Journal of Chromatography B, 877 (2009) 2330–2336

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

ARTICLE INFO

Article history:
Available online 28 March 2009

Keywords:
Steroid
Anabolic
Urine
Validation
Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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.

☆ This paper is part of a special issue entitled "Method Validation, Comparison and Transfer", guest edited by Serge Rudaz and Philippe Hubert.

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

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.03.033

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

Table 1
Chromatographic and MS/MS data of the analytes: retention time, mass to charge ratio (precursor and most abundant product ions), optimal collision energy and tube lens.

Compound	Rt (min)	Precursor ion $[M+H]^+$	Product ions (m/z)	CE (eV)	Tube lens (V)
α/β-Zearalanol	15.17/12.92	323.2	123.1[a]	36	112
			149.1	31	112
α/β-Zearalenol	15.72/13.71	321.2	175.2[a]	34	105
			267.3	21	105
Zearalenone	16.58	319.1	185.1[a]	29	102
			187.2	30	102
α/β-Boldenone	16.15/14.28	287.2	121.1[a]	34	82
			135.3	20	82
α-Nortestosterone	17.07	275.2	109.1[a]	29	81
			145.2	28	81
Stanozolol	15.17	329.2	81.1[a]	46	126
			95.1	38	126
16β-OH-Stanozolol	12.81	345.2	81.0[a]	37	162
			95.0	39	162
Compound	**Rt (min)**	**Precursor ion $[M-H]^-$**	**Product ions (m/z)**	**CE (eV)**	**Tube lens (V)**
Diethylstilbestrol	15.86	267.1(−)	222.1[a]	41	101
			237.1	38	101
Dienestrol	16.44	265.1(−)	93.0[a]	35	95
			236.2	27	95
Hexestrol	16.90	269.2(−)	119.0[a]	39	89
			133.0	20	89
β-Estradiol	15.17	271.2(−)	145.2[a]	39	118
			183.3	49	118
Ethynylestradiol	15.15	295.2(−)	145.3[a]	36	112
			159.1	39	112

CE: collision energy.
[a] The most abundant ion (also used for analyte quantification).

signals with APCI. Hence APCI was applied for the rest of the study. MS/MS product ion scans were then recorded in full scan. Finally, all the analyses, were carried out by multiple reaction monitoring (MRM) mode monitoring the product ions of the steroids in order to obtain higher detection specificity and sensitivity. Table 1 lists the parent ions and the product ions of each compound with their optimum selected collision energy.

3.2. Optimization of sample preparation

Most of the anabolic steroids and metabolites in urine are excreted as glucuronide and sulfate conjugates. Although the direct quantification of conjugated steroids has been reported in the literature [37–40] the simultaneous analysis of both the conjugates and the free steroids faces limitations due to the different chromatographic properties, and the different ionisation properties of the conjugated steroids. In-source decomposition of glucuronide conjugates represents a challenge in LC–MS based metabolism studies. In-source decomposition of steroids (β-estradiol) [41] and other drugs [41–43] has been reported to be dependent to the type of the analyte (aglycon) and the experimental conditions (both mobile phase composition and ionization potential) thus giving reason for further research. Therefore typically conjugates are hydrolysed prior to LC–MS/MS analysis. For this, purpose an enzymatic hydrolysis procedure using Helix pomatia juice (which contains β-glucuronidase and sulphatase) was used [16,27,33].

In many papers after hydrolysis the sample is cleaned up directly with SPE, however we found that employing a liquid–liquid extraction (LLE) step enhances sample clean-up. In order to extract the steroids from urine, TBME and diethyl ether were tested in varying amounts. Extraction with TBME gave better recoveries and was selected for the rest of the study. Next a second extraction step was applied to remove non-polar interferences. For this reason we needed a non-polar solvent to remove these interferences and as such hexane and pentane were tried against mixtures of methanol and acetonitrile in water in different organic content ratios. The combination of a methanol/water mixture (4/1, v/v) (as the polar solvent) with hexane (as the non-polar solvent) gave very good separation of the two phases (no emulsion formation) and the highest extraction recoveries (see Fig. 1). These steps proved to be essential when very dirty samples had to be analyzed.

