JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Anabolic Steroids in Muscle Tissue by Liquid Chromatography–Tandem Mass Spectrometry

GEORGE KAKLAMANOS,[†] GEORGIOS THEODORIDIS,*[,‡] IOANNIS N. PAPADOYANNIS,[‡] AND THEMISTOKLIS DABALIS[†]

Veterinary Laboratory of Serres, Terma Omonoias, 62110 Serres, Greece, and Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University, Thessaloniki, 54124 Greece

A specific and sensitive method based on liquid chromatography–tandem mass spectrometry using atmospheric pressure chemical ionization (LC-APCI-MS/MS) has been developed for the determination of four anabolic steroids [trenbolone, methylboldenone, methyltestosterone, and norethandrolone] in bovine muscle. Methyltestosterone-d_3 was used as internal standard. The procedure involved enzymatic hydrolysis, extraction with *tert*-butyl methyl ether, defattening, and final cleanup with solid-phase extraction with Oasis HLB cartridges. The analytes were analyzed by reversed-phase LC-MS/MS, acquiring two diagnostic product ions from the chosen precursor $[M + H]^+$ for the unambiguous confirmation of hormones. The method was validated according to the European Commission Decision 2002/657/EC for the detection and confirmation of residues in products of animal origin. The limits of detection (LOD) and limits of quantitation (LOQ) were found to be 0.3 ng/g and 1.0 ng/g, respectively. The accuracy and precision have been determined, with recoveries ranging from 83% to 104% and the CV factor not exceeding the value of 7%. The decision limits CCα were calculated and ranged from 0.05 to 0.15 ng/g while the detection capabilities CCβ ranged from 0.09 to 0.25 ng/g. The method proved to be sensitive and reliable and thus renders an appropriate means for residue analysis studies.

KEYWORDS: Steroids; trenbolone; methylboldenone; methyltestosterone; norethandrolone; LC-MS/MS

INTRODUCTION

The use of anabolic steroids as growth promoters in livestock farming has been a known problem for 30 years. A wide range of different anabolic steroids have been used in livestock farming for increasing the muscular mass of the animals, leading to various products of animal origin. As a result the public health of consumers is placed in risk. Because of the above, the use of growth-promoting drugs for fattening livestock has been banned in the European Union since 1986 (*1*). Commission Decision 98/179/EC lays down the detailed rules on official sampling for the monitoring of certain substances and residues thereof in live animals and animal products. Therefore, it is imperative to have analytical methods with enough selectivity for the unambiguous detection and confirmation of these substances, which must be in compliance with the criteria of the European Commission Decision 2002/657/EC (*2*). The discovery of methylboldenone, 17α-methyltestosterone, and norethandrolone in illegal preparations combined with the fact that these compounds are rarely detected in muscle samples (within control programs) prompted us to develop the present research on the detection of steroids in muscle tissue.

Several techniques have been developed for the determination of the above-mentioned steroids in biological samples. As such, high-performance liquid chromatography (HPLC) (*3–6*), gas chromatography (GC), GC coupled with mass spectrometry (GC-MS) (*7–19*), and LC coupled with mass spectrometry (LC-MS) have been utilized (*20–28*).

GC-MS is a sensitive and suitable technique for the assay of hormones and has been the major tool for this assay. However, the methodology can prove to be time-consuming because it requires derivatization to reduce analyte polarity and enhance thermal stability and chromatographic behavior (*7–19*). A further drawback is that derivatization reactions, e.g., toward pentafluoropropionyl derivatives, do not provide the same yields for all steroids; in fact, some of the steroids (methyltestosterone, trenbolone, norethandrolone, and others) do not derivatize well, and there is no universal reagent to overcome these issues (*24*). The best alternative to GC-MS is HPLC-MS and HPLC-MS/MS in the MS2 mode. These configurations can reach acceptable detection limits and most importantly can provide the necessary detection specificity (*20–27*).

In general, the target specimen is serum, urine, and hair samples no matter the chromatographic method (GC or LC

* Corresponding author. Tel: 0030-2310-997718. Fax: 0030-2310-997719. E-mail: gtheodor@chem.auth.gr.
[†] Veterinary Laboratory of Serres.
[‡] Aristotle University.

