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Short communication

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

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ABSTRACT

In the present paper we report the LC–MS/MS determination of residues of 12 anabolic steroids in bovine serum, as an expansion of our work protocols for steroids determination in biological matrices. Steroids analyzed included α-zearalanol, β-zearalanol, α-trenbolone, β-trenbolone, methyltestosterone, α-estradiol, β-estradiol, ethynylestradiol, α-boldenone, β-boldenone, α-nortestosterone and β-nortestosterone. Following protein precipitation, serum samples were cleaned up by solid-phase extraction using Oasis HLB and Amino cartridges. Atmospheric pressure chemical ionization (APCI) in both positive and negative ionization modes was used and mass spectrometry detection was carried out in multiple reaction monitoring mode following two or (in most cases) three product ions per precursor ion. The method was validated in accordance with the Commission Decision 2002/657/EC. The decision limit (CCα) values obtained, ranged from 0.01 to 0.07 ng/ml and the detection capability (CCβ) values obtained ranged from 0.02 to 0.12 ng/ml. The recoveries ranged from 70.2% to 118.2%. The developed method is suitable for routine and confirmatory purposes such as control of illegal use in livestock production.

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1. Introduction

Administration of anabolic steroids for fattening livestock has been prohibited in the European Community to protect consumers from possible harmful effects [1]. Monitoring the illegal use of such substances requires reproducible, sensitive and selective methods in compliance with the criteria of the Commission Decision 2002/657/EC [2]. GC–MS has been used extensively to identify steroids in various matrices (meat, fat, urine and blood serum) [3,4]. LC–MS methods have been mostly applied to the analysis of steroids in urine, meat and fat, however few reports deal with serum or plasma analysis [5–11]. Usually, anabolic steroids are implanted in the animal's ear so to achieve longer term release in the bloodstream [12]. Analysis of blood serum from dosed animals would provide a "snapshot" of its metabolic profile virtually showing an image of the concentration of biomolecules (including drugs and their metabolites) at the moment of blood withdrawal. Multi-methods for the determination of anabolic steroids in serum would provide a useful tool for the control of animal "doping". Interesting approaches are reported in the literature to achieve these ends, including liquid–liquid extraction (LLE) [11], combinations of liquid–liquid extraction (LLE) with solid-phase extraction (SPE) [5], on-line coupling of SPE with LC–MS–MS [10], or combination of derivatization (to enhance detection sensitivity) with LLE, SPE and UPLC–MS–MS [9].

Our laboratory has developed methodologies for the determination of steroids in meat [13], fat [14] and urine [15]. The present study is an expansion of our previous works and aims to cover the analysis of serum to complete the list of necessary analytical protocols in our laboratory. The intention of this research was to develop a specific and sensitive LC–MS/MS method for the analysis of blood serum from livestock for the determination of anabolic steroids that are most widely used for fattening. The experimental protocol was based on our previous methods [13,15] with small modification and utilised APCI (and not ESI which is most often reported) and a simple clean-up process for the pre-treatment of real samples.

2. Experimental

2.1. Chemicals and reagents

Solvents, acids and salts were from Merck (Darmstadt, Germany), Sigma (Sigma–Aldrich, Steinhem, Germany) and Panreac (Barcelona, Spain) [15]. Pure water was produced by a Pure Lab system (Sation 9000, Spain). SPE cartridges Oasis HLB (60 mg, 3 ml) were purchased from Waters (Milford, MA, USA) and Amino

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and Discovery DSC-18 cartridges (500 mg, 3 ml) from Supelco (Bellefonte, PA, USA). Hormone standards were provided from NARL (Pymble, NSW, Australia), Cerilliant (Promochem, Wesel, Germany) and from Sigma (Sigma-Aldrich, Steinhem, Germany). Internal standards were all obtained from RIVM (Bilthoven, The Netherlands). Each compound was dissolved in methanol to make a stock solution at a concentration of 1 mg/ml and was stored at −20 °C. Solutions were diluted to the required concentrations with methanol and were stored at 4 °C.