A final SPE step was needed for effective clean-up of urine samples. Solid phase extraction cartridges, including Discovery DSC-18 (500 mg, 3 ml, Supelco) and Oasis HLB (60 mg, 3 ml, Waters) were tested. For the washing step different combinations of methanol and water were tested. In both SPE cartridges the best results were obtained with the application of a dual washing step with methanol/water at 5/95 (v/v, 1st step) and 40/60 (v/v, 2nd step). This washing scheme was found to enhance clean-up without eluting the steroids. Next the pH of the washing step was studied; applying alkaline washing resulted to clean chromatograms, without additional interferences from the matrix. For the elution of the steroids methanol, acetonitril, acetone and combinations of them with water were tested. Finally using the Oasis cartridge acetone as the elution solvent provided the highest recovery; for the Discovery DSC-18 cartridge methanol/water (80/20, v/v) provided the best results as the elution solvent. Overall the Oasis cartridge gave better recoveries and more satisfactory peak shapes at the final chromatogram and was thus finally selected for the rest of the study.

The SPE procedure was also tested at an ASPEC XL (Gilson, USA) automated system for solid phase extraction. This device can host up to 40 SPE cartridges (of 3 ml volume) and thus can process up to 40 samples simultaneously. The results from the test were sat-

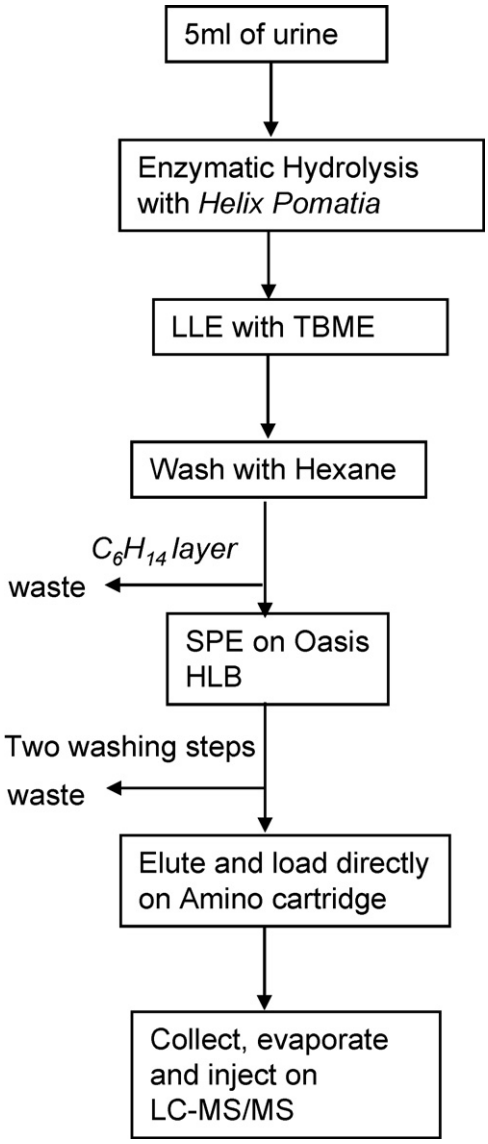

Fig. 1. Analytical procedure for the pre-treatment (hydrolysis, LLE, SPE) of urine samples for the determination of anabolic steroids. LLE: Liquid–liquid extraction, TBME: tetr-butylmethyl ether, Oasis HLB: Oasis hydrophilic–lipophilic balance sorbent.

isfactory, giving an opportunity for analyzing a larger amount of samples in an automated way.

3.3. Validation

It is necessary to ensure the quality and comparability of the analytical results generated by laboratories. This should be achieved by using quality assurance systems and specifically by applying methods validated according to common procedures and performance criteria and by ensuring traceability to common standards. The European Commission Decision 2002/657/EC provides rules for the analytical methods and specifies common criteria for the interpretation of analytical results of official control laboratories in order to ensure a harmonized implementation. Moreover, approved laboratories for official residue control of residues in products of animal origin must prove their competence by regular and successful participation in adequate proficiency testing schemes recognized or organized by the Community reference laboratories.

