Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 789 (2003) 219–226
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Confirmatory analysis of 17β-boldenone, 17α-boldenone and androsta-1,4-diene-3,17-dione in bovine urine by liquid chromatography–tandem mass spectrometry

Rosa Draisci*, Luca Palleschi, Emanuele Ferretti, Luca Lucentini, Fernanda delli Quadri

Laboratorio di Medicina Veterinaria, Istituto Superiore di Sanità, V.le Regina Elena, 299, 00161, Rome, Italy

Received 3 September 2002; received in revised form 8 January 2003; accepted 8 January 2003

Abstract

A sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for confirmatory analysis of 17β-boldenone (17β-BOL), 17α-boldenone (17α-BOL) and androsta-1,4-diene-3,17-dione (ADD) in bovine urine was developed. [$^2H_2$]17β-Testosterone (17β-T-d_2) was used as the internal standard. Sample preparation involved enzymatic hydrolysis and purification on a C_{18} solid-phase extraction column. Chromatographic separation of the analytes was obtained using an RP-C_{18} HPLC column. LC–MS–MS detection was carried out with an atmospheric pressure chemical ionisation (APCI) source equipped with a heated nebulizer (HN) interface operating in the positive ion mode. For unambiguous hormone confirmation, three analyte precursor–product ion combinations were monitored during multiple-reaction monitoring (MRM) LC–MS–MS analysis. Overall recovery (%), repeatability (relative standard deviations, RSD, %) and within-laboratory reproducibility (RSD, %) ranged from 92.2 to 97.7%, from 6.50 to 2.94% and from 13.50 to 5.04%, respectively, for all analytes. The limit of quantification in bovine urine was 0.20 ng ml^{-1} for 17β-BOL and ADD and 0.50 ng ml^{-1} for 17α-BOL. The validated method was successfully applied for determination of 17β-BOL, 17α-BOL and ADD in a large number of bovine urine samples collected within the national Official Residue Control Program.

Keywords: Boldenone; Androsta-1,4-diene-3,17-dione

1. Introduction

17β-Boldenone (androsta-1,4-dien-17β-ol-3-one, 17β-BOL) is a synthetic androgenic steroid hormone with anabolic properties [1], first synthesized in 1956 by dehydrogenation of the male hormone testosterone with selenium dioxide [2]. It is illegally used as a growth promoter in cattle husbandry [3–5] and as a performance enhancer in race horses [6] and for athletes [7].

In view of the intrinsic properties of hormones and related scientific findings, the use of natural and synthetic hormones for growth promotion purposes in animals destined for meat production is prohibited in the European Union (EU) to avoid consumer

*Corresponding author. Tel./fax: +39-06-4990-2327.
E-mail address: draisci@iss.it (R. Draisci).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00072-2

exposure to unforeseeable risks from the intake of hormone residues and their metabolites [8,9].

Injectable esters of 17β-BOL have been previously identified at application sites in cattle, thus proving their illegal use [3–5]. 17β-Boldenone esters are hydrolysed to 17β-BOL following intramuscular injection [10]. 17α-Boldenone (17α-BOL), considered to be the main metabolite of 17β-BOL in equine [11,12] and cattle urine [4,10,13,14], is found together with 17β-BOL in bovine urine following illegal administration.

In addition, androsta-1,4-diene-3,17-dione (ADD), the ''dione'' form of boldenone, has been identified as a metabolite in the urine of veal calves treated with 17β-BOL [13,14] or its esters [4] and as the most prominent in vitro metabolite in microsomal incubations [14]. ADD is the direct precursor of 17β-BOL in humans [12] due to the enzymatic action of the 17β-hydroxysteroid dehydrogenase. The same mediator enzyme is present in cattle [15], thus suggesting that boldenone could be the product of metabolic ADD conversion in cattle. In fact, we have found in other in vivo studies still in progress that oral administration of ADD to veal calves (age< 6 months) leads to the presence of 17α-BOL, 17β-BOL and ADD in bovine urine.

