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Quantitation of anabolic hormones and their metabolites in bovine serum and urine by liquid chromatography–tandem mass spectrometry

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Abstract

A specific and sensitive method based on tandem mass spectrometry with on-line high-performance liquid chromatography using atmospheric pressure chemical ionisation (LC–APCI-MS–MS) for the quantitation of anabolic hormone residues (17β-19-nortestosterone, 17β-testosterone and progesterone) and their major metabolites (17α-19-nortestosterone and 17α-testosterone) in bovine serum and urine is reported. [$^2H_2$]17β-Testosterone was used as internal standard. The analytes were extracted from urine (following enzymatic hydrolysis) and serum samples by liquid–liquid extraction and purified by C_{18} solid-phase extraction. Ionisation was performed in a heated nebulizer interface operating in the positive ion mode, where only the protonated molecule, $[M+H]^+$, was generated for each analyte. This served as precursor ion for collision-induced dissociation and two diagnostic product ions for each analyte were identified for the unambiguous hormone confirmation by selected reaction monitoring LC–MS–MS. The overall inter-day precision (relative standard deviation) ranged from 6.37 to 2.10% and from 6.25 to 2.01%, for the bovine serum and urine samples, respectively, while the inter-day accuracy (relative error) ranged from −5.90 to −3.18% and from −6.40 to −2.97%, for the bovine serum and urine samples, respectively. The limit of quantitation of the method was 0.1 ng/ml for all the hormones in bovine serum and urine. On account of its high sensitivity and specificity the method has been successfully used to confirm illegal hormone administration for regulatory purposes.

Keywords: Food analysis; Hormones

1. Introduction

The use of natural and synthetic hormones for growth promotion purposes in meat producing animals is prohibited in the European Union (EU) [1] to protect consumers from possible harmful effects due to the intake of hormone residues and their metabolites.

Very recently, a risk to the consumer has been identified for a number of hormones, such as 17β-testosterone and progesterone, as endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects could be envisaged, particularly in prepubertal children [2]. In view of the intrinsic properties of the hormones and in consideration of the epidemiological findings, no threshold level and therefore no acceptable daily intake (ADI) can be established [2].

On account of possible illegal uses of endogenous steroids, such as 17β-testosterone and progesterone, the Italian legislation [3] has fixed ''action limits'' in order to discriminate between the physiological

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concentrations in bovine plasma or serum and the hormone levels depending on illegal administrations. In order to check the compliance with regulations, an enforcement system has been set up in each of the EU member States. The control may be either direct, such as identification or quantitation of residues of the parent compound or the metabolites in the muscle tissue or edible organs, or indirect, by their detection in injection sites, blood, urine and faeces. 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.

Various methods for the detection of anabolic agents in biological fluids have been proposed.

Radioimmunoassay (RIA) has been widely used for routine screening [4–6] since this technique is rapid and sensitive and permits inexpensive screening of a large number of samples; however, its reduced specificity depending on cross-reactivity and drawbacks due to the use of a radioactive agent led to the proposal of enzyme immunoassay (EIA) as a profitable alternative method [7,8]. On the other hand gas chromatography (GC) coupled to mass spectrometry (MS) and tandem mass spectrometry (MS–MS) has been used as confirmatory method because the information on the molecular structure of the analyte, obtained by electron impact (EI) ionisation [9–16] or negative chemical ionisation (NCI) [17,18], result in higher specificity.

The combination of liquid chromatography (LC) with MS and 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.

The development of *soft* ionisation techniques employed in LC–MS and LC–MS–MS methods is of great interest to research groups for bovine hormone residue determination purposes [19–23].

In a previous paper [21] we reported the use of micro-LC–MS–MS with an atmospheric pressure ionisation (API) source and an ion spray (IS) interface for the specific direct detection of natural (17β-testosterone and progesterone) and synthetic (17β-19-nortestosterone, methyltestosterone and medroxyprogesterone) hormone residues in bovine blood. Recently, we have shown the feasibility of using LC–MS–MS with an atmospheric pressure chemical ionisation (APCI) source and a heated nebulizer (HN) interface, compatible with flow-rates of conventional high-performance liquid chromatography (HPLC), for the quantitation of 17β-estradiol in bovine serum [23].

In this study we investigated the possibility of using LC–MS–MS via HN interface for the detection and quantitation of 17β-19-nortestosterone (17β-NT), 17β-testosterone (17β-T), progesterone (P) and their major metabolites 17α-19-nortestosterone (17α-NT), 17α-testosterone (17α-T) in complex biological matrices, such as bovine serum and urine, using [$^2H_2$]17β-testosterone (17β-T-d_2) as deuterated internal standard (I.S.).

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, methanol, ammonium acetate, sodium acetate and hydrochloric acid were HPLC-grade and purchased from Carlo Erba (Milan, Italy). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). β-Glucuronidase/arylsulphatase (*Helix pomatia*) from Boehringer Mannheim (Germany) were used as supplied. 17β-NT, 17α-T, 17β-T and P were provided by Sigma (St. Louis, MO, USA); 17α-NT was purchased from Steraloids (Wilton, NH, USA); deuterated internal standard 17β-T-d_2 (>95% isotopic purity) was obtained from RIVM (Bilthoven, The Netherlands). Individual hormone standard stock solutions of 0.2 mg/ml were prepared in methanol. Individual and composite working standard solutions were prepared daily by appropriate dilution of the standard stock solutions with methanol. All solutions were stored at 4°C and were stable for at least 1 month.