10.1021/jf0713455 CCC: $37.00
Published on Web 09/24/2007

coupled to MS). For muscle tissue, which is a complex matrix, a few applications have been reported, but the inspection of muscle tissue has an important role. Recently, Shao et al. utilized electrospray ionization (ESI) LC-MS for the determination of 11 illegal natural and synthetic steroids in meat, liver, kidney, and milk (*26*). The sample preparation includes overnight hydrolysis and cleanup with Oasis HLB, silica, and aminopropyl solid-phase extraction cartridges. Further to this, Xu et al. used ESI LC-MS/MS for the determination of 10 steroids in animal tissue (*28*). The sample preparation includes 3–4 h hydrolysis, *tert*-butyl methyl ether (TBME) extraction, and cleanup with C18 cartridges.

In the present paper we report a study using LC-MS/MS with atmospheric pressure chemical ionization (APCI) for the determination of four anabolic steroids, trenbolone, methylboldenone, methyltestosterone, and norethandrolone, including additional analytes compared to the above papers. In most of the published LC-MS works, the ESI mode is applied at the interface between the LC and the MS. Atmospheric pressure chemical ionization (APCI) is by its nature a different technique for the ionization of the compounds compared to ESI. The two modes differ considerably on the ionization mechanism and thus the ionization efficiency for a given compound. Hence, large differences are observed to the signal intensity of the analytes. The developed LC (APCI)-MS/MS method was applied to confirm and quantify the presence of the steroids in bovine muscle. Positive APCI and detection in the MS2 mode in the mass spectrometer gave superior results compared to ESI. Gradient elution gives the advantage of cleaning up interferences that elute close to the retention time of the analytes. The sample preparation protocol consisted of a 2 h enzymatic hydrolysis of the muscle tissue (used for hydrolysis of proteins), extraction with TBME, defattening, and final cleanup on SPE. Sample preparation often proves a critical issue in LC-MS and GC-MS trace analysis. The developed methodology gave satisfactory recoveries and clean final extracts. As a whole the method proved to be reliable and reached the required sensitivity. Hence, it provides a suitable means for the determination and confirmation of steroid residues in muscle and can be used for residue control programs.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. 17β-Trenbolone was purchased from NARL (Pymble, NSW, Australia) and norethandrolone from Cerilliant (Promochem, Wesel, Germany), methylboldenone was obtained from RIVM (Bilthoven, The Netherlands), and 17α-methyltestosterone and methyltestosterone-d_3 were from Riedel de Haen (Sigma-Aldrich, Steinheim, Germany).

Methanol (HPLC grade) and tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany), TBME, hexane, and acetone were from Riedel de Haen (Sigma-Aldrich, Steinheim, Germany), and concentrated ammonia (25%) was from Panreac (Barcelona, Spain). The enzyme protease subtilisin was purchased from Fluka (Sigma-Aldrich, Steinheim, Germany).

Tris buffer, 0.1 M (pH 9.5), was prepared by dissolving 12.1 g of Tris in 1000 mL of water. Ultrapure water was produced with a Pure Laboratory system (Sation 9000, Spain). Ammonium/water solution (2%) was prepared by adding 8 mL of 25% ammonium in 92 mL of water. Oasis HLB (60 mg, 3 mL) cartridges were obtained from Waters (Milford, MA).

Stock standard solutions of 17β-trenbolone, norethandrolone, methylboldenone, 17α-methyltestosterone, and methyltestosterone-d_3 (1 mg/mL) were prepared in methanol and stored at -20 °C in the dark. 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.

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

Instrumentation. LC-MS/MS analysis was performed on a Thermo Finnigan LCQ mass spectrometer (ion trap) equipped with a vacuum degasser (SCM 1000), a pump system (P4000), an autosampler (AS 3000), and a Gateway 2000 computer with Xcalibur data acquisition software (ThermoFinnigan, San Jose, CA).

