food chain. At farm level, misuse of EGAs in living animals is being monitored by analysis of the animal's excreta (urine, faeces). Out of analytical point of view, urine is preferred to faeces because of its homogeneity. Furthermore, after administration, EGAs are metabolised into more hydrophilic structures to advance elimination out of the animal's body, by which detection of EGAs' residues and their degradation products in aqueous matrices becomes an option [10].

Development of procedures for the determination of residual substances in urine has always been a challenge, as urine is a reservoir of the body's waste products possibly affecting unequivocal detection of the target analytes. Prior to this study, an anabolic steroid residue analysis of urine was performed by hydrolysis of 25 mL bovine urine with *Helix pomatia* juice ($62 \pm 2\,^{\circ}C$, 120 min), followed by a diethyl ether liquid–liquid extraction and fractionation with high performance liquid chromatography (HPLC). Selectively chosen HPLC fractions were combined afterwards to be evaporated and derivatized with $MSTFA^{++}$ (Table 1). Finally, three GC–MS^2 analyses (one run for each combined HPLC fraction) were required to obtain the results for only one urine sample. It took at least 48 h until the analytical results could be passed to the inspection services. And, as urine is sometimes hard to sample resulting in little urine volumes, analysis could not be resumed because of a lack of laboratory sample volume.

In this experimental setup, the extraction and clean-up part of the conventional procedure was optimised because of its rate-limiting part in the conventional methodology.

2. Experimental

2.1. Chemical reagents and reference standards

Reference steroid standards, i.e. hexestrol, diethylstilbestrol, dienestrol, α-nortestosterone, β-nortestosterone, α-boldenone, β-boldenone, methylandrostanediol, methandriol, ethylestranediol, methylboldenone, methyltestosterone, ethinylestradiol, α-zeranol, β-zeranol, α-trenbolone, norgestrel, norethandrolone, chloroandrostenedione, fluoxymesterone, androsterone and equilinine, were obtained from Steraloids (Wilton, NY, USA), Sigma (St. Louis, MO, USA) or the National Reference Laboratory (WIV, Brussels, Belgium). Equilinine and androsterone were used as respectively internal and external reference standard. The EGAs' stock solutions (200 $\mu g\,mL^{-1}$ anabolic steroid in absolute ethanol) and a working solution containing all EGAs at National MRPL concentration level were stored at $4\,^{\circ}C$ when frequently used. If not, storage at $-18\,^{\circ}C$ was recommended.

All reagents and solvents used were of analytical grade quality and provided by Merck (Darmstadt, Germany). Sodium acetate buffer (pH 5.2 ± 0.5) was made out of 150 mL acetic acid 0.4 M and 1 L sodium acetate 0.4 M. Sodium carbonate solution (pH 10.2 ± 0.5) was prepared by mixing 100 mL $NaHCO_3$ (10%, w/v in water) and 500 mL Na_2CO_3 (10%, w/v in water). Both the sodium acetate buffer and the sodium carbonate solution were adjusted to the desired pH with hydrochloric acid 2 M or sodium hydroxide 5 M. Abalone acetone powder from abalone entrails (glucuronidase activity 286,000 units g^{-1}; sulphatase activity 18,500 units g^{-1}) was purchased from Sigma (St. Louis, MO, USA). The derivatization reagent $MSTFA^{++}$, needed to obtain GC–MS^n suitable EGAs (enol-trimethylsilyl ethers), was prepared by dissolving 100 mg ammonium iodide (NH_4I) (Sigma, St. Louis, MO, USA) and 0.2 mL ethanethiol (Acros, Geel, Belgium) in 5 mL *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), followed by dilution of 1.5 mL of this solution with 10 mL MSTFA.

3. Apparatus and materials

3.1. Extraction and clean-up

Following devices were used for extraction and clean-up: a balance, a mini-shaker, a centrifuge, a rotary vacuum evaporator, a water bath, a vacuum sample processing station and a nitrogen evaporator.

Next to Nunc tubes (Nalge Nunc International, Rochester NY, USA) and amber 0.7 mL autosampler vials, glassware and other recipients were selectively chosen to be suitable in each step of the procedure. Solid phase extraction columns were purchased at IST International (Mid Glamorgan, UK): Isolute C_{18} reversed phase columns (500 mg–6 mL) and Isolute aminopropyl (NH_2) columns (100 mg–1 mL).

3.2. GC–MS^n apparatus

A POLARIS ion trap mass spectrometer, coupled to a ThermoQuest CE Trace GC gas chromatograph (Thermo Finnigan, Austin, TX, USA) with a split/splitless injector, was used to perform the GC–MS^n analyses. Samples were injected using a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium or hydrogen gas was used as GC carrier gas at a flow-rate of 1 mL min^{-1}. The hydrogen carrier gas was made out