2.2. Samples

Blank control samples of bovine serum and urine were collected, in compliance with the procedures provided by the national program for residue control in Italy, from cattle purposely born and fattened for this study. Two independent pools of serum and urine, obtained from various bovine specimens, were assayed by selected reaction monitoring (SRM) LC–

MS–MS and the absence of the analyte under investigation was verified.

Eighteen serum and urine samples were collected from animals of both sexes at different farms and slaughterhouses as part of the national program for residue control in Italy. All samples were stored at −20°C until assayed.

2.3. Sample preparation procedure

2.3.1. Serum samples

An aliquot (2.0 ml) of serum was fortified with 4 ng of I.S. and 15.0 ml of acetate buffer solutions (ABS) 0.15 *M*, pH 5, and the mixture was sonicated with an ultrasonic bath for 5 min. The sample was purified by solid-phase extraction (SPE) using a C_{18} cartridge (Baker C_{18}, 500 mg, 3 ml cartridges) which had previously been conditioned with 2.5 ml of methanol and 5.0 ml of water. After sample loading, the cartridge was washed with 5.0 ml of ABS, 10.0 ml of water and 2.5 ml of methanol–water (40:60, v/v). Finally analytes were eluted with 4.0 ml of methanol, the solvent removed under nitrogen stream and the residue dissolved in 100 μl of methanol. A 5-μl volume of the solution was injected into the LC–MS–MS system.

2.3.2. Urine samples

An aliquot (2.0 ml) of urine was fortified with 10 ng of I.S. and added with 20 μl of the crude enzyme solution (*Helix pomatia*) and the mixture was incubated for 12 h at 37°C. The hydrolysate was cooled at room temperature and 15.0 ml of ABS 0.15 *M*, pH 5, was added. Analytes were then extracted as above described for the serum samples.

2.4. Liquid chromatography–mass spectrometry

Analyses were carried out with a Phoenix 20 CU LC pump (Fisons, Milan, Italy) liquid chromatograph. A Valco (Valco, Houston, TX, USA) injection valve equipped with a 5-μl internal loop was used for injection by flow injection analysis (FIA)–MS, FIA–MS–MS and LC–MS–MS. Chromatographic separations were obtained under isocratic conditions using a reversed-phase Kingsorb C_{18} column (Phenomenex, Torrance, CA, USA) (250×2 mm I.D., 5 μm) at room temperature, with a mobile phase of acetonitrile–water (70:30, v/v) containing 2 m*M* ammonium acetate, and at a flow-rate of 150 μl/min.

Mass spectral analyses were performed on a PE-Sciex API III plus triple-quadrupole (PE-Sciex, Thornill, Canada) equipped with an APCI source and a HN operating in the positive ion mode. Ultra-high-purity nitrogen was used as curtain gas (0.6 l/min), while air was used as nebulizer gas (400 kPa) and auxiliary gas (1.5 l/min). The HN temperature was set at 350°C and the discharge current at 4 μA. The orifice potential voltage (OR) was set at 90 V for all the analytes. Full-scan mass spectra were acquired in the positive ion mode from *m/z* 200–400. In the MS–MS experiments, product ion mass spectra were acquired in positive ion mode by colliding quadrupole 1 (Q1) selected precursor ion, with argon (gas thickness $300 \cdot 10^{13}$ molecules/cm^2) in quadrupole 2 (Q2) operated in radio frequency (RF)-only mode, and scanning the third quadrupole mass spectrometer, Q3, from *m/z* 50–350. The MS and MS–MS experiments were run with a resolution of 0.8 u measured at half peak height for both the mass-resolving quadrupoles. A collision energy of 25 eV was chosen for the collision-induced dissociation (CID) experiments. The protonated molecule, $[M+H]^+$, at *m/z* 275 for 17α-NT and 17β-NT, *m/z* 289 for 17α-T and 17β-T, *m/z* 315 for P and *m/z* 291 for 17β-T-d_2, was the precursor ion for CID and two product ions for each anabolic hormone were identified for SRM LC–MS–MS analyses. The double precursor–product ion combinations of *m/z* 275→109 and *m/z* 275→83 were used for both 17α-NT and 17β-NT, *m/z* 289→109 and *m/z* 289→97 for both 17α-T and 17β-T, *m/z* 315→109 and *m/z* 315→97 for P, *m/z* 291→111 and *m/z* 291→99 for 17β-T-d_2. The dwell time for each monitored transition was 150 ms. Peak-area ratios of the analyte to internal standard were computed using MacQuan version 1.3 software from PE-Sciex.

2.5. Calibration and quantitation

In order to evaluate the extraction efficiency of the analytes, standard curves were prepared daily in the range 2–600 ng/ml by plotting peak area ratios of the analyte to I.S. versus hormone concentrations using a least-squares linear regression model.

Calibration curves were prepared daily by spiking

serum and urine control samples with mixtures of the anabolic compounds to obtain concentration in the range 0.1–30 ng/ml. Estimates of the amount of the analyte in fortified and real samples were interpolated from these calibration graphs, constructed in the same way as standard curves.

Three replicates of serum and urine blank control samples, fortified with mixtures of the hormones to obtain concentrations of 0.1, 2.0, 10.0 and 30.0 ng/ml, were prepared and analysed on each of three days for each concentration to evaluate extraction efficiency, accuracy and precision of the method.