A number of studies have investigated the possible endogenous presence of 17α-BOL in animals destined for human consumption. 17α-BOL has been detected by Arts et al. in urine samples of cattle declared to be untreated at levels ranging from 0.1 to 2.7 $\mu g\ l^{-1}$. 17α-BOL has also been quantified in two of 20 urine samples from untreated calves at concentrations of 1.8 and 1.2 $\mu g\ l^{-1}$ [16]. However, the conclusion of a study investigating the identification of metabolites of boldenone in cattle urine and faeces was that there was no direct evidence that 17α-BOL or 17β-BOL is of endogenous origin [14].

Intramuscular administration of 200 [4] and 700 mg [13,14] of 17β-boldenone undecanoate to bovines resulted in the excretion of 17α-BOL in urine at concentrations of 0.7–0.2 $\mu g\ l^{-1}$ [4] and up to 988 $\mu g\ l^{-1}$ [13,14], whereas 17β-BOL was detected at levels of <0.1–3 $\mu g\ l^{-1}$ [4] and up to 160 $\mu g\ l^{-1}$ [13,14] respectively. The compounds disappeared gradually in time after the end of the treatment, with the highest concentrations observed within 48 h of treatment. ADD and a number of 17β-BOL metabolites have also been observed in the urine of treated animals at levels of up to 46 $\mu g\ l^{-1}$ during the first days after administration [13,14].

A number of analytical assays for the detection of 17β-BOL, 17α-BOL and ADD in urine samples have been developed for the purposes of pharmacokinetic studies and surveillance of illegal use in animal production.

An enzyme linked immunosorbent assay (ELISA) was developed for routine screening of the anabolic steroid boldenone in equine blood and urine [17], proving high sensitivity and sample throughput. However, cross-reactivity among 17β-BOL and its metabolites and/or analogues is frequently a concern with this assay. GC–MS is currently applied to identify17β-BOL [4,13,14,18] together with its metabolites, 17α-BOL [4,13,14] and ADD [13,14], although it requires cumbersome and time-consuming derivatisation steps. A simplified, rapid, specific and sensitive alternative to GC–MS methods, with simple extraction procedures and no need for derivatisation reactions, is offered by the combination of LC–MS and LC–MS–MS.

Applications of LC using mass spectrometry were proposed for the determination of boldenone in animal kidney fat [19] and urine [1,10,20,21]. 17β-BOL with another 35 anabolic steroids has been detected by LC–APCI-MS–MS in kidney fat, even though sample preparation was obtained by preparative HPLC [19].

Boldenone sulphoconjugate was confirmed in equine urine after administration of boldenone undecylenate, by LC–MS–MS with an ionspray interface [1,20]. Recently, the use of LC–MS–MS with an electrospray [10] and heated nebulizer [21] interface has been reported for the detection of 17β-BOL and 17α-BOL in bovine urine.

The present study investigated the possibility of using LC–MS–MS via a heated nebulizer (HN) interface for the simultaneous detection and quantification of 17β-boldenone, 17α-boldenone and androsta-1,4-diene-3,17-dione in bovine urine, using [$^2H_2$]17β-testosterone (17β-T-d_2) as the deuterated internal standard (I.S.). The validated method was successfully applied for the determination of 17β-BOL, 17α-BOL and ADD in bovine samples collected within the national Official Residue Control Program.

2. Experimental

2.1. Chemicals and reagents

All solvents were HPLC or analytical grade and purchased from Farmitalia Carlo Erba (Milan, Italy). Water was purified by MILLI-Q System (Millipore, Bedford, MA, USA). β-Glucuronidase/arylsulphatase (*Helix pomatia*) from Merck (Darmstadt, Germany) was used as supplied.

17α-BOL and 17β-T-d_2 were provided by RIVM (Bilthoven, The Netherlands). 17β-BOL was purchased from Riedel-de Haen (Seelze, Germany) and ADD from Steraloids (Newport, RI, USA).