LC-MS/MS Analyses. A reversed-phase NUCLEOSIL 100-5 C_{18} column (250 mm $\times$ 4.6 mm i.d., 5 μm; Macherey Nagel, Duren, Germany) was used for the analyses. The mobile phase was composed of deionized 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), where it was held isocratic for 25 min ($t = 37$ min). The gradient was then returned to 40% solvent B for an additional 4 min before the next injection. The flow rate was kept at 0.7 mL/min. The injection volume was 26 μL throughout the study.

The atmospheric pressure ionization (API) source was operated in the positive APCI mode. A capillary temperature at 150 °C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 80 and 10 arbitrary units, respectively. The vaporizer temperature was set at 450 °C, the discharge current at 5 μA, the capillary voltage at 46 V, and the tube lens offset at 0 V. Detection of the steroids was performed in the MS2 mode with two events and maximum ion time set at 200 ms.

Sample Preparation. For each tissue muscle sample, a mass of 100 g was homogenized, and a test portion of 5.0 g was weighed into a 50 mL centrifuge tube. The internal standard was added at the concentration of 5 ng/g and mixed with the test portions at least 30 min prior to the addition of 15 mL of 0.1 M Tris buffer (pH 9.5), containing 5 mg of subtilisin. The mixture was incubated for 2 h at 50 °C. After cooling to room temperature, the mixture was extracted twice with 10 mL of TBME (10 min rotating and centrifuged at 3000 rpm). The combined extracts were evaporated in a water bath (55 °C) under a stream of nitrogen. After addition of 4 mL of methanol/water (4/1 v/v) the mixture was washed twice with 6 and 4 mL of hexane for defattening. The tube was vortexed for 30 s and was subsequently centrifuged for 5 min at 3000 rpm. The hexane layers were decanted, and the methanol/water mixture was collected and combined. The resulting solution was next evaporated (water bath at 55 °C and nitrogen stream) to reduce its volume to a final volume of 0.5 mL. After the addition of 3 mL of 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 a washing step with 3 mL of 5% methanol in 2% ammonium/water, 3 mL of 40% methanol in 2% ammonium/water, and 3 mL of water, the analytes were eluted with 4 mL of acetone. The extract was evaporated to dryness in a water bath at 55 °C under a stream of nitrogen. The residue was dissolved in 600 μL of methanol, transferred to an injection vial, evaporated under a stream of nitrogen at 55 °C to dryness, redissolved in 30 μL of methanol, and analyzed directly on the LC-MS/MS system.

RESULTS AND DISCUSSION

Liquid Chromatography–Mass Spectrometry Conditions. Preliminary experiments were performed to select the most suitable column for the separation of the steroids. Four C_{18} columns were tried: Kingsorb (Phenomenex, Torance, CA), Altima (Alltech, Deerfield, IL), Hypersil ODS (Hypersil, Runcorn, U.K.), and Nucleosil 100-5 (MN, Duren, Germany). The tested columns showed varying selectivity for this separation as a result of the different characteristics and chemical properties of their stationary phases (silica chemistry, degree of silanization, carbon loading, etc.). The best overall results

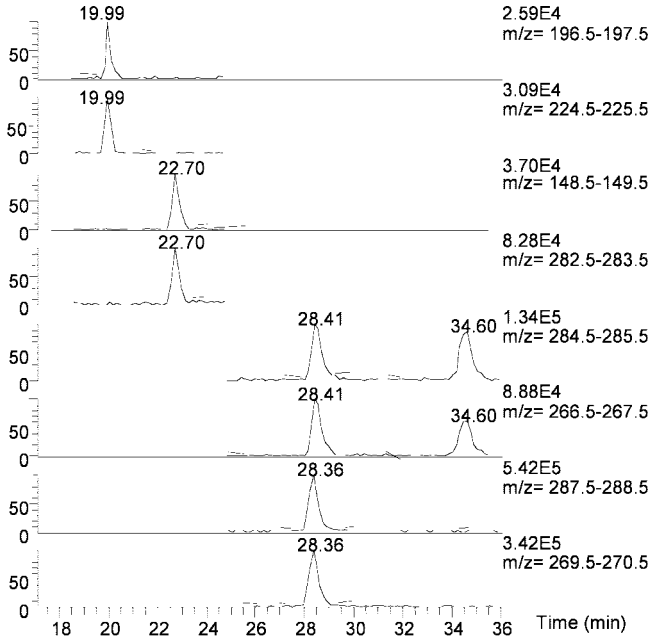

Figure 1. SRM chromatogram (MS/MS) in positive APCI of a standard solution containing 17β-trenbolone, methylboldenone, methyltestosterone, and norethandrolone at a concentration of 1 ng/g with the ISTD methyltestosterone-d_3 at 5 ng/g. Peaks: ISTD, methyltestosterone-d_3, 28.36 min; 17β-trenbolone, 19.99 min; methylboldenone, 22.70; methyltestosterone, 28.41 min; norethandrolone, 34.60 min.