The method validation was done according to the European Commission Decision 2002/657/EC [2]. Three experiments were performed on 3 different days, Exp1, Exp2 and Exp3. A homogeneous sample (pooled sample from different bovine animals) was made and divided in 63 sub-samples. 21 fortified samples were analysed on each day for 3 days. The samples were fortified as follows: 1 sample not spiked (blank), 6 samples spiked at a level of 1* Validation Level (VL) which was set at the Minimum Required Performance Limit (MRPL), 6 samples spiked at a level of 1.5*VL, 6 samples spiked at a level of 2*VL, 1 sample spiked at a level of 3*VL and 1 sample spiked at a level of 5*VL ($n = 21$). The validation level was 1 ng/ml for diethylstilbestrol, dienestrol, hexestrol, β-estradiol, ethynylestradiol, β-boldenone, α-boldenone, α-nortestosterone and 2 ng/ml for α-zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, zearalenone, stanozolol and 16β-OH-stanozolol.

For the construction of the calibration curves the area of the selected ion of the analyte and internal standard are calculated and their ratio was used as the response variable. A calibration curve is constructed by linear curve fitting using least squares linear regression calculation. Ten points are used for the calibration curve of the standard solutions at concentrations 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 10 ng/ml with the internal standards at concentration 4 ng/ml. Method linearity was satisfactory for all analytes and the correlation coefficients (r^2) were greater than 0.995.

Fig. 2 shows the chromatogram of a spiked sample of the steroids, containing the internal standards at a concentration of 4 ng/ml in MRM mode. From the three experiments on 3 different days the precision and accuracy were determined. Accuracy (the closeness of agreement between a test result and the accepted value) is determined by determining trueness and precision. Since certified reference materials are not available, recovery was determined and was found in the range 79–119%. Precision (the closeness of agreement between independent test results obtained under stipulated conditions) is often expressed as the repeatability. The repeatability (intra-day) was also satisfactory with the CV ranging from 2% to 14% as shown in Table 2. These results, which characterise the analytical variability, indicated that the method can be used on a routine basis.

A fourth experiment Exp4 was applied in order to check the specificity and the ruggedness of the method. Ten blank urine samples collected from different animals (differing in race, age, sexes and were fed with different types of feed) were analysed to look for possible matrix interferences. No interfering peaks were detected. Also, the same blank urine samples ($n = 10$) were spiked at 1*VL, giving satisfactory recoveries from 76% to 119% and CV from 3% to 12%, resulting that the ruggedness of the method is acceptable.

The decision limit CCα is defined as the limit at and above which it can be concluded (with an error probability of α) that a sample is non-compliant. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the intercept equals the decision limit. The detection capability CCβ is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability of β. This β error should be less than or equal to 5%. The corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability. From the calibration curves constructed for the spiked samples at the three experiments the values of the decision limits and detection capabilities for all analytes were calculated as shown in Table 3.

The uncertainty of measurement for all compounds was calculated. The expanded uncertainty (U) provides an interval within the value of the measurement and is believed to lie with a higher level of confidence. It is obtained by multiplying the combined standard uncertainty $u_c(y)$ with a coverage factor k. The choice of the fac-

tor k is based on the level of confidence desired. For a confidence level of 95% the k is 2. Possible sources of error is contamination, inhomogenity, reading errors, weighing, pipettes, purity of standards, construction of calibration curve, interference, etc. To determine the measurement uncertainty, in the case of this validation the variances are the reproducibility and the matrix effects. The matrix effect is determined by subtracting the repeatability of experiment 4 with the reproducibility variance of experiment 1–3. The calculated expanded uncertainties as a relative value (U%) for the anabolic steroids are shown in Table 4.

The possible uncertainties from the preparation of the stock solutions (weighing/volumetric flask/purity), the pipettes and the construction of the calibration curves were investigated. The results showed that the combined standard uncertainties were not signifi-