Individual stock solutions of 4 μg ml^{-1} were prepared in methanol. Individual and composite working standard solutions were prepared daily by appropriate dilution of the standard stock solutions with methanol. Stability of the analytes in solution was observed for 4 weeks in the dark at −20 °C and no degradation phenomena were observable during identification and/or quantification.

2.2. Samples

Blank urine samples were collected on a farm from cattle (n=25 animals) under dietary control and assayed by multiple reaction monitoring (MRM) LC–MS–MS. The absence of 17β-BOL, 17α-BOL and ADD was verified.

Incurred bovine urine samples were collected from cattle as part of the national Official Residue Control Program. All samples were stored in the dark at ≈−20 °C until assayed.

2.3. Sample preparation

An aliquot (2.0 ml) of urine was fortified with 5 ng of 17β-T-d_2 (I.S.). Six milliliters of acetate buffer solution (ABS) (0.15 *M*), pH 5.0 and 50 μl of β-glucuronidase/arylsulphatase enzyme solution (*Helix pomatia*) were added. The mixture was left in an ultrasonic bath for 5 min and incubated for 12 h at 37 °C. It was then centrifuged for 10 min at 3000 *g*.

The sample was purified by solid-phase extraction (SPE) using a C_{18} cartridge (Baker C_{18}, 500 mg, 3 ml) previously 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, 15.0 ml of water and 3.0 ml of methanol–water (70:30, v/v). Finally, the analytes were eluted with 3.0 ml of methanol, the solvent removed using an evaporation block at 40 °C under nitrogen and the residue dissolved in 100 μl of methanol. Five microliters of this solution was injected into the LC–MS–MS.

2.4. LC–MS and LC–MS–MS

Analyses were carried out on a Varian LC 9010 pump (Varian, Turin, Italy) liquid chromatograph. A Rheodyne 9725 sample injector equipped with a 5 μl external loop (Rheodyne, Rohnert Park, CA, USA) was used for the 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 HPLC column (250×2.1 mm I.D., 5 μm) Allure C_{18} (Restek, Bellefonte, PA, USA) with a C_{18} guard column (4×2 mm I.D.) (SecurityGuard, Phenomenex, USA) at room temperature, with a mobile phase of acetonitrile–water (60:40, v/v) containing 5 m*M* ammonium acetate and at a flow-rate of 130 μl min^{-1}.

Mass spectral analyses were carried out on a PE-SCIEX API III plus triple-quadrupole (PE-Sciex, Thornhill, Ontario, Canada) equipped with an atmospheric pressure chemical ionisation (APCI) source and heated nebulizer (HN) interface operating in positive ion mode at 350 °C. Ultra-high-purity nitrogen was used as the curtain gas (0.6 l min^{-1}), and air was used as the nebulizer (400 kPa) and auxiliary gas (1.5 l min^{-1}). Discharge current was set at 4 μA.

The effect of varying orifice potential voltage (OR) on ion intensity was investigated for each analyte between 50 and 100 V. Selected ion monitoring (SIM) FIA–MS analyses at m/z 287, 285 and 291 were carried out to obtain the maximum signal intensity for the protonated molecular ion of each analyte. ORs of 50 V for ADD, 60 V for 17α- and 17β-BOL and 70 V for 17β-T-d_2 (I.S.) were found to offer the best compromise in terms of signal-to-noise ratio.

Full-scan mass spectra were acquired in positive ion mode from m/z 200 to 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

300×10^{13} molecules cm^{-2}) in quadrupole 2 (Q2) operated in radio frequency (RF)-only mode, scanning the third quadrupole mass spectrometer, Q3, from m/z 50 to 300. A collision energy of 25 eV was chosen for the collision-induced-dissociation (CID) experiments. The protonated molecule, $[M+H]^+$, at m/z 287 for 17α-BOL and 17β-BOL, at m/z 285 for ADD and at m/z 291 for 17β-T-d_2, was the precursor ion for CID and two product ions for each anabolic hormone were identified to carry out MRM LC–MS–MS analyses. Three precursor–product ion combinations of m/z 287→121, m/z 287→135, and m/z 287→173 were used for both 17α-BOL and 17β-BOL, m/z 285→121, m/z 285→147 and m/z 285→151 for ADD, m/z 291→99 and m/z 291→111 for 17β-T-d_2. The dwell time for each monitored transition was 150 ms. Peak-area ratios of analyte to internal standard were calculated using MacQuan version 1.3 software from PE-Sciex.