3. Results and discussion

Positive-ion FIA–MS was initially performed by adopting a mixture of acetonitrile–water (70:30, v/v) containing 2 m*M* ammonium acetate and at a flow-rate of 150 μl/min. Representative HN full-scan (mass range *m/z* 200–400) mass spectra as obtained by FIA in the MS positive ion mode for the analytes under investigation are shown in Fig. 1. The formation of the protonated molecule, $[M+H]^+$, of 17α-NT and 17β-NT (*m/z* 275), 17α-T and 17β-T (*m/z* 289), P (*m/z* 315) and 17β-T-d_2 (*m/z* 291), was observed with negligible fragmentation.

Following experiments were performed to obtain spectra with maximum intensities of the protonated molecular ion of each analyte by selected ion monitoring (SIM) FIA–MS analyses at *m/z* 275, 289, 291 and 315. The effect of varying orifice potential voltage was investigated between the range 60–100 V. An OR of 90 V was adopted for all the hormones as the best compromise in terms of signal-to-noise ratio. Although the simplicity of the FIA–MS spectra is useful for the identification of the analytes based on their molecular-related ions, they do not provide further structural information. Tandem mass spectrometry was therefore used in order to obtain additional structural information by detecting diagnostic product ions obtained by CID of the precursor ion.

The protonated molecule, $[M+H]^+$, served as the precursor ion for CID in the MS–MS experiments, carried out by FIA–MS–MS on the individual hormone standard solutions. Fig. 2 shows the positive product ion mass spectra (mass range *m/z* 50–350) of the protonated molecules, $[M+H]^+$, of 17α-NT, 17β-NT, 17α-T, 17β-T, P and 17β-T-d_2. Comparison of the spectra reveals the production of the most abundant product ions at *m/z* 109 and 83 for 17α-NT and 17β-NT, at *m/z* 109 and 97 for 17α-T, 17β-T and P, at *m/z* 111 and 99 for 17β-T-d_2. Transitions of the respective protonated molecules to these product ions were therefore selected according to the SRM technique.

In order to achieve targeted analyses and maximum sensitivity as well as for quantitative purposes SRM LC–MS–MS analyses were finally performed using a reversed-phase Kingsorb C_{18} column at room temperature, with a mobile phase of acetonitrile–water (70:30, v/v) containing 2 m*M* ammonium acetate, at a flow-rate of 150 μl/min.

Under the adopted conditions, the separation of 17β-NT (5.6 min), 17β-T and 17β-T-d_2 (6.1 min), 17α-NT (6.4 min), 17α-T (7.2 min), and P (11.4 min) observed in SRM LC–MS–MS profiles of a standard mixture was not excellent (data not shown). However, the specificity of MS–MS reduces the need for complete chromatographic resolution of individual compounds and the focus in developing the confirmatory LC–MS–MS method was rather on providing simple and fast treatment of samples and reducing the analytical run time by APCI-compatible mobile phases. Specificity of the SRM LC–MS–MS method was also proved by processing and analysing serum and urine blank (Figs. 3A and 4A) control samples. No interference was noticed around the hormones retention times either in serum or urine samples.

Calibration graphs were constructed by plotting peak-area ratios versus hormone concentrations using a least-squares linear regression model; the linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) greater than 0.995 for all curves.

Fortified bovine serum and urine samples were prepared at four different concentrations (0.1, 2.0, 10.0 and 30.0 ng/ml) and analysed to determine the extraction efficiency of the hormones and to evaluate the intra- and inter-day precision and accuracy of the analytical method. Representative chromatograms of serum and urine spiked control samples are reported in Figs. 3B and 4B, respectively. The relative retention time, for each analyte, corresponded to that