Table 1. Parent and Most Abundant Precursor Ions and Their Optimal Collision Energy

compound	parent ion $[M + H]^+$	precursor ions (m/z)	collision energy (eV)
17β-trenbolone	271	197	36
		225[a]	36
methylboldenone	301	283[a]	36
		149	36
methyltestosterone	303	285[a]	36
		267	36
norethandrolone	303	285[a]	36
		267	36
methyltestosterone-d_3	306	288[a]	36
		270	36

[a] The most abundant ion.

were obtained from the Nucleosil 100-5 C_{18} column, which provided sharper peaks with the strongest detection signals.

Acquisition parameters of the mass spectrometer were optimized in the 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 preliminarily on the standard compounds in full scan in the positive mode to choose an abundant precursor $[M + H]^+$. MS-MS product ion scans were then recorded in full scan. Finally, all of the analyses were carried out by LC-MS/MS in the MS2 mode, monitoring the product ions of the steroids to obtain a high specificity and sensitivity. The collision energy found to give the highest sensitivity for the analytes was at 36 eV. **Table 1** lists the parent ions, and the product ions of each compound are presented. The chromatogram of a standard solution containing 17β-trenbolone, norethandrolone, methylboldenone, 17α-methyltestosterone, and methyltestosterone-d_3 at a concentration of 1 ng/g in the MS2 mode is shown in **Figure 1**.

Optimization of Sample Preparation. The enzymatic hydrolysis is a necessary step for the hydrolysis of muscle tissue proteins. For that, an enzymatic hydrolysis procedure using Tris buffer and subtilisin (protease) was used (*10*).

For the extraction of the steroids from muscle tissue, TBME and petroleum ether were tested in different amounts. Extraction with TBME gave better recoveries. The use of TBME for extraction of steroids has also been reported in previous papers (*10, 28*). After the extraction of the steroids we must remove the fat that remains from the evaporation of the extraction solvent. To facilitate that, liquid–liquid extraction (LLE) procedures were tested. We needed a polar solvent in which the steroids would be soluble and a second nonpolar

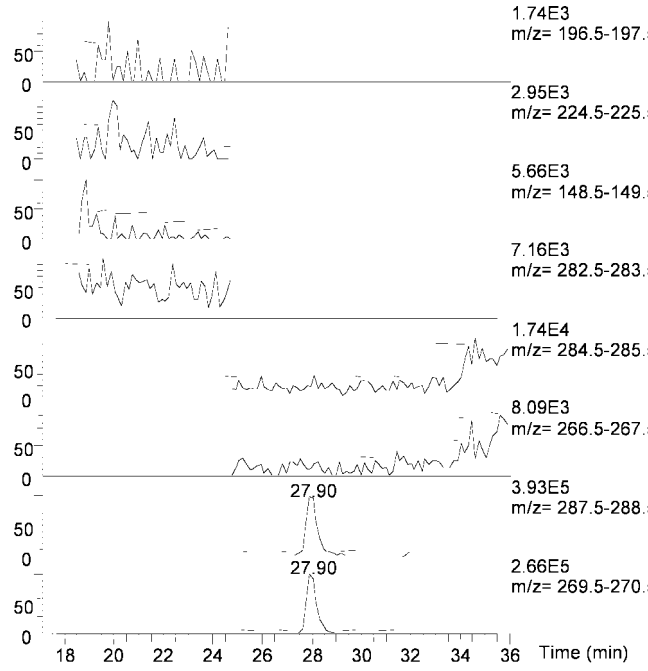

Figure 2. SRM chromatogram (MS/MS) in positive APCI of a blank meat sample. Peaks: ISTD, methyltestosterone-d_3, 27.90 min.