2.5. Calibration, repeatability, within-laboratory reproducibility and recovery

Blank urine samples (2 ml) were fortified with analytes and I.S. (5 ng), resulting in three analytical series each with four hormone concentrations (i.e. 0.2, 5.0, 12.0 and 40.0 ng ml^{-1} for 17β-BOL and ADD; 0.5, 5.0, 12.0 and 40.0 ng ml^{-1} for 17α-BOL) and six samples per concentration. The series were analysed on each of three different days to evaluate the analytical method's repeatability (within-day), within-laboratory reproducibility (different operators and environmental conditions) and recovery (internal standard-corrected).

Matrix calibration curves were prepared daily by spiking blank urine samples (2 ml) with 5 ng of I.S. followed by mixtures of the anabolic compounds to obtain concentrations in the range 0.2–50.0 ng ml^{-1} for 17β-BOL and ADD and 0.5–50.0 ng ml^{-1} for 17α-BOL. Estimates of analyte concentration in the validation and incurred samples were interpolated from these calibration curves, constructed by plotting peak area ratios of the analyte to I.S. versus hormone concentrations using a least-squares linear regression model.

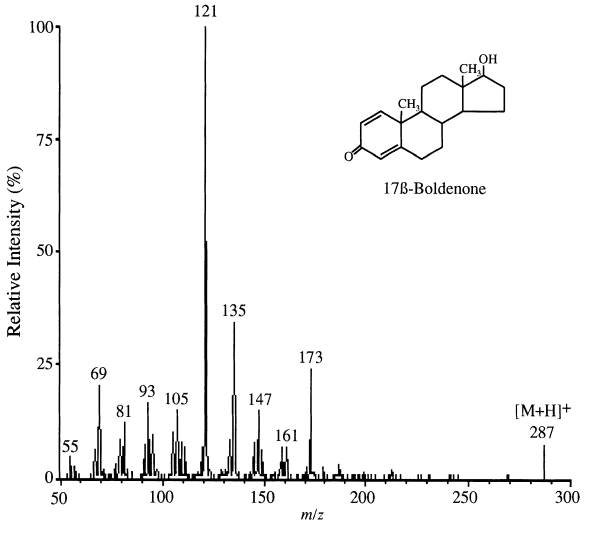

Fig. 1. Positive product ion mass spectrum of 17β-BOL, with the $[M+H]^+$ ion at m/z 287 as precursor. Conditions: FIA; mobile phase: acetonitrile–water (60:40, v/v) containing 5 m*M* ammonium acetate; flow-rate 130 μl min^{-1}; OR was set at 60 V. Argon was used as the collision gas. CID was carried out with a collision energy of 25 eV.

3. Results and discussion

Figs. 1 and 2 show the positive-product ion mass spectra (mass range m/z 50–300) of the protonated molecule $[M+H]^+$ of 17β-BOL and ADD, respectively from experiments carried out by FIA–MS–MS on the individual hormone standard solutions. Spectral comparison reveals the production of the most abundant product ions at m/z 121, 135, 173 for 17α- and 17β-BOL, at m/z 121, 147 and 151 for ADD and at m/z 99 and 111 for 17β-T-d_2. Transitions of the respective protonated molecules to these product ions were therefore selected according to the MRM technique.

Good separation of 17β-BOL ($t_r=7.3$ min), ADD ($t_r=8.8$ min), 17α-BOL ($t_r=9.0$ min) and 17β-T-d_2 ($t_r=9.2$ min), was obtained in MRM LC–MS–MS profiles of a standard analyte mixture (data not shown).