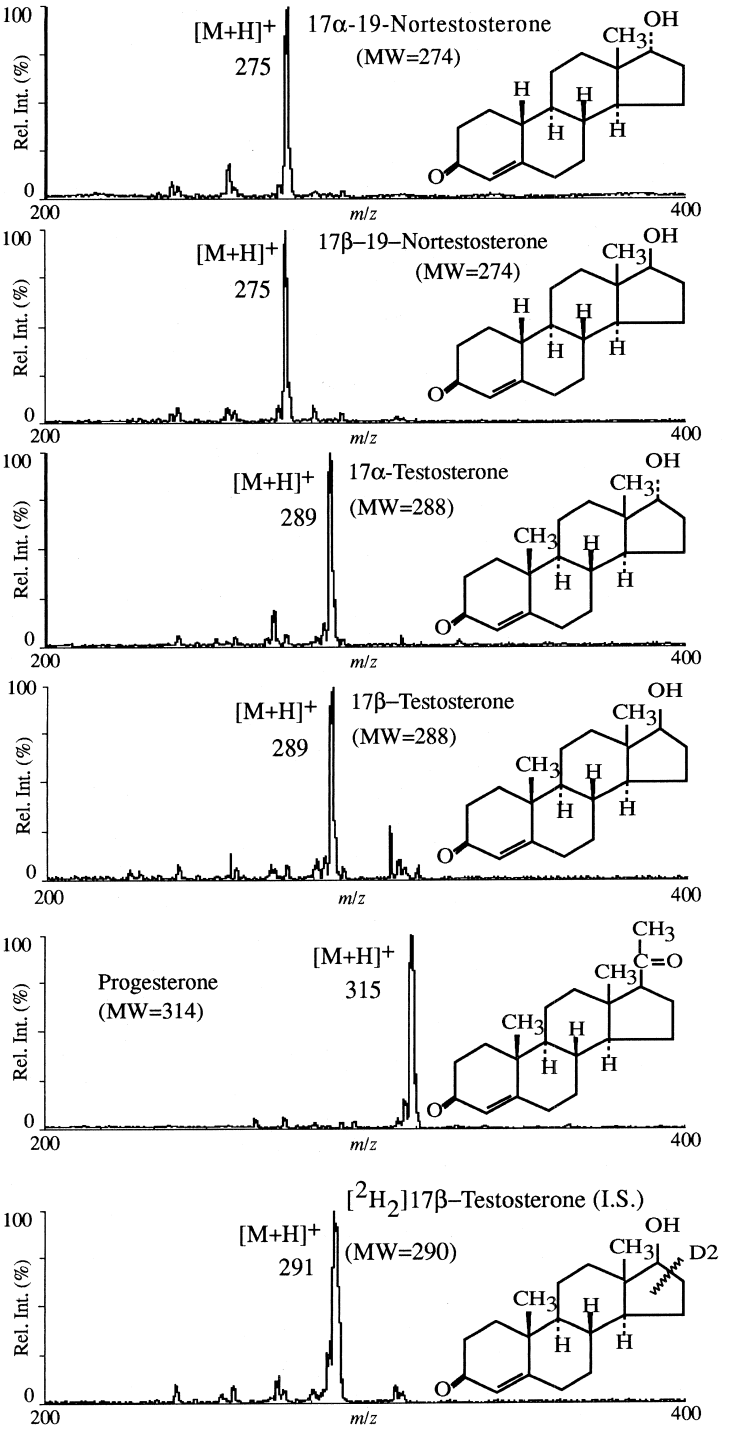

Fig. 1. Positive ion mass spectra of 17α-19-nortestosterone, 17β-19-nortestosterone, 17α-testosterone, 17β-testosterone, progesterone and [$^2H_2$]17β-testosterone. Conditions: FIA; mobile phase: acetonitrile–water (70:30, v/v) containing 2 m*M* ammonium acetate; flow-rate 150 μl/min; OR was set at 90 V.