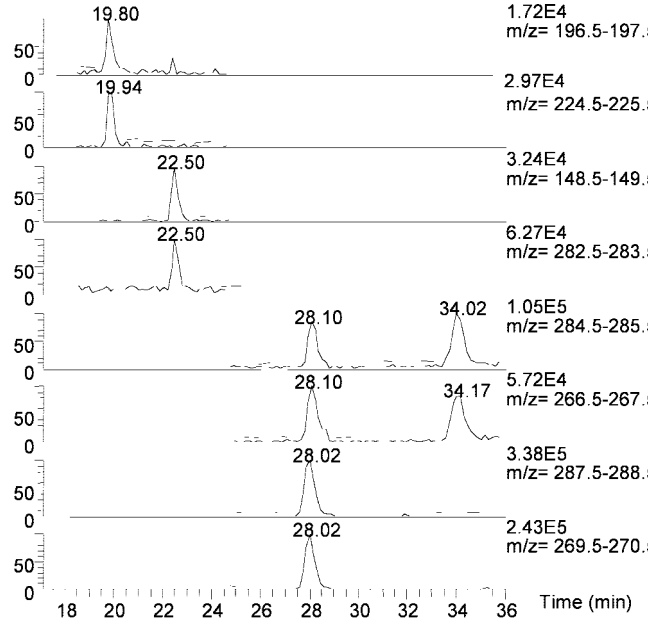

Figure 3. SRM chromatogram (MS/MS) in positive APCI of a spiked meat sample containing 17β-trenbolone, methylboldenone, methyltestosterone, and norethandrolone at a concentration of 1 ng/g with the ISTD methyltestosterone-d_3 at 5 ng/g. Peaks: IS, methyltestosterone-d_3, 28.02 min; 17β-trenbolone, 19.94 min; methylboldenone, 22.50; methyltestosterone, 28.10 min; norethandrolone, 34.02 min.

Table 2. Precision and Accuracy Data for the Steroids Obtained from the Analysis of Spiked Muscle Samples on Experiments 1–3

		repeatability					
		expt 1		expt 2		expt 3	
compound	spiked (ng/g)	accuracy (%)	CV (%)	accuracy (%)	CV (%)	accuracy (%)	CV (%)
17β-trenbolone	1	101.4	0.78	101.1	2.23	103.7	2.62
	1.5	99.7	2.32	98.9	1.15	102.0	1.16
	2	99.6	2.43	100.8	0.85	102.0	1.33
methylboldenone	1	97.8	1.84	95.2	4.70	98.2	3.19
	1.5	96.1	0.92	92.8	4.91	91.7	2.08
	2	83.3	3.37	84.4	4.92	88.1	2.56
methyltestosterone	1	99.7	2.06	99.5	3.18	96.8	1.58
	1.5	100.2	0.62	99.1	0.56	100.2	5.12
	2	96.6	1.24	97.6	0.92	97.7	0.24
norethandrolone	1	97.8	6.86	93.9	6.98	98.0	3.25
	1.5	90.1	0.48	88.7	3.13	94.9	6.11
	2	94.6	1.40	93.8	0.55	93.4	2.01

solvent in which the fat would partition. The choice of the two solvents determines the selectivity and the efficiency of LLE. We tried different combinations of methanol and acetonitrile in water with hexane and pentane. Methanol/water (4/1 v/v) with hexane gave a very good separation of the two phases and the highest steroid recoveries.

A final SPE step is needed for effective cleanup of the muscle tissue 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. The best choice was found to be the application of mixtures of methanol/water (40/60 v/v) and methanol/water (5/95 v/v) for both cartridges. This washing was found to enhance cleanup without eluting the steroids. Next, the pH of the washing step was studied; applying alkaline washing resulted in clean chromatograms, without additional interferences from the matrix. Alkaline washing, which made the difference, has not been reported in previous papers. For the elution of the steroids methanol, acetonitrile, 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.