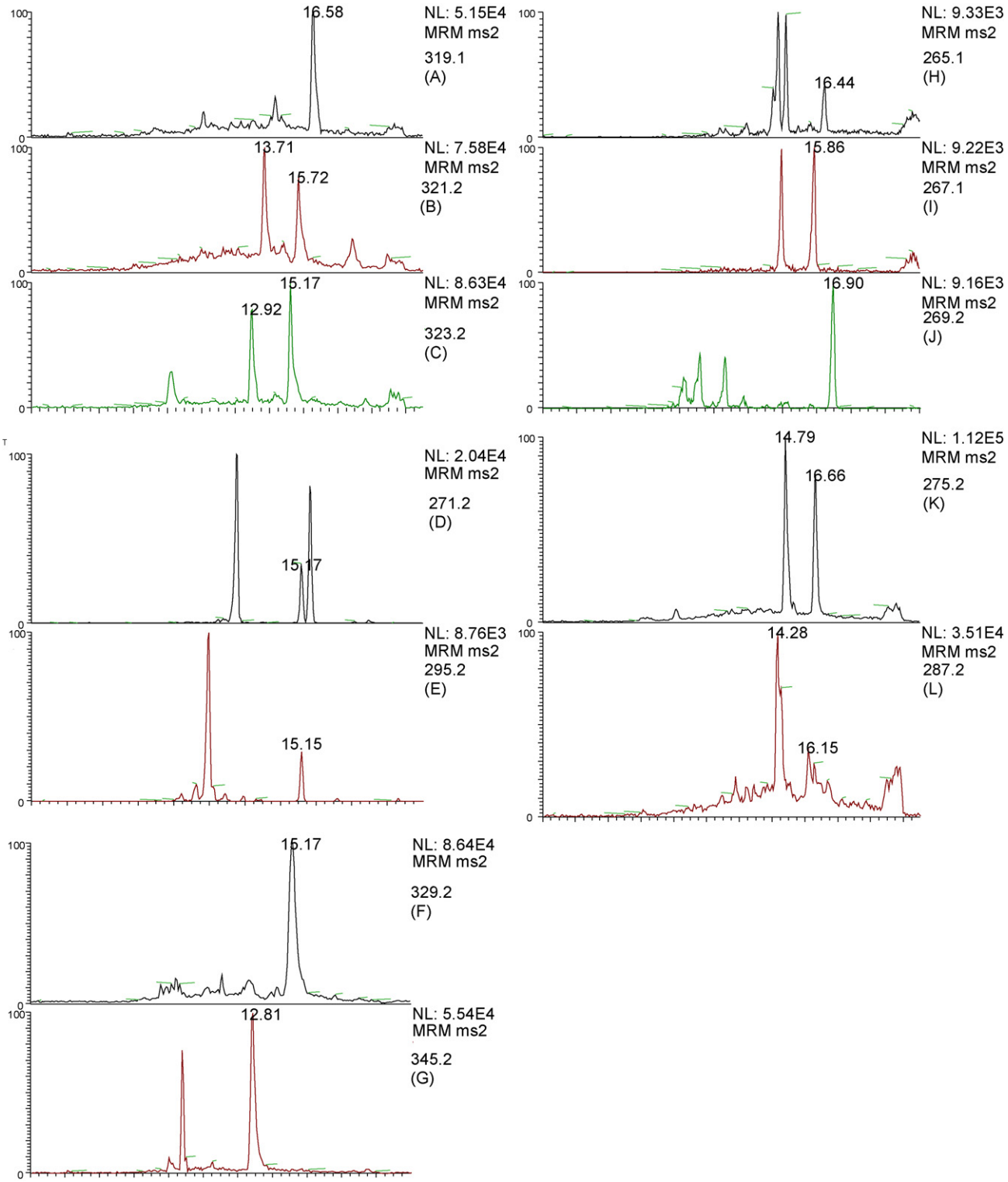

Fig. 2. MRM chromatogram (MS/MS) in APCI of a spiked urine sample containing, zearalenone (A), β/α-zearalenol (B), β/α-zearalanol (C) at a concentration of 2 ng/ml; β-estradiol (D), ethynylestradiol (E) at a concentration of 1 ng/ml; stanozolol (F), 16β-OH-stanozolol (G) at a concentration of 2 ng/ml; dienestrol (H), diethylstilbestrol (I), hexestrol (J) at a concentration of 1 ng/ml; α-nortestosterone (K), and β/α-boldenone (L), at a concentration of 1 ng/ml.

Table 2
Precision and accuracy data for the steroids obtained from the analysis of spiked urine samples on Exp1/2/3 (intra-day and inter-day).