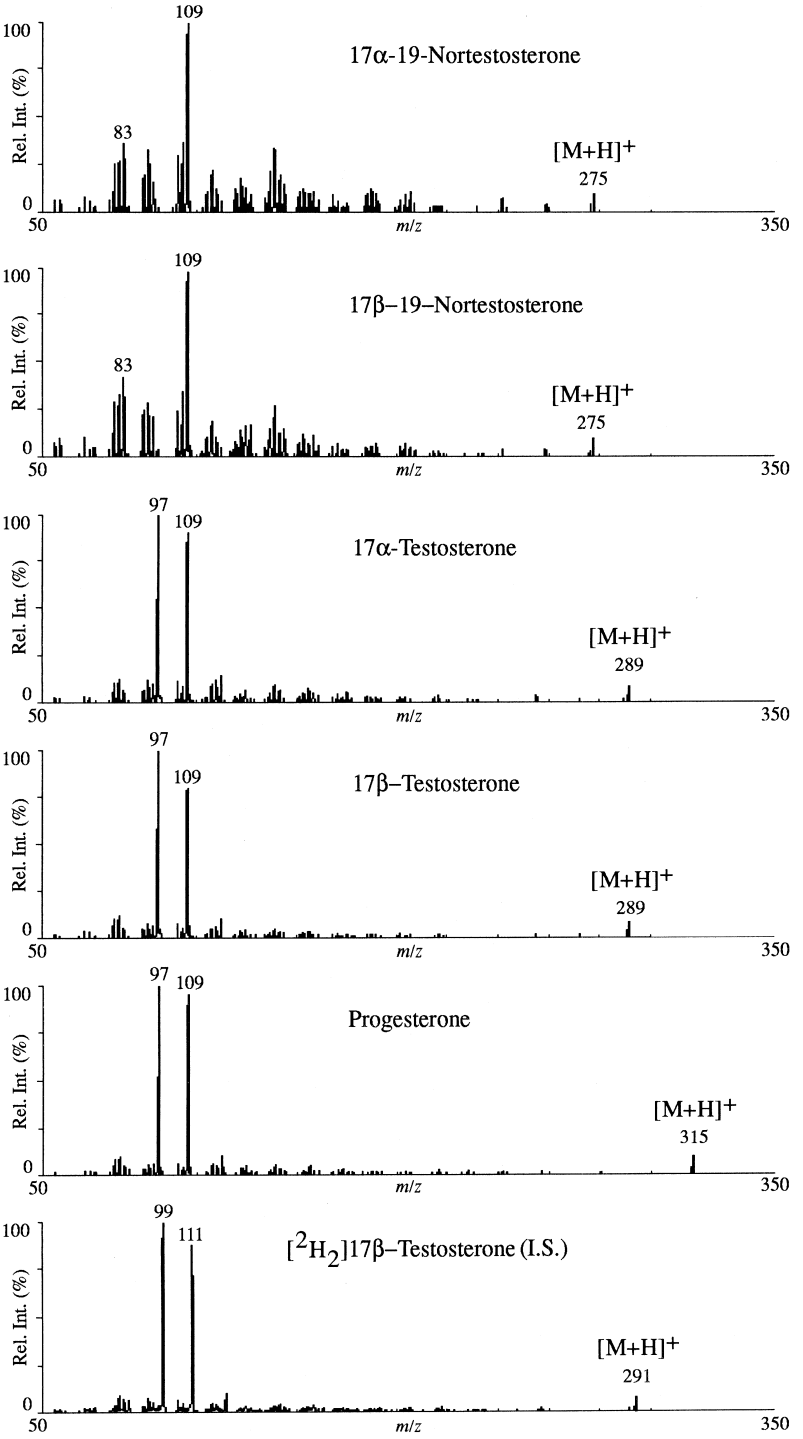

Fig. 2. Positive product ion mass spectra of 17α-19-nortestosterone, 17β-19-nortestosterone, 17α-testosterone, 17β-testosterone, progesterone and [$^2H_2$]17β-testosterone, with the $[M+H]^+$ ion as precursor, at m/z 275 for 17α-NT and 17β-NT, at m/z 289 for 17α-T and 17β-T, at m/z 315 for P, and at m/z 291 for 17β-T-d_2. Conditions: FIA; mobile phase: acetonitrile–water (70:30, v/v) containing 2 m*M* ammonium acetate; flow-rate 150 μl/min; OR was set at 90 V. Argon was used as the collision gas. CID was carried out with a collision energy of 25 eV.

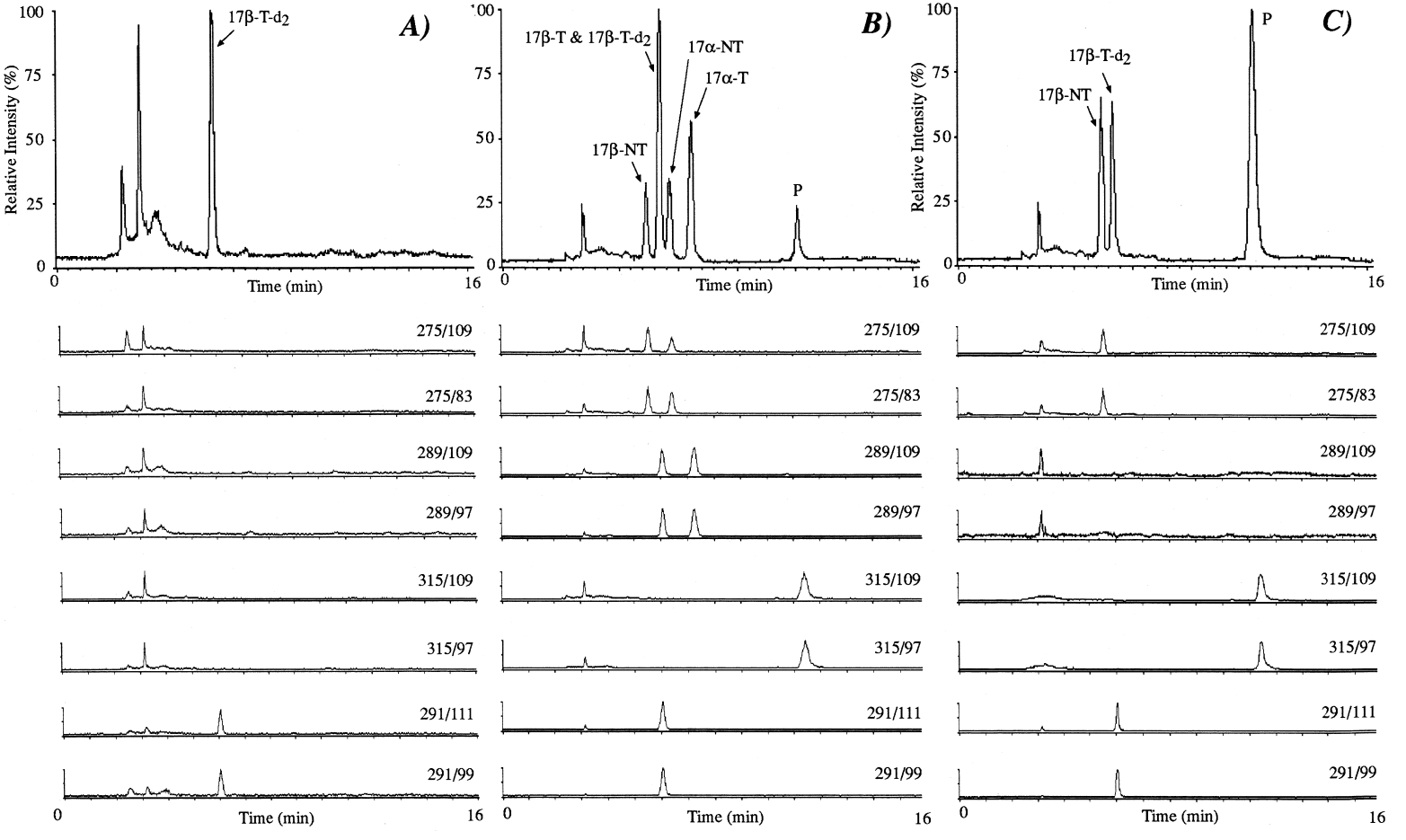

Fig. 3. SRM LC–MS–MS chromatograms of (A) extract of blank control bovine serum spiked with 2 ng/ml of 17β-T-d_2 as I.S.; (B) extract of blank control bovine serum spiked with 2 ng/ml of 17β-NT, 17β-T, 17β-T-d_2, 17α-NT, 17α-T and P; (C) extract of a positive bovine serum sample (female>6 months old) containing 17β-NT (4.2 ng/ml) and P (6.7 ng/ml). Precursor–product ion combinations used in SRM detection are shown. Conditions: isocratic HPLC analysis; column reversed-phase Kingsorb C_{18} column (250×2 mm, 5 μm); mobile phase: acetonitrile–water (70:30, v/v) containing 2 m*M* ammonium acetate; flow-rate 150 μl/min; OR was set at 90 V. Argon was used as the collision gas. CID was carried out with a collision energy of 25 eV.