Validation. The method validation was done according to the European Commission Decision 2002/657/EC (*2*). The validation program ResVal version 2.0 obtained from the Community Reference Laboratory CRL in hormones (RIVM, Bilthoven, The Netherlands) was followed and used for the calculations (*29*). Three experiments were performed on three different days, day 1, day 2, and day 3. A homogeneous sample was made and divided in 63 subsamples. Twenty-one fortified samples were analyzed on each day for 3 days. The samples were fortified as follows: one sample not spiked (blank), six samples spiked at a level of 1 ng/g, six samples spiked at a level of 1.5 ng/g, six samples spiked at a level of 2 ng/g, one sample spiked at a level of 3 ng/g, and one sample spiked at a level of 5 ng/g ($n = 21$).

For the construction of the calibration curves the areas of the selected ion of the analyte and the internal standard are calculated, and their ratio was used as the response variable. A calibration curve is constructed by linear curve fitting using the least squares linear regression calculation. Six points are used for the calibration curve of the standard solutions at concentrations of 0, 0.5, 1, 2, 4, and 10 ng/g with the internal standard at a concentration of 5 ng/g. The linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) being greater than 0.995 for all curves. For the spiked muscle samples the calibration curves were calculated with six points at concentrations of 0, 1, 1.5, 2, 3, and 5 ng/g with the internal standard at a concentration of 5 ng/g. The correlation coefficients (r^2) were greater than 0.99 for all curves.

Figure 2 shows the chromatogram of a blank sample, and **Figure 3** shows the chromatogram of a spiked sample of the steroids, containing the internal standard (ISTD) at a concentration of 5 ng/g in SRM (single reaction monitoring) mode. It can be seen that, in the blank tissue sample, no traces of the selected ions are found in the retention time of the analytes (see also **Table 1**). Comparing **Figure 1** with **Figure 3**, one can see that the peaks of the steroids are unobstructed and have the same scale in the extracted muscle tissue sample (**Figure 3**) as in the reference sample (**Figure 1**). This indicates that detection in the mass spectrometer is selective and that the method recovery is satisfactory.

From the three experiments on three different days the precision and accuracy were determined. Accuracy means the closeness of agreement between a test result and the accepted value. It is determined by determining trueness and precision. Trueness can only be established by means of certified reference materials (CRM). If no CRM is available, instead of trueness, the recovery can be determined, which means the percentage of the true concentration of a substance recovered during the analytical procedure. Precision means the closeness of agreement between independent test results obtained under stipulated conditions. The measure of precision usually is expressed in terms of imprecision and computed as the standard deviation of the result. Repeatability means precision under repeatability conditions, which means conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment. The recovery ranged from 83.3% to 103.7%. The accuracy was evaluated, and CV was calculated at 7% as shown in **Table 2**. These results indicated that the method has acceptable precision and satisfactory recoveries to be used on a routine basis.

The limit of detection (LOD) was checked with spiked muscle samples on the basis of a signal to noise ratio (S/N) of 3:1 and was found to be 0.3 ng/g for all analytes. The limit of quantification (LOQ) was found to be 1 ng/g for all analytes, resulting in a signal to noise ratio (S/N) of 10:1.

Table 3. Calculated CCα and CCβ for the Analytes

compound	CCα (ng/g)	CCβ (ng/g)
17β-trenbolone	0.08	0.14
methylboldenone	0.15	0.25
methyltestosterone	0.05	0.09
norethandrolone	0.13	0.23

The specificity was checked in two different manners. First, 20 different blank bovine muscle tissues were analyzed to look for possible matrix interferences. No interfering peaks were detected. Second, blank bovine muscle tissues were spiked with the following related compounds: dexamethasone, flumethasone, and triamcinolone acetonide. This was done to look for compounds that could possibly interfere with the detection of the compounds under investigation. The chromatograms of this experiment were very similar to the ones obtained by analyzing blank samples. From these two experiments it could be concluded that the method was specific.

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 noncompliant. 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**.

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 $\pm$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 $\pm$20%. All criteria were fulfilled for the analysis of the spiked muscle samples.

Real Sample Analysis. The method was applied to 48 tissue samples, 33 bovine muscle samples, 14 chicken muscle tissue samples, and 1 rabbit muscle tissue sample. 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 analyzed and found not containing any of the monitored steroids.