2.2. Instrumentation and LC–MS/MS conditions

HPLC analyses were carried out on a Surveyor MS pump with a degasser and Surveyor plus autosampler (ThermoElectron, San Jose, CA, USA). The chromatographic program and MS conditions were based on work previously done for the analysis of steroids in urine and meat [12,14]. Separations were obtained with a Hypersil ODS column (ThermoElectron, 150 mm × 4.6 mm i.d., 5 μm) under gradient conditions with water and methanol, at a flow rate of 0.7 ml/min, with the injection volume set at 15 μl. Data were acquired using a ThermoElectron TSQ Quantum mass spectrometer (ThermoElectron, San Jose, CA, USA) operating in positive and negative APCI ionization mode.

2.3. Sample preparation

5 ml of a serum sample were fortified with 2 ng/ml of a mixture of internal standards d3 17β-estradiol, d3 testosterone, d4 α/β-zearalanol and d3 methyltestosterone. An amount of 2 ml acetate buffer 2 M (pH = 5.2) and 650 μl of methanol were added to the sample. The mixture was vortexed for 30 s, centrifuged for 5 min at 3000 rpm and the supernatant was transferred to a clean tube. The sample was purified by SPE as reported by our group for the analysis of steroids in urine [15]. The SPE process in brief was as follows: the sample was loaded on an Oasis cartridge which was next washed with 3 ml of methanol:water (5:95 2% ammonia, v/v), 3 ml of methanol:water (40:60 2% ammonia, v/v) and 3 ml of water. The analytes were eluted with 3 ml of acetone:methanol (80:20, v/v) directly onto and through an amino cartridge and the eluate was evaporated to dryness, re-dissolved in methanol and analyzed in LC–MS/MS.

3. Results and discussion

3.1. Method development

Our intention was to develop a LC–MS/MS method for the sensitive and specific determination of hormones in serum of animal origin. For this experiment, hormones that are most widely used illegally (such as steroids and resorcylic acid lactones) were selected as the target analyte group. According to results reported by EU Member States in the framework of older but also recent Annual National Plans, there are findings for androgens such as α/β-boldenone, α/β-nortestosterone, α/β-estradiol, trenbolone and for RALs (α-zearalanol/β-zearalanol) in bovine animals [16]. So, in comparison to previous works from our group the selection of analytes was adapted to meet the needs for the analysis of the specific specimen, hence some analytes were added in the target list because their determination in blood samples is of interest; α/β-trenbolone, methyltestosterone, α-estradiol and β-nortestosterone which were not analyzed with our previous method in urine [15] are now analyzed in serum; α-estradiol and α-trenbolne which were not analyzed with our previous method in meat [13] are now analyzed in serum.

In the present communication the developed method could determine 12 anabolic steroids with high sensitivity. This number is higher than the number of analytes typically reported in published works (up to 8 steroids detected in serum of livestock origin [4–7]). Utilization of APCI instead of ESI improved detection sensitivity in accordance to previous findings from our lab [13,15]. Most of the steroids were detected in positive ionization mode, except of estradiol and ethynylestradiol, which were more efficiently ionized in negative mode. The most appropriate product ions were selected and all mass spectrometer parameters were optimized to improve their intensities. The product ions for all precursor ions of the analytes were used to set up a multiple reaction monitoring (MRM) method to enhance detection specificity and sensitivity. In this study the addition of a third transition ion was tested. The measurement of the ion ratio of two product ions is important for the confirmation of the identity of illicit substances; hence regulatory legislation requires the detection of two product ions at the minimum. In real practice this may lead to unexpected false positive results in the analysis of unknown samples of high complexity. Therefore, there is recently a preference for using an increased number of 'product ions'. During MRM optimisation, 8 of

Table 1
Retention time, precursor and product ions and optimal collision energies for all analytes.