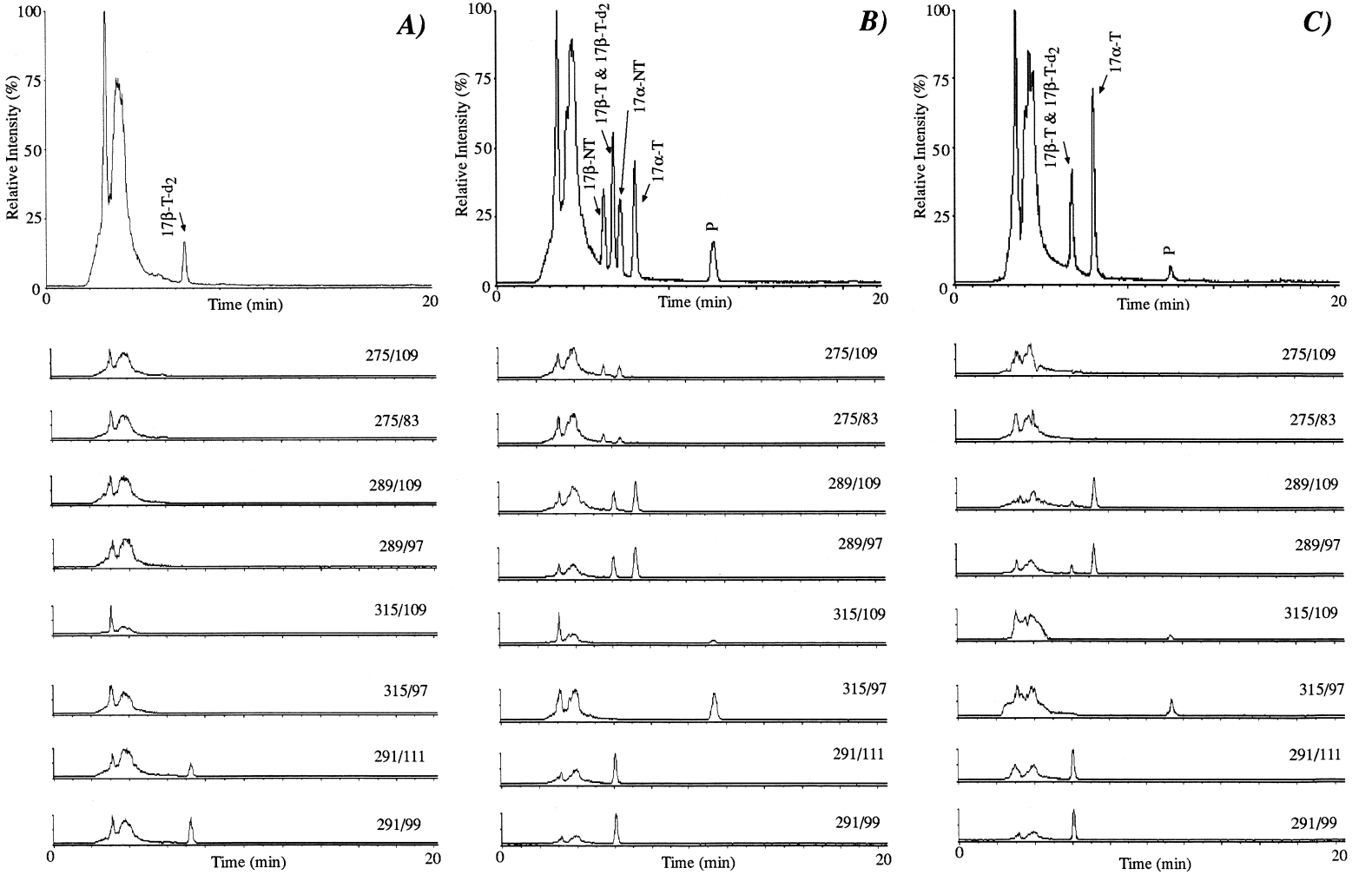

Fig. 4. SRM LC–MS–MS chromatograms of (A) extract of blank control bovine urine spiked with 5 ng/ml of 17β-T-d_2 as I.S.; (B) extract of blank control bovine urine spiked with 5 ng/ml of 17β-NT, 17β-T, 17β-T-d_2, 17α-NT, 17α-T and P; (C) extract of a bovine urine sample (male>6 months old) containing 17β-T (1.6 ng/ml), 17α-T (10.2 ng/ml) and P (2.1 ng/ml). Precursor–product ion combinations used in SRM detection are shown. Conditions: isocratic HPLC analysis; column reversed-phase Kingsorb C_{18} column (250×2 mm, 5 μm); mobile phase: acetonitrile–water (70:30, v/v) containing 2 m*M* ammonium acetate; flow-rate 150 μl/min; OR was set at 90 V. Argon was used as the collision gas. CID was carried out with a collision energy of 25 eV.

of the calibration standard within a tolerance of ±0.4%.