Specificity of the MRM LC–MS–MS method was also demonstrated by the preparation and analysis of 20 blank samples with (Fig. 3a) and without internal standard. No interference was observed around the hormone retention times in urine samples.

Matrix calibration curves were prepared daily by

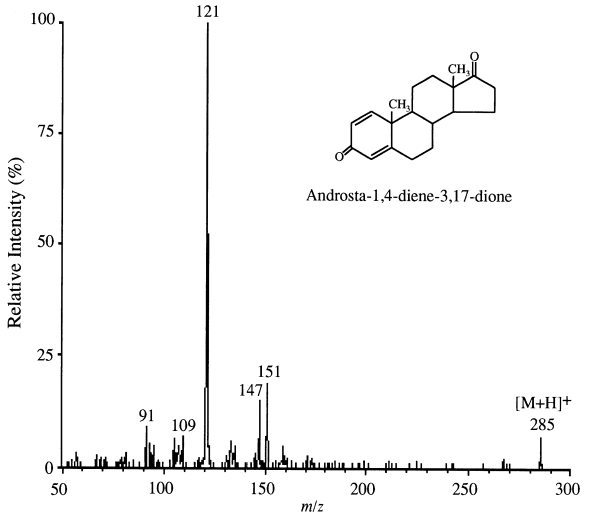

Fig. 2. Positive product ion mass spectrum of ADD, with the $[M+H]^+$ ion at m/z 285 as precursor. Conditions: FIA; mobile phase: acetonitrile–water (60:40, v/v) containing 5 m*M* ammonium acetate; flow-rate 130 μl min^{-1}; OR was set at 50 V. Argon was used as the collision gas. CID was carried out with a collision energy of 25 eV.

spiking blank urine samples with I.S. (2.5 ng ml^{-1}) and mixtures of the anabolic compounds to obtain concentrations of 0.2–50.0 ng ml^{-1} for 17β-BOL and ADD and 0.5–50.0 ng ml^{-1} for 17α-BOL. Linearity was good for all analytes throughout the range of tested concentrations, as proved by the correlation coefficients (r^2) greater than 0.996 for all curves.

Fortified bovine urine samples at 0.2, 5.0, 12.0 and 40.0 ng ml^{-1} for 17β-BOL and ADD and at 0.5, 5.0, 12.0 and 40.0 ng ml^{-1} for 17α-BOL were prepared and analysed in order to determine the analytical method's repeatability, within-laboratory reproducibility and recovery. Representative chromatograms of urine samples fortified at 5.0 ng ml^{-1} of 17α-BOL, 17β-BOL, ADD, containing 2.5 ng ml^{-1} of 17β-T-d_2 (I.S.), are reported in Fig. 3b. The relative retention time for each analyte corresponded to that of the standard within a tolerance of ±0.6%, in accordance with the criteria laid down by the Commission Decision 2002/657/EC [22].

The method's repeatability, within-laboratory reproducibility and recovery were assessed by analysing six replicates of bovine urine fortified at 0.2, 5.0, 12.0 and 40.0 ng ml^{-1} for 17β-BOL and ADD and at 0.5, 5.0, 12.0 and 40.0 ng ml^{-1} for 17α-BOL on each of three different days. Repeatability (within-day) and within-laboratory reproducibility (different operators and environmental conditions) were determined by calculating relative standard deviations (RSD, %) for the repeated measurements, while mean percentages of recovery (internal standard-corrected, %) were assessed from the agreement between measured and nominal concentrations of the fortified samples.