Compound	Spiked (ng/ml)	Repeatability (intra-day)						Inter-day (n = 3)	
		Exp1		Exp2		Exp3		Accuracy (%)	CV (%)
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)		
α-Zearalanol	2	104	8	95	6	102	5	101	5
	3	96	4	96	3	91	4	94	3
	4	97	6	95	7	86	2	92	6
β-Zearalanol	2	95	7	97	4	98	5	97	2
	3	92	3	98	4	95	5	95	3
	4	88	9	98	4	101	3	98	7
α-Zearalenol	2	85	6	94	5	72	7	84	13
	3	81	7	84	3	71	7	79	8
	4	90	10	82	8	98	6	90	8
β-Zearalenol	2	93	6	96	4	90	5	93	3
	3	95	9	88	4	81	8	88	8
	4	93	5	93	2	86	3	91	5
Zearalenone	2	122	6	112	5	115	5	117	4
	3	101	3	100	2	101	6	101	1
	4	97	5	102	7	107	5	102	5
Diethylstilbestrol	1	87	4	106	5	83	6	92	3
	1.5	91	3	100	9	94	7	95	4
	2	98	4	91	8	104	4	98	6
Dienestrol	1	90	3	107	4	108	9	102	10
	1.5	91	7	97	3	107	9	98	8
	2	89	4	94	5	104	10	96	8
Hexestrol	1	95	6	102	2	117	11	104	11
	1.5	95	8	97	5	110	11	101	8
	2	94	4	96	4	111	14	100	9
β-Estradiol	1	101	4	100	3	102	5	101	1
	1.5	104	4	103	3	108	6	105	2
	2	101	5	101	6	105	5	102	2
Ethynylestradiol	1	99	9	102	7	98	8	100	2
	1.5	104	7	94	6	108	7	102	7
	2	107	4	98	7	107	2	104	5
α-Boldenone	1	81	7	79	6	87	3	82	5
	1.5	85	5	79	5	84	6	83	4
	2	77	5	84	6	86	4	82	5
β-Boldenone	1	120	7	121	9	118	8	120	1
	1.5	109	9	109	3	109	2	109	0
	2	104	4	104	1	107	2	105	2
α-Nortestosterone	1	100	2	113	4	105	4	106	6
	1.5	105	2	119	2	108	5	110	7
	2	104	2	119	2	111	4	112	7
Stanozolol	2	96	5	98	6	88	5	94	6
	3	91	5	96	5	84	7	90	7
	4	91	6	91	5	90	3	90	2
16β-OH-Stanozolol	2	100	2	102	4	98	3	100	2
	3	97	5	102	3	101	11	100	3
	4	97	4	99	2	105	1	100	4

cant to the combined standard uncertainty from the reproducibility of the method.

In accordance with the Commission Decision 2002/657/EC a sample can be confirmed as positive when the following criteria are met. The relative retention time of the analyte (RRT) 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 ion, must correspond to those of the reference analyte, either from calibration standards or from incurred samples, at comparative concentrations and measured under the same condition, within the needed tolerances. The ion ratios of the two product ions (relative intensities >50%) of each analyte, signal 2/signal 1 (most abundant), must not exceed the tolerance of ±20%. All criteria were fulfilled for the analysis of the spiked urine samples.

3.4. Real samples analysis

The method was applied to 230 urine samples. These samples had been collected from veterinary directories of the Greek ministry of Rural Development and Food. All samples were processed according to the method described. The samples were analysed and found not containing any of the monitored steroids. Subsequently samples were also spiked at the MRPL level for all compounds and analysis was performed again. In the spiked samples quantification of the steroids at the spiking level was achieved.

3.5. Participation in proficiency test

Participation in proficiency testing allows laboratories to evaluate the reliability of the results they are producing. Also, the

Table 3
Calculated CCα and CCβ for the analytes.

Compound	CCα (ng/ml)	CCβ (ng/ml)
α-Zearalamol	0.22	0.38
β-Zearalanol	0.17	0.29
α-Zearalenol	0.28	0.49
β-Zearalenol	0.19	0.32
Zearalenone	0.26	0.44
Diethylstilbestrol	0.11	0.19
Dienestrol	0.12	0.21
Hexestrol	0.15	0.26
β-Estradiol	0.11	0.19
Ethynylestradiol	0.16	0.27
α-Boldenone	0.10	0.17
β-Boldenone	0.10	0.18
α-Nortestosterone	0.06	0.11
Stanozolol	0.22	0.37
16β-OH-Stanozolol	0.15	0.25

Table 4
Expanded uncertainties (U%) for the analytes.