Compound	Rt (min)	Precursor ion $[M+H]^+$	Product ions (m/z)	C.E. (eV)
α/β-Zearalanol	14.04/11.91	323.20	123.154[a]	36
			149.106	31
			189.165	32
α/β-Trenbolone	13.52/12.91	271.17	253.112[a]	22
			165.009	54
			199.109	28
Methyltestosterone	15.82	303.20	109.111[a]	31
			97.088	33
α/β-Boldenone	14.97/13.31	287.20	121.115[a]	34
			135.253	20
α/β-Nortestosterone	15.34/13.89	275.20	109.128[a]	29
			145.165	28
			239.304	21

Compound	Rt (min)	Precursor ion $[M-H]^-$	Product ions (m/z)	C.E. (eV)
α/β-Estradiol	14.81/14.45	271.18	145.170[a]	39
			183.295	49
			239.362	37
Ethynylestradiol	14.38	295.19	145.308[a]	36
			159.115	39

[a] The most abundant ion (also used for analyte quantification).

Table 2
Precision and accuracy for the anabolic steroids calculated on the three experiments.

Compound	Spiked (ng/ml)	Repeatability					
		Exp.1		Exp.2		Exp.3	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
α-Zearalanol	0.4	98.8	13.9	92.8	10.7	87.6	13.0
	0.6	91.5	16.6	92.0	10.7	89.4	6.0
	0.8	94.5	9.0	90.1	4.2	102.0	8.3
β-Zearalanol	0.4	99.2	11.9	104.4	3.8	93.1	12.6
	0.6	98.4	8.4	94.0	4.3	110.0	6.3
	0.8	97.9	4.5	105.2	9.0	110.4	8.0
α-Trenbolone	0.2	102.3	8.0	102.1	3.2	102.7	3.3
	0.3	115.7	4.5	102.4	11.4	102.6	9.3
	0.4	103.8	3.1	108.5	5.4	109.4	6.1
β-Trenbolone	0.2	99.8	4.8	102.5	2.8	102.0	2.6
	0.3	103.9	3.3	105.2	9.4	104.9	7.5
	0.4	101.4	4.1	104.2	3.8	104.3	5.2
Methyltestosteronene	0.2	93.8	3.8	96.4	7.0	95.7	8.9
	0.3	100.7	2.7	98.8	2.7	99.8	1.9
	0.4	97.4	4.5	101.1	5.7	100.2	5.2
α-Estradiol	0.1	93.9	13.3	85.7	6.9	82.6	10.6
	0.15	96.6	10.4	98.6	7.3	98.2	6.5
	0.2	95.4	8.5	100.7	4.5	91.4	4.1
β-Estradiol	0.1	78.0	18.1	87.5	5.2	90.5	15.0
	0.15	84.9	16.2	88.0	9.3	94.2	12.3
	0.2	88.8	10.9	98.9	6.5	90.4	4.0
Ethynylestradiol	0.1	71.8	18.1	73.8	12.6	92.0	7.0
	0.15	78.2	18.8	79.7	15.0	85.2	12.5
	0.2	70.2	12.4	71.8	17.8	76.0	10.7
α-Boldenone	0.4	114.3	8.3	114.2	3.1	118.2	6.5
	0.6	115.6	8.3	117.3	5.9	105.9	7.6
	0.8	97.8	4.0	108.6	3.8	108.9	4.9
β-Boldenone	0.4	104.4	5.7	106.9	10.3	109.5	7.6
	0.6	98.9	5.3	98.3	2.7	99.0	3.5
	0.8	98.6	3.1	101.4	2.5	103.2	2.6
α-Nortestosterone	0.1	112.1	2.2	98.8	10.4	108.3	8.2
	0.15	101.8	6.8	108.5	4.5	106.2	1.9
	0.2	98.5	5.1	107.7	3.8	98.2	6.3
β-Nortestosterone	0.1	112.3	7.9	101.5	8.4	111.0	6.4
	0.15	102.5	5.4	107.4	7.2	101.2	3.8
	0.2	94.9	4.2	107.4	3.4	98.1	5.1

the 12 analyzed steroids provided a third product ion worth investigating as analytical trace. Hence these 8 analytes were monitored with three ions, thus enhancing method specificity and identification potential in real life unknown samples. The rest four analytes were monitored with two product ions in accordance to regulatory authority legislation. The precursor and product ions for each analyte as well as the corresponding optimal collision energies are shown in Table 1.