Table 1
LOQ, repeatability, within-laboratory reproducibility and recovery for 17α-BOL, 17β-BOL and ADD in bovine urine samples

Analyte	LOQ (ng ml^{-1})	Spike level (ng ml^{-1})	Repeatability (RSD, %) ($n=6$)	Reproducibility (RSD, %) ($n=18$)	Recovery (%) ($n=18$)
17α-BOL	0.50	0.50	6.03	11.82	95.5
		5.0	5.78	10.23	95.8
		12.0	5.51	9.17	96.6
		40.0	2.94	5.04	97.1
17β-BOL	0.20	0.20	6.17	10.42	95.9
		5.0	4.89	8.95	96.4
		12.0	4.56	7.36	96.6
		40.0	3.12	5.22	97.7
ADD	0.20	0.20	6.50	13.50	92.2
		5.0	5.09	10.75	94.2
		12.0	4.88	9.88	94.8
		40.0	3.41	6.22	95.1

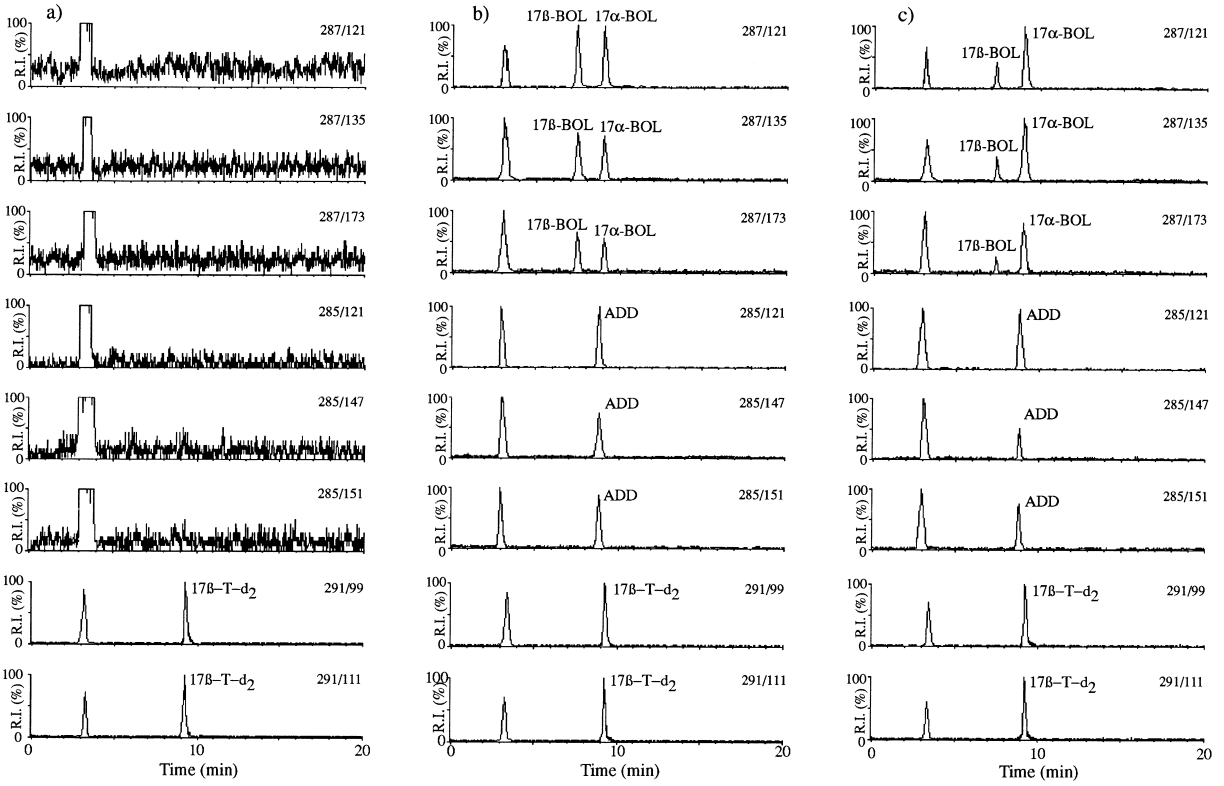

Fig. 3. MRM LC–MS–MS chromatograms of: (a) extract of blank bovine urine spiked at 2.5 ng ml^{-1} of 17β-T-d_2 as I.S.; (b) extract of blank control bovine urine spiked at 5.0 ng ml^{-1} of 17α-BOL, 17β-BOL, ADD and 2.5 ng ml^{-1} of 17β-T-d_2 (I.S.); (c) extract of an incurred bovine urine sample containing 17α-BOL (4.6 ng ml^{-1}), 17β-BOL (1.5 ng ml^{-1}), ADD (2.9 ng ml^{-1}) and 17β-T-d_2 (2.5 ng ml^{-1}). Precursor–product ion combinations used in MRM detection are shown. Conditions: isocratic HPLC analysis; reversed-phase Allure C_{18} (250×2.1 mm I.D., 5 μm) column; mobile phase: acetonitrile–water (60:40, v/v) containing 5 m*M* ammonium acetate; flow-rate 130 μl min^{-1}; OR was set at 50 V for ADD, 60 V for 17α- and 17β-BOL and 70 V for 17β-T-d_2 (I.S.). Argon was used as the collision gas. CID was carried out with a collision energy of 25 eV.

Overall recovery (%), repeatability (RSD, %) and within-laboratory reproducibility (RSD, %) ranged from 92.2 to 97.7%, from 6.50 to 2.94% and from 13.50 to 5.04%, respectively, for all analytes (Table 1).

The limit of quantification (LOQ) in bovine urine was 0.20 ng ml^{-1} for 17β-BOL and ADD and 0.50 ng ml^{-1} for 17α-BOL. These values are thought to be satisfactory, considering the complexity of the biological matrices, and make the procedure described suitable for control purposes.

The stability assessment of analytes in matrix under storage conditions was carried out by comparing their concentration in the stored samples with their original concentrations in the fresh materials. Their stability under storage conditions was verified for 4 weeks in the dark at −20 °C. No degradation phenomena were observable during identification and/or quantification.