CONCLUSIONS

The aim of this work was to develop a specific sensitive and reliable LC-MS/MS method for the determination and confirmation of the anabolic steroids 17β-trenbolone, norethandrolone, methylboldenone, and 17α-methyltestosterone in bovine muscle. The method has proven to be highly specific and sensitive. Data obtained showed a satisfactory precision and accuracy with the recoveries ranging from 83% to 104% and the coefficient of variation (CV) not exceeding the value of 7%. The presence of steroids was confirmed, according to the criteria of the European Commission Decision, with the results not exceeding the needed tolerances resulting in the unambiguous detection of the analyte. The method is therefore suitable for laboratories involved in official residue control analyses.

LITERATURE CITED

(1) EC Directive 86/469 concerning the examination of animals and fresh meat for the presence of residues. *Off. J. Eur. Commun.* **1986**, *L275*, 36.

(2) 657/2002/EC Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.* **2002**, *L221*, 8–36.

(3) Yoshioka, N.; Akiyama, Y.; Takeda, N. Determination of α- and β-trenbolone in bovine muscle and liver by liquid chromatography with fluorescence detection. *J. Chromatogr. B* **2000**, *739*, 363–367.

(4) Marwah, A.; Marwah, P.; Lardy, H. Development and validation of a high performance liquid chromatograph assay for 17α-methyltestosterone in fish feed. *J. Chromatogr. B* **2005**, *824*, 107–115.

(5) Dickson, L. C.; MacNeil, J. D.; Reid, J.; Neiser, C. D.; Fesser, A. C. E. Internal validation of screening methodology for residues of epi-trenbolone, trenbolone, epi-nortestosterone and nortestosterone in bovine urine using immunoaffinity cartridges for sample clean-up. *Anal. Chim. Acta* **2002**, *473*, 111–118.

(6) Gonzalo-Lumbreras, R.; Izquierdo-Hornillos, R. High-performance liquid chromatographic optimization study for the separation of natural and synthetic anabolic steroids. Application to urine and pharmaceutical samples. *J. Chromatogr. B* **2000**, *742*, 1–11.

(7) Hsu, S. H.; Eckerlin, R. H.; Henion, J. D. Identification and quantitation of trenbolone in bovine tissue by gas chromatography-mass spectrometry. *J. Chromatogr.* **1988**, *424*, 219–229.

(8) Marchand, P.; Le Bizec, B.; Gade, C.; Monteau, F.; Andre, F. Ultra trace detection of a wide range of anabolic steroids in meat by gas chromatography coupled to mass spectrometry. *J. Chromatogr. A* **2000**, *867*, 219–233.

(9) Impens, S.; De Wasch, K.; Cornelis, M.; De Brabander, H. F. Analysis on residues of estrogens, gestagens and and androgens in kindey fat and meat with gas chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2002**, *970*, 235–247.

(10) Stolker, A. A. M.; Linders, H. M. A.; Van Ginkel, L. A.; Brinkman, U. A. T. Application of the revised EU criteria for the confirmation of anabolic steroids in meat using GC-MS. *Anal. Bioanal. Chem.* **2004**, *378*, 1313–1321.

(11) Ayotte, C.; Goudreault, D.; Charlebois, A. Testing for natural and synthetic anabolic agents in human urine. *J. Chromatogr. B* **1996**, *687*, 3–25.

(12) Casademont, G.; Perez, B.; Regueiro, J. A. G. Simultaneous determination, in calf urine, of twelve anabolic agents as heptafluorobutyryl derivatives by capillary gas chromatography-mass spectrometry. *J. Chromatogr. B* **1996**, *686*, 189–198.

(13) Stolker, A. A. M.; Van Ginkel, L. A.; Stephany, R. W.; Maxwell, R. J.; Parks, O. W.; Lightfield, A. R. Supercritical fluid extraction of methyltestosterone, nortestosterone and testosterone at low ppb levels from fortified bovine urine. *J. Chromatogr. B* **1999**, *726*, 121–131.

(14) Buiarelli, F.; Cartoni, G. P.; Amendola, L.; Botre, F. Screening and confirmation analysis of anabolic agents in human urine by gas chromatography-hybrid mass spectrometry (high resolution-time of flight). *Anal. Chim. Acta* **2001**, *447*, 75–88.