For the pre-treatment of serum different analytical and sample preparation procedures have been proposed in the literature. Draisci et al. [5] used a combination of LLE and SPE on C18 cartridges to achieve the determination of 5 compounds in bovine serum at 0.1 ng/ml as the limit of quantification. On-line coupling of SPE with LC-triple quadrupole linear ion trap mass spectrometry [10] was used for the determination of 13 anabolic steroids in human serum at detection limits 0.02–9 ng/ml. Recently a sample preparation process consisting of LLE, derivatization and SPE was employed for UPLC/MS/MS determination of testosterone and dihydrotestosterone in human urine [9]. Derivatization was employed in order to reduce the limit of quantitation at 0.01 ng/ml.

Steroids are lipophilic compounds that bind strongly to blood proteins. Removal of proteins from a sample is important, in order to avoid pressure build-up in the LC system. Protein crash by addition of organic solvents and centrifugation is a common strategy to remove proteins from blood-derived samples. However, protein precipitation may lead to analyte co-precipitation and loss: analytes bound strongly to hydrophobic pockets in the protein structure may plummet along the protein crash and thus be removed from the sample. In this case a way to overcome this is to try to disrupt analyte–protein interactions before the protein crash or to apply different protein removal methods. Alteration of the pH, or addition of small volume of organic solvents may achieve liberation of organic analytes from the proteins. In the present work we studied these approaches with the aim of increased detection sensitivity. We found that loading the sample directly on the SPE column, without a protein precipitation step, resulted to signal interferences and increase on the pressure of the LC system. We also found that the combination of buffer and methanol addition resulted in limited protein crash (probably of heavy hydrophobic protein chains) and satisfactory analyte recovery. Subsequently the sample was further purified in SPE. Different SPE cartridges were studied alone or in combination. The combination of the extraction mechanisms of Oasis HLB and Amino cartridges gave superior results [see also reference 15] compared to combinations of the above cartridges with Discovery DSC-18 cartridges or application of single cartridge SPE.

Our overall goal was to develop a generic method that would be applied with minor modifications to the analysis of anabolic steroids in different biological matrices (meat, urine, serum). This would need a basic platform to be selected and LC-APCI–MS/MS provided the best results in this aspect. Further to this, sample preparation was studied with the goal to reach a robust, reliable and amenable to automation methodology. The applied SPE protocol was applicable to all matrices, that could be easily transferred in the automated system used for solid phase extraction in our laboratory (ASPEC XL, Gilson, USA). As a result, a significant gain in

productivity was reached along with simplification, reduction of cost and the ability to analyse a large number of different types of samples.

3.2. Method validation

As with the previously developed methodologies for urine and meat [13,15] the method was validated according to the European Commission Decision 2002/657/EC [2] concerning linearity, accuracy, precision, decision limit (CCα), detection capability (CCβ), specificity and measurement of uncertainty (U%). A homogeneous sample was produced by a pooled blood serum from samples from different male bovine animals. In male animals the amount of the natural occurred testosterone is much higher than estradiol. In the pooled sample, no natural hormones were detected. The sample was fortified with each analyte at 1*Validation Level (VL), 1.5*VL and 2*VL. Six replicates at each of the three fortification levels were analyzed. Also, the pooled sample was fortified at 3*VL and 5*VL. The 20 sub-samples with a blank sample (n = 21) were analyzed in three separate experiments. For the anabolic steroids α/β-estradiol, ethynylestradiol, α/β-nortestosterone the VL was set at 0.1 ng/ml, for α/β-trenbolone, methyltestosterone at 0.2 ng/ml and for α/β-boldenone, α/β-zearalanol at 0.4 ng/ml.

The calibration curve of the standard solutions had a dynamic range from 0 to 2 ng/ml using 9 calibration points (0, 0.05, 0.1, 0.2, 0.5, 0.8, 1, 1.5 and 2 ng/ml) with the internal standards at concentration 2 ng/ml. The regression coefficients (r^2) for the analytes were greater than 0.992 for all calibration curves. The accuracy and precision of the method was determined from the serum samples fortified at 1*VL, 1.5*VL, 2*VL (n = 18) in the three separate experiments. The recovery ranged from 70.2% to 118.2%. The accuracy was evaluated and CV ranged from 1.9% to 18.8% as shown in Table 2. As a result, the method provides acceptable precision and satisfactory recoveries.