Representative MRM LC–MS–MS chromatographic profiles of an incurred bovine urine sample containing 17α-BOL (4.6 ng ml^{-1}), 17β-BOL (1.5 ng ml^{-1}), ADD (2.9 ng ml^{-1}), and 17β-T-d_2 (2.5 ng ml^{-1}) are shown in Fig. 3c. Importantly, the suitability of the MRM LC–MS–MS to identify 17β-BOL and 17α-BOL in samples of bovine urine in violation of EU law was therefore shown.

The MRM LC–MS–MS method was used by this

National Reference Laboratory (NRL) to analyse bovine urine samples obtained within the national Official Residue Control Program.

In recent years, the NRL has found 17α-BOL in more than 100 cattle, even at levels of over 50 ppb (average 11 ppb). More than 60% of farms investigated have been found positive for 17β-BOL, with maximum levels above 1.5 ppb (average 0.5 ppb). In addition, more than 60% of these cattle have also been found positive for ADD, with levels even above 30 ppb (average 3 ppb).

4. Conclusion

The aim of this research was to develop a specific, sensitive and reliable LC–APCI-MS–MS method for the simultaneous confirmatory analysis of 17β-BOL, 17α-BOL and ADD in bovine urine.

The unambiguous confirmation of the presence of these hormones in bovine urine in the proposed method results from retention time information, the presence of the protonated molecule of the analyte, and the employment of a triple precursor–product ion reaction. The developed method therefore complies with the criteria laid down by the recent Commission Decision 2002/657/EC [22] for confirmatory methods of substances listed in Group A of Annex I of Council Directive 96/23/EC [23], such as anabolic steroids, requiring analyte identification based on the presence of at least one precursor and two transition product ions for LC–MS–MS technique.

The MRM LC–MS–MS method was successfully used by this National Reference Laboratory to analyse a large number of bovine urine samples, over a wide range of boldenone concentrations, within the national Official Residue Control Program.

We found that the simplified sample preparation and the specificity and sensitivity of the developed analytical method made it particularly suitable for routine control of the illegal use of 17β-boldenone and its metabolites in livestock production.

The method is an indispensable tool in the task of improving knowledge required for risk assessment related to the presence of boldenone in food of animal origin.