Compound	*U%*
α-Zearalamol	13
β-Zearalanol	12
α-Zearalenol	18
β-Zearalenol	13
Zearalenone	12
Diethylstilbestrol	19
Dienestrol	21
Hexestrol	25
β-Estradiol	10
Ethynylestradiol	16
α-Boldenone	11
β-Boldenone	11
α-Nortestosterone	15
Stanozolol	14
16β-OH-stanozolol	10

results from a proficiency test permit to assess the bias regarding the consensual value reported by the participating laboratories. We participated, in 2007, at the proficiency test "Estradiol in lyophilized urine", organized by the Community Reference Laboratory CRL in hormones (RIVM, Bilthoven, The Netherlands) among the NRLs (National Reference Laboratories). The aim of this study was to assess the ability to identify and to quantify residues of 17β-estradiol in bovine urine. The number of participants that submitted results where 15, 12 submitted confirmatory results and 3 did not send any results. Fourteen laboratories included a hydrolysis step in the sample preparation prior to extraction and just as much labs extracted the analytes by solid phase extraction. Only seven labs extracted the analytes by liquid/liquid extraction and five used a preparative HPLC-technique too. Two laboratories used LC–MS or LC–MS/MS detection and 13 used GC-HRMS or GCMS. The results were satisfactory at low (mean = 1.98, *z*-score = 0.10, CV = 4%) and high concentrations for β-estradiol (mean = 6.00, *z*-score = 0.64, CV = 0%). The mean value for all laboratories was 1.95 (CV = 24%) at low concentrations and 4.82 (CV = 21%) at high concentrations.

4. Conclusions

The aim of this work was to develop a specific and sensitive multi-method for the quantification and confirmation of 15 anabolic steroids in bovine urine. The method has proven to be highly selective and sensitive. Data obtained showed satisfactory precision and accuracy. The presence of steroids was confirmed, according to the criteria of the European Commission Decision 2002/657/EC, achieving the unambiguous detection of the analytes. The developed method is therefore suitable for laboratories involved in official residue control programs.