From the matrix calibration curves constructed for the fortified samples at the 3 experiments (6 points) the values of the decision limits (CCα) and detection capabilities (CCβ) for all analytes were calculated. The calculated CCα and CCβ values are shown in Table 3. CCβ values for all 12 compounds are below 0.12 ng/ml.

Table 3
CCα, CCβ values and measurement of uncertainty (U%) for the analytes.

Compound	CCα (ng/ml)	CCβ (ng/ml)	U%
α-Zearalanol	0.07	0.12	20.52
β-Zearalanol	0.06	0.11	20.50
α-Trenbolone	0.02	0.04	14.86
β-Trenbolone	0.02	0.03	10.19
Methyltestosterone	0.02	0.03	8.60
α-Estradiol	0.01	0.02	20.80
β-Estradiol	0.02	0.03	21.38
Ethynylestradiol	0.03	0.05	28.14
α-Boldenone	0.06	0.10	16.41
β-Boldenone	0.03	0.06	9.00
α-Nortestosterone	0.01	0.02	14.22
β-Nortestosterone	0.01	0.02	20.27

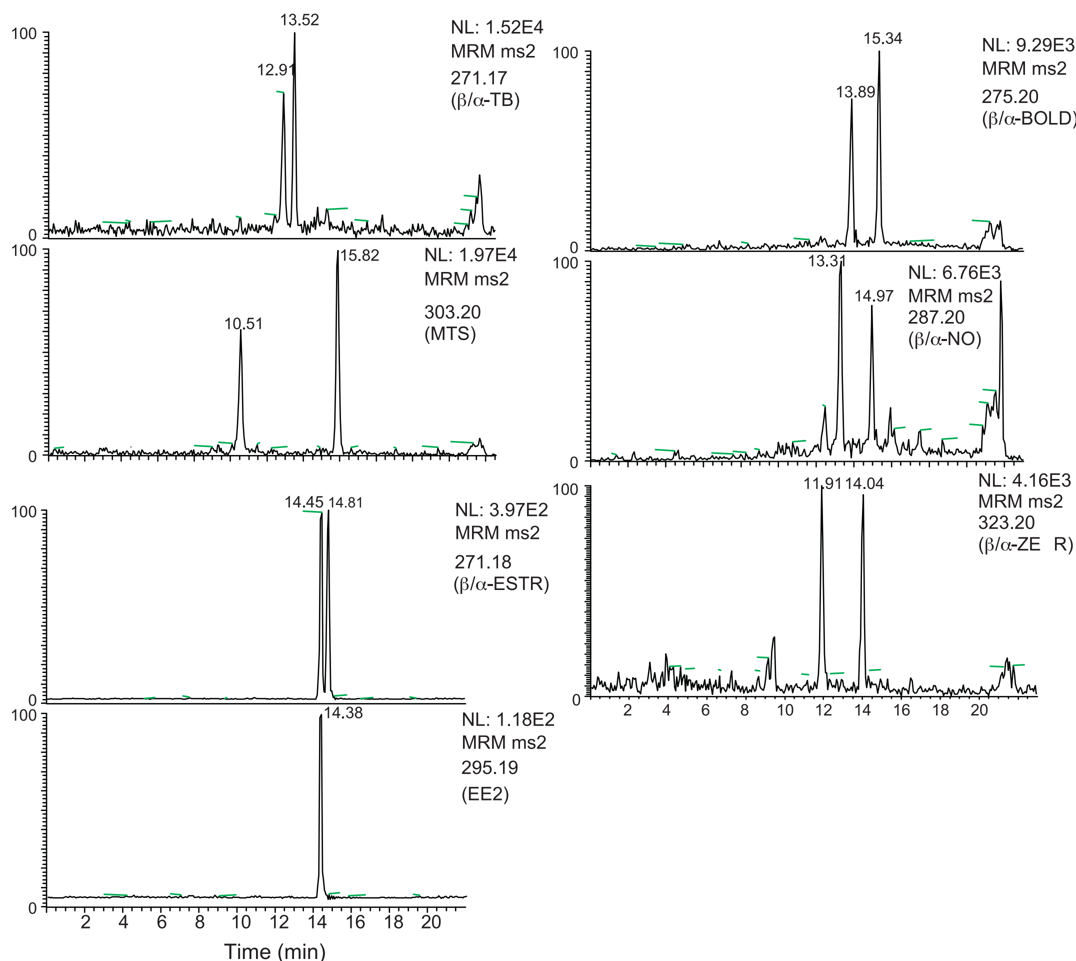

Fig. 1. MRM chromatogram of a spiked serum sample containing β/α-trenbolone (β/α-TB); methyltestosterone (MTS) at a concentration of 0.2 ng/ml, β/α-estradiol (β/α-ESTR); ethynylestradiol (EE2) at a concentration of 0.1 ng/ml; β/α-boldenone (β/α-BOLD) at a concentration of 0.4 ng/ml; β/α-nortestosterone (β/α-NO) at a concentration of 0.1 ng/ml and β/α-zearalanol (β/α-/ZEαR) at a concentration of 0.4 ng/ml.

For the evaluation of methods specificity 20 blank serum samples from different animals were analyzed at a separate experiment. No interfering peaks were observed at the retention time for any of the product ions of the analytes. Additionally, the same blank serum samples (20) were spiked at 1*VL, and the samples were assayed. No false negative results were observed and no significant matrix interferences were observed. The measurement of uncertainty was estimated taking in account the variances of the reproducibility and the matrix effects. Table 3 shows the measurement uncertainties (*U*%) for all compounds. Finally, all fortified samples were confirmed according to the 2.5% relative retention time tolerance and the 20% tolerance of the ion ratios of the two product ions.

Fig. 1 shows MRM mass chromatograms of a spiked sample.

3.3. Application to real samples

The method was applied for the analysis of 240 serum samples collected from veterinary directories, in compliance with the procedures provided by the national program for residue control in Greece. No traces of the 12 steroids were detected in any of the analyzed samples. Our laboratory also participated in the proficiency test "estradiol in lyophilized plasma" (2007) organized by the Community Reference Laboratory for hormones (RIVM, Bilthoven, The Netherlands). This