(15) Marcos, J.; De la Torre, X.; Gonzalez, J. C.; Segura, J.; Pascual, J. A. Liquid chromatography clean-up method to improve identification of anabolic agents in human urine by gas chromatography-mass spectrometry. *Anal. Chim. Acta* **2004**, *522*, 79–88.

(16) Blokland, M. H.; Van Rossum, H. J.; Herbold, H. A.; Sterk, S. S.; Stephany, R. W.; Van Ginkel, L. A. Metabolism of methyltest-

osterone, norethandrolone and methylboldenone in a heifer. *Anal. Chim. Acta* **2005**, *529*, 317–323.
(17) Fedeniuk, R. W.; Boison, J. O.; MacNeil, J. D. Validation of a gas chromatography-mass spectrometry method for the determination of pg/ml levels of 17α-trenbolone in bovine serum. *J. Chromatogr. B* **2004**, *802*, 307–315.
(18) Rambaud, L.; Bichon, E.; Cesbron, N.; Andre, F.; Le Bizec, B. Study of 17*β*-estradiol-3-benzoate, 17α-methyltestosterone and medroxyprogesterone acetate fixation in bovine hair. *Anal. Chim. Acta* **2005**, *532*, 165–176.
(19) Marcos, V.; Perogordo, E.; Espinosa, P.; De Pozuelo, M. M.; Hooghuis, H. Multiresidue analysis of anabolic compounds in bovine hair by gas chromatography-tandem mass spectrometry. *Anal. Chim. Acta* **2004**, *507*, 219–227.
(20) Hewitt, S. A.; Blanchflower, W. J.; McCaughey, W. J.; Elliott, C. T.; Kennedy, D. G. Liquid chromatography-thermospray mass spectrometric assay for trenbolone in bovine bile and faeces. *J. Chromatogr.* **1993**, *639*, 185–191.
(21) Horie, M.; Nakazawa, H. Determination of trenbolone and zeranol in bovine muscle and liver by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A* **2000**, *882*, 53–62.
(22) Van Poucke, C.; Van Peteghem, C. Development and validation of a multi-analyte method for the detection of anabolic steroids in bovine urine with liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* **2002**, *772*, 211–217.
(23) Buiarelli, F.; Cartoni, G. P.; Coccioli, F.; De Rosi, A.; Neri, B. Determination of trenbolone and its metabolite in bovine fluids by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* **2003**, *784*, 1–15.
(24) Yu, N. H.; Ho, E. N. M.; Leung, D. K. K.; Wan, T. S. M. Screening of anabolic steroids in horse urine by liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2005**, *37*, 1031–1038.
(25) Guan, F.; Uboh, C. E.; Soma, L. R.; Luo, Y.; Rudy, J.; Tobin, T. Detection, quantification and confirmation of anabolic steroids in equine plasma by liquid chromatography and tandem mass spectrometry. *J. Chromatogr. B* **2005**, *829*, 56–68.
(26) Shao, B.; Zhao, R.; Meng, J.; Xue, Y.; Wu, G.; Hu, J.; Tu, X. Simultaneous determination of residual hormonal chemicals in meat, kidney, liver tissues and milk by liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta* **2005**, *548*, 41–50.
(27) Guan, F.; Soma, L. R.; Luo, Y.; Uboh, C. E.; Peterman, S. Collision-induced dissociation pathways of anabolic steroids by electrospray ionazation tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 477–489.
(28) Xu, C. L.; Chu, X. G.; Penga, C. F.; Jina, Z. Y.; Wang, L. Y. Development of a faster determination of 10 anabolic steroids residues in animal muscle tissues by liquid chromatography tandem mass spectrometry. *J. Pharm. Biomed. Anal.* 200*41*, 616–621.
(29) Blockland, M.; Herbold, H. ResVal 2.0 validation program from CRL in hormones (RIVM, Bilthoven, The Netherlands), **2003–2004**.

Received for review May 8, 2007. Revised manuscript received July 30, 2007. Accepted July 30, 2007.

JF0713455