References

[1] EC Directive 86/469, Off. J. Eur. Commun. L275 (1986) 36.
[2] 657/2002/EC Commission Decision, Off. J. Eur. Commun. L221 (2002) 8.
[3] N. Yoshioka, Y. Akiyama, N. Takeda, J. Chromatogr. B 739 (2000) 363.
[4] A. Marwah, P. Marwah, H. Lardy, J. Chromatogr. B 824 (2005) 107.
[5] L.C. Dickson, J.D. MacNeil, J. Reid, C.D. Neiser, A.C.E. Fesser, Anal. Chim. Acta 473 (2002) 111.
[6] R. Gonzalo-Lumbreras, R. Izquierdo-Hornillos, J. Chromatogr. B 742 (2000) 1.
[7] S.H. Hsu, R.H. Eckerlin, J.D. Henion, J. Chromatogr. 424 (1988) 219.
[8] P. Marchand, B. Le Bizec, C. Gade, F. Monteau, F. Andre, J. Chromatogr. A 867 (2000) 219.
[9] S. Impens, K. De Wasch, M. Cornelis, H.F. De Brabander, J. Chromatogr. A 970 (2002) 235.
[10] A.A.M. Stolker, H.M.A. Linders, L.A. Van Ginkel, U.A.Th. Brinkman, Anal. Bioanal. Chem. 378 (2004) 1313.
[11] C. Ayotte, D. Goudreault, A. Charlebois, J. Chromatogr. B 687 (1996) 3.
[12] G. Casademont, B. Perez, J.A.G. Regueiro, J. Chromatogr. B 686 (1996) 189.
[13] A.A.M. Stolker, L.A. Van Ginkel, R.W. Stephany, R.J. Maxwell, O.W. Parks, A.R. Lightfield, J. Chromatogr. B 726 (1999) 121.
[14] F. Buiarelli, G.P. Cartoni, L. Amendola, F. Botre, Anal. Chim. Acta 447 (2001) 75.
[15] J. Marcos, X. De la Torre, J.C. Gonzalez, J. Segura, J.A. Pascual, Anal. Chim. Acta 522 (2004) 79.
[16] M.H. Blokland, H.J. Van Rossum, H.A. Herbold, S.S. Sterk, R.W. Stephany, L.A. Van Ginkel, Anal. Chim. Acta 529 (2005) 317.
[17] R.W. Fedeniuk, J.O. Boison, J.D. MacNeil, J. Chromatogr. B 802 (2004) 307.
[18] L. Rambaud, E. Bichon, N. Cesbron, F. Andre, B. Le Bizec, Anal. Chim. Acta 532 (2005) 165.
[19] V. Marcos, E. Perogordo, P. Espinosa, M.M. De Pozuelo, H. Hooghuis, Anal. Chim. Acta 507 (2004) 219.
[20] M.R. Fuh, M.R. Huang, T.Y. Lin, Talanta 64 (2004) 408.
[21] J. Seo, H.Y. Kim, B.C. Chung, J. Hong, J. Chromatogr. A 1067 (2005) 303.
[22] S. Impens, J. Van Loco, J.M. Degroodt, H. De Brabander, Anal. Chim. Acta 586 (2007) 43.
[23] L. Rambaud, F. Monteau, Y. Deceuninck, E. Bichon, F. Andre, B. Le Bizec, Anal. Chim. Acta 586 (2007) 93.
[24] S.A. Hewitt, W.J. Blanchflower, W.J. McCaughey, C.T. Elliott, D.G. Kennedy, J. Chromatogr. 639 (1993) 185.
[25] M. Horie, H. Nakazawa, J. Chromatogr. A 882 (2000) 53.
[26] C. Van Poucke, C. Van Peteghem, J. Chromatogr. B 772 (2002) 211.
[27] F. Buiarelli, G.P. Cartoni, F. Coccioli, A. De Rosi, B. Neri, J. Chromatogr. B 784 (2003) 1.
[28] N.H. Yu, E.N.M. Ho, D.K.K. Leung, T.S.M. Wan, J. Pharm. Biomed. Anal. 37 (2005) 1031.
[29] F. Guan, C.E. Uboh, L.R. Soma, Y. Luo, J. Rudy, T. Tobin, J. Chromatogr. B 829 (2005) 56.
[30] B. Shao, R. Zhao, J. Meng, Y. Xue, G. Wu, J. Hu, X. Tu, Anal. Chim. Acta 548 (2005) 41.
[31] F. Guan, L.R. Soma, Y. Luo, C.E. Uboh, S. Peterman, J. Am. Soc. Mass Spectrom. 17 (2006) 477.
[32] C.L. Xu, X.G. Chu, C.F. Penga, Z.Y. Jina, L.Y. Wang, J. Pharm. Biomed. Anal. 41 (2006) 616.
[33] R. Draisci, L. Palleschi, E. Ferretti, L. Lucentini, P. Cammarata, J. Chromatogr. A 870 (2000) 511.
[34] K. Deventer, P. Van Eenoo, F.T. Delbeke, Biomed. Chromatogr. 20 (2006) 429.
[35] C. Blasco, C. Van Poucke, C. Van Peteghem, J. Chromatogr. A 1154 (2007) 230.
[36] B. Hauser, T. Deschner, C. Boesch, J. Chromatogr. B 862 (2008) 100.
[37] D.J. Borts, L.D. Bowers, J. Mass Spectrom. 35 (2000) 50.
[38] E. Strahm, I. Kohler, S. Rudaz, S. Martel, P.-A. Carrupt, J.-L. Veuthey, M. Saugy, C. Saudan, J. Chromatogr. A 1196 (2008) 153.
[39] F. Pu, A.R. McKinney, A.M. Stenhouse, C.J. Suann, M.D. McLeod, J. Chromatogr. B 813 (2004) 214.
[40] F. Buiarelli, F. Coccioli, M. Merolle, B. Neri, A. Terracciano, Anal. Chim. Acta 526 (2004) 113.
[41] Z. Yan, G.W. Caldwell, W.J. Jones, J.A. Masucci, Rapid Commun. Mass Spectrom. 17 (2003) 1433.
[42] G.S. Walker, J. Atherton, J. Bauman, C. Kohl, W. Lam, M. Reily, Z. Lou, A. Mutlib, Chem. Res. Toxicol. 20 (2007) 876.
[43] H. Keski-Hynnilä, PhD thesis, University of Helsinki, 2002.