Ongoing research activities by our group aim at clarifying the origin and levels of 17β-BOL, 17α-BOL and ADD in bovine matrices, in relation both to their endocrine status and to livestock technologies.

References

[1] L.O.G. Weidolf, T.M. Chichila, J.D. Henion, J. Chromatogr. 433 (1988) 9.
[2] C. Meystre, H. Frey, W. Voser, A. Wettstein, Helv. Chim. Acta 88 (1956) 734.
[3] K. Vanoosthuyze, E. Daeseleire, A. Van Overbeke, C. Van Peteghem, A. Ermens, Analyst 119 (1994) 2655.
[4] C.J.M. Arts, R. Schilt, M. Schreurs, L.A. van Ginkel, in: A. Ruiter (Ed.), Residue of Veterinary Drugs in Food, Proceedings of the EuroResidue III, Veldhoven, The Netherlands, 6–8 May 1996, Utrecht University, Faculty of Veterinary Medicine, 1996, p. 212.
[5] R.W. Stephany, APMIS 109 (Suppl. 103) (2001) 357.
[6] T. Tobin, Drugs and the Performance Horse, Thomas, Springfield, IL, 1981, p. 149.
[7] M. Donike, H. Geyer, A. Gotzman, M. Kraft, F. Mandel, E. Nolteernsting, G. Opfermann, G. Sigmund, W. Schänzer, G. Zimmermann, Dope Analysis, International Athletic Foundation World Symposium on Doping in Sport, Florence, 1987.
[8] http://europa.eu.int/comm/food/fs/sc/scv/out21_en.pdf. Report of the Scientific Committee on Veterinary Measures Relating to the Public Health, European Commission DG XXIV, 30 April 1999.
[9] Commission of the European Communities, Council Directive 96/22/CE, Off. J. Eur. Commun. Legis. L125 (1996) 3.
[10] C. Van Poucke, C. Van Peteghem, J. Chromatogr. B 772 (2002) 211.
[11] M.C. Dumasia, E. Houghton, Biomed. Environ. Mass Spectrom. 17 (1988) 383.
[12] W. Shanzer, M. Donike, Biol. Mass Spectrom. 21 (1992) 3.
[13] M. Van Puymbroeck, L. Leyssens, D. Vanderzande, J. Gelan, J. Raus, Analyst 123 (1998) 2449.
[14] M. Van Puymbroeck, M.E. Kuilman, R.F. Maas, R.F. Witkamp, L. Leyssens, D. Vanderzande, J. Gelan, J. Raus, Analyst 123 (1998) 2681.
[15] M.A. Juarez-Oropeiza, V. Lopez, G. Alvarez-Fernandez, Y. Gomez, E. Pedernera, J. Exp. Zool. 271 (1995) 373.
[16] S. Sterk, F. van Tricht, A. van Soeren-Kieft, H. Herbold, R. Stephany, L. van Ginkel, Fresenius J. Anal. Chem. 360 (1998) 454.
[17] H.W. Hagedorn, R. Schulz, G. Jaeschke, Biomed. Chromatogr. 8 (2) (1994) 63.
[18] H.F. De Brabander, P. Batjoens, D. Courtheyn, J. Vercammen, K. De Wasch, J. Chromatogr. A 750 (1996) 105.
[19] P.E. Joos, M. Van Ryckeghem, Anal. Chem. 71 (1999) 4701.
[20] L.O. Weidolf, E.D. Lee, J.D. Henion, Biomed. Environ. Mass Spectrom. 15 (1988) 283.

[21] R. Draisci, C. Marchiafava, P. Cammarata, A. Cecilia, L. Achene, The 11th Nordic Conference on Mass Spectrometry, Loen, Norway, 18–21 August 2001, p. 62.
[22] Commission of the European Communities, Commission Decision 2002/657/EC, Off. J. Eur. Commun. Legis. L221 (2002) 8.
[23] Commission of the European Communities, Council Directive 96/23/CE, Off. J. Eur. Commun. Legis. L125 (1996) 3.