The extraction efficiency of the analytes and the I.S. were determined by comparing the peak areas of fortified samples with those of the corresponding standards and of I.S. Three replicates at each concentration were analysed on each day for 3 days ($n=9$) and the following mean percentages of extraction efficiencies were estimated: 81.5±6.9% and 83.9±5.8% for 17α-NT, 83.1±5.5% and 85.6±5.2% for 17β-NT, 81.8±6.9% and 84.1±5.8% for 17α-T, 82.8±6.1% and 85.3±5.8% for 17β-T, 72.1±7.8% and 75.4±6.2% for P, 82.4±5.5% and 87.9±4.7% for 17β-T-d_2, for bovine serum and urine samples, respectively.

The inter-day precision and accuracy of the method were obtained by analysing three replicates at 0.1, 2.0, 10.0 and 30.0 ng/ml on each of 3 days for bovine serum and urine. Precision was determined by calculating the relative standard deviation (RSD, %) for the repeated measurements and the accuracy (relative error, RE, %) was calculated by the agreement between the measured and the nominal concentrations of the fortified samples. For inter-day

Table 1
Inter-day precision and accuracy for hormones in bovine serum samples

Hormone	Parameter	Validation sample level (ng/ml)			
		0.1	2.0	10.0	30.0
17α-NT	Average (ng/ml)	0.09	1.88	9.61	28.93
	SD (ng/ml)	0.01	0.10	0.21	0.68
	Precision (RSD, %)	6.37	5.31	2.20	2.35
	Accuracy (RE, %)	−5.80	−5.90	−3.87	−3.56
	n	9	9	9	9
17β-NT	Average (ng/ml)	0.09	1.90	9.55	28.89
	SD (ng/ml)	0.01	0.09	0.20	0.66
	Precision (RSD, %)	6.24	4.83	2.12	2.28
	Accuracy (RE, %)	−5.50	−4.80	−4.46	−3.69
	n	9	9	9	9
17α-T	Average (ng/ml)	0.09	1.92	9.62	29.04
	SD (ng/ml)	0.01	0.08	0.22	0.64
	Precision (RSD, %)	6.26	4.37	2.30	2.21
	Accuracy (RE, %)	−5.80	−3.95	−3.77	−3.19
	n	9	9	9	9
17β-T	Average (ng/ml)	0.09	1.89	9.65	28.99
	SD (ng/ml)	0.01	0.08	0.20	0.63
	Precision (RSD, %)	6.12	4.38	2.10	2.18
	Accuracy (RE, %)	−5.20	−5.30	−3.46	−3.36
	n	9	9	9	9
P	Average (ng/ml)	0.09	1.90	9.61	28.95
	SD (ng/ml)	0.01	0.10	0.22	0.66
	Precision (RSD, %)	5.84	5.36	2.31	2.28
	Accuracy (RE, %)	−5.90	−4.85	−3.88	−3.50
	n	9	9	9	9
17β-T-d_2	Average (ng/ml)	0.09	1.91	9.68	29.02
	SD (ng/ml)	0.01	0.09	0.20	0.69
	Precision (RSD, %)	6.35	4.86	2.11	2.38
	Accuracy (RE, %)	−5.50	−4.30	−3.18	−3.25
	n	9	9	9	9

assays, the overall precision ranged from 6.37 to 2.10% and from 6.25 to 2.01%, for the serum and urine samples, respectively, while the accuracy ranged from −5.90 to −3.18% and from −6.40 to −2.97%, for the serum and urine samples, respectively (Tables 1 and 2). These values were considered satisfactory, on account of the complexity of the biological matrices.

The limit of quantitation (LOQ), defined as the lowest concentration on the calibration graph at which an acceptable accuracy and precision is obtained, was 0.1 ng/ml for all the analytes under investigation in both the biological fluids, which makes the procedure described suitable for control purposes.

The SRM LC–MS–MS method was then used to analyse real samples collected from animals of both sexes, as part of the national program for hormone control in Italy (Table 3). 17β-NT was found in 3/9 of the serum samples, 17β-T was detected in 6/9 serum samples and P was present in 4/9 serum samples. For the urine samples 17β-NT and 17α-NT were detected in 2/9 samples, 17β-T and 17α-T were detected in 7/9 samples and P in 3/9 samples.