study focused on the quantification and confirmation of residues 17β-estradiol in bovine plasma. Although the method was developed and validated in blood serum we used it nonetheless without any modification for the analysis of blood plasma during this proficiency test. Serum and plasma differ in the fact that plasma contains fibrin and other soluble clotting elements, which are removed from serum during blood clotting. The rest of the blood content (other macromolecules and smaller metabolites) are found in both serum and plasma, hence it is common practice in life sciences and more specifically in analytical separations to treat both specimens in the same fashion. Results from the proficiency test in plasma were satisfactory: *z*-scores at low (0.1 ng/ml/*z*-score = 0.67, CV = 3%) and high concentrations (1.0 ng/ml/*z*-score = −0.18, CV = 11%), indicating that the developed method can be equally applied to plasma and serum.

The current analytical methodology provided highly sensitive analysis of the steroids in serum with very low limits with the CCβ ranging from 0.02 to 0.12 ng/ml. Compared to our previous results in meat (CCβ = 0.05–0.24) and urine (CCβ = 0.11–0.49) but also to the reports published in the literature for the analysis of serum the current method provided higher sensitivity. The analysis of serum offers an interesting alternative for monitoring anabolic steroids in trace levels in living animals also due to the fact that the analytical procedure is simpler compared to the analysis of meat or urine. A further concern however remains in the metabolic fate and renal plasma clearance rate of steroids. This should be taken into account, because blood derived specimens (such as serum or plasma) provide a narrower time frame compared to urine or fat/meat, for the detection of steroids but also other pharmaceuticals or xenobiotics.

4. Conclusions

A method employing LC-APCI–MS–MS and simple sample preparation for the determination of 12 anabolic steroids in bovine serum was developed and validated over the range from 0 to 2 ng/ml. The method is sensitive, accurate and selective, and thus suitable for throughput quantitative analysis. The developed method complies with the criteria of Decision 2002/657/EC, in which analysis for the anabolic steroids requires identification of the analyte based on presence of at least one precursor and two product ions. Hence the method is valuable for routine control of the illegal use of anabolic steroids and their metabolites in livestock production.

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