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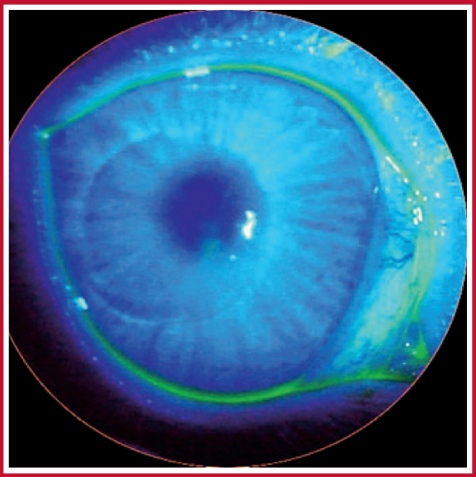

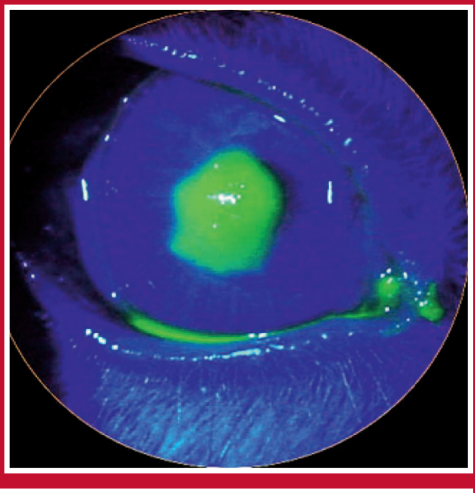

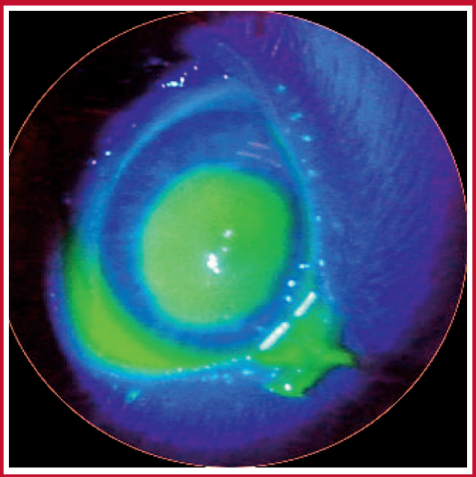

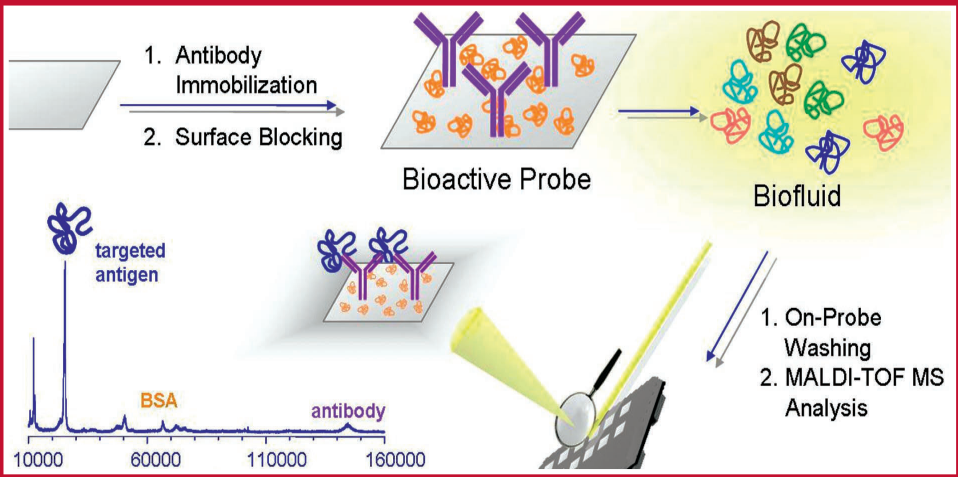

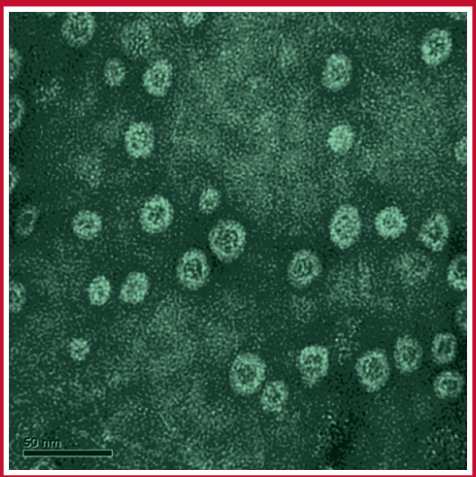

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DOI 10.1002/pmic.200601039 *Proteomics* 2007, *7*, 3184–3193

RESEARCH ARTICLE

Integrated analytical approach in veal calves administered the anabolic androgenic steroids boldenone and boldione: urine and plasma kinetic profile and changes in plasma protein expression

Rosa Draisci[1], Clara Montesissa[2], Barbara Santamaria[3], Chiara D'Ambrosio[4], Giovanni Ferretti[1], Roberta Merlanti[2], Carolina Ferranti[1], Marco De Liguoro[2], Claudia Cartoni[1], Erika Pistarino[3], Lino Ferrara[4], Micaela Tiso[3], Andrea Scaloni[4] and M. Elisabetta Cosulich[3, 5]

[1] Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy
[2] Department of Public Health, Comparative Pathology and Veterinary Hygiene, Agripolis, Legnaro, Italy
[3] Department of Chemistry and Industrial Chemistry, University of Genoa, Genoa, Italy
[4] Proteomic and Mass Spectrometry Laboratory, ISPAAM, National Research Council, Naples, Italy
[5] Department of Biochemistry "A. Castellani", University of Pavia, Pavia, Italy

Surveillance of illegal use of steroids hormones in cattle breeding is a key issue to preserve human health. To this purpose, an integrated approach has been developed for the analysis of plasma and urine from calves treated orally with a single dose of a combination of the androgenic steroids boldenone and boldione. A quantitative estimation of steroid hormones was obtained by LC-APCI-Q-MS/MS analysis of plasma and urine samples obtained at various times up to 36 and 24 h after