Table 2
Inter-day precision and accuracy for hormones in bovine urine samples

Hormone	Parameter	Validation sample level (ng/ml)			
		0.1	2.0	10.0	30.0
17α-NT	Average (ng/ml)	0.09	1.90	9.66	29.09
	SD (ng/ml)	0.01	0.10	0.20	0.62
	Precision (RSD, %)	6.16	5.37	2.09	2.15
	Accuracy (RE, %)	−5.80	−4.95	−3.37	−3.02
	n	9	9	9	9
17β-NT	Average (ng/ml)	0.09	1.87	9.65	29.08
	SD (ng/ml)	0.01	0.10	0.19	0.59
	Precision (RSD, %)	5.91	5.40	2.01	2.04
	Accuracy (RE, %)	−5.30	−6.40	−3.48	−3.07
	n	9	9	9	9
17α-T	Average (ng/ml)	0.09	1.91	9.62	29.06
	SD (ng/ml)	0.01	0.09	0.20	0.60
	Precision (RSD, %)	6.25	4.81	2.10	2.07
	Accuracy (RE, %)	−5.60	−4.45	−3.80	−3.12
	n	9	9	9	9
17β-T	Average (ng/ml)	0.09	1.89	9.68	29.10
	SD (ng/ml)	0.01	0.08	0.20	0.64
	Precision (RSD, %)	6.01	4.44	2.11	2.19
	Accuracy (RE, %)	−5.10	−5.35	−3.19	−2.99
	n	9	9	9	9
P	Average (ng/ml)	0.09	1.89	9.64	29.09
	SD (ng/ml)	0.01	0.10	0.21	0.66
	Precision (RSD, %)	5.81	5.45	2.21	2.26
	Accuracy (RE, %)	−5.40	−5.45	−3.60	−3.03
	n	9	9	9	9
17β-T-d_2	Average (ng/ml)	0.09	1.90	9.70	29.07
	SD (ng/ml)	0.01	0.08	0.20	0.59
	Precision (RSD, %)	5.96	4.47	2.08	2.04
	Accuracy (RE, %)	−6.10	−4.85	−2.97	−3.10
	n	9	9	9	9

Table 3
Hormone concentrations in real samples of bovine serum and urine provided by the national program for hormone control in Italy, assayed by LC–MS–MS

Samples	17β-NT (ng/ml)		17α-NT (ng/ml)	17β-T (ng/ml)		17α-T (ng/ml)	P (ng/ml)	
	Determined values	Maximum physiological limit[a]	Determined values	Determined values	Maximum physiological limit[a]	Determined values	Determined values	Maximum physiological limit[a]
Serum								
M>6 m	1.9	–		n.d.	30.0		n.d.	1.5
M>6 m	n.d.	–		2.4	30.0		1.7	1.5
M>6 m	n.d.	–		1.4	30.0		2.7	1.5
M>6 m	n.d.	–		2.2	30.0		3.4	1.5
M>6 m	2.3	–		n.d.	30.0		n.d.	1.5
F>6 m	4.2	–		n.d.	0.5		6.7	14.0
F>6 m	n.d.	–		2.3	0.5		n.d.	14.0
F<6 m	n.d.	–		0.6	0.5		n.d.	1.0
F<6 m	n.d.	–		2.6	0.5		n.d.	1.0
Urine								
M>6 m	n.d.		n.d.	1.6		10.2	2.1	
M>6 m	0.5		4.9	n.d.		n.d.	n.d.	
M>6 m	0.8		8.4	n.d.		n.d.	n.d.	
M<6 m	n.d.		n.d.	0.7		6.2	0.3	
M<6 m	n.d.		n.d.	4.0		24.1	1.4	
M<6 m	n.d.		n.d.	0.9		8.1	n.d.	
F>6 m	n.d.		n.d.	0.5		4.8	n.d.	
F<6 m	n.d.		n.d.	0.4		4.2	n.d.	
F<6 m	n.d.		n.d.	0.6		5.9	n.d.	

[a] From reference; M: male; m: months; F: female; n.d.: not detected.

Representative SRM LC–MS–MS chromatographic profiles of a bovine serum sample (female> 6 months old) (Fig. 3C) show presence of 17β-NT (4.2 ng/ml) and P (6.7 ng/ml). The level of 17β-NT, as determined by the SRM LC–MS–MS, confirmed an illegal administration of this synthetic anabolic compound, whereas the concentration of progesterone was considered in compliance with the maximum physiological limits [3]. Representative SRM LC–MS–MS chromatograms of a bovine urine sample (male>6 months old) containing P (2.1 ng/ml), 17β-T (1.6 ng/ml) and 17α-T (10.2 ng/ml) are shown in Fig. 4C. Unsurprisingly, 17α-T, the main metabolite of 17β-T in cattle urine, was found to have a higher concentration (i.e., 10.2 ng/ml) than the precursor compound.

Importantly, the suitability of the SRM LC–MS–MS to assess physiological concentrations of anabolic hormones in bovine urine was shown.

4. Conclusion

The aim of this research was to develop a specific, sensitive and reliable LC–APCI-MS–MS method for the measurement of anabolic hormone residues (17β-NT, 17β-T and P) and their metabolites (17α-NT and 17α-T) in bovine serum and urine.

The unambiguous confirmation of the presence of the hormones both in bovine serum and urine in the proposed method results from the retention time information, the presence of the protonated molecule of the analyte, and the employment of a double precursor–product ion reaction, thus achieving a confidence in identification higher than 99.99% [24].

The developed method complies with the criteria proposed by the recent revision of the Commission Decision 93/256/EEC [25] for confirmatory methods of substances listed in Group A of Annex I of Council Directive 96/23/EC [26], such as anabolic

steroids, requiring the identification of the analyte based on the presence of at least one precursor and two transition product ions for LC–MS–MS.

The ability to perform multi-residue analyses involving simple extraction procedures and highly selective and sensitive determination by SRM LC–MS–MS, along with the widespread presence of benchtop LC–MS^n apparatus in laboratories, makes this analytical method particularly valuable for routine control of the illegal use of anabolic hormones and their potentially toxic metabolites in livestock production.

Research activities by our group are ongoing in order to obtain more detailed profiles of the hormones here investigated and of their metabolites in the biological fluids of interest, which would allow further development in the control strategies of illegal treatments.

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