treatment, respectively. These experiments demonstrated that boldione was never found, while boldenone α- and β-epimers were detected in plasma and urine only within 2 and 24 h after drug administration, respectively. Parallel proteomic analysis of plasma samples was obtained by combined 2-DE, MALDI-TOF-MS and μLC-ESI-IT-MS/MS procedures. A specific protein, poorly represented in normal plasma samples collected before treatment, was found upregulated even 36 h after hormone treatment. Extensive mass mapping experiments proved this component as an N-terminal truncated form of apolipoprotein A1 (ApoA1), a protein involved in cholesterol transport. The expression profile of ApoA1 analysed by Western blot analysis confirmed a significant and time dependent increase of this ApoA1 fragment. Then, provided that further experiments performed with a growth-promoting schedule will confirm these preliminary findings, truncated ApoA1 may be proposed as a candidate biomarker for steroid boldenone and possibly other anabolic androgens misuse in cattle veal calves, when no traces of hormones are detectable in plasma or urine.

Received: December 20, 2006
Revised: March 22, 2007
Accepted: May 18, 2007

Keywords:
Illicit treatment / Mass spectrometry / Steroids

Correspondence: Professor M. Elisabetta Cosulich, Department of Chemistry and Industrial Chemistry, University of Genoa, Via Dodecaneso 31, 16100 Genoa, Italy
E-mail: cosulich@chimica.unige.it
Fax: +39-010-3538763

Abbreviations: ADD, 1,4-androstadiene-3,17-dione; **APCI,** atmospheric pressure chemical ionization, **ApoA1,** apolipoprotein A1; **α-Bol,** 1,4-androstadiene-17α-ol-3-one; **β-Bol,** 1,4-androstadiene-17β-ol-3-one; **β-T-d_2,** 17β-testosterone-d_2; **HDL,** high density lipoproteins

1 Introduction

Steroids hormones are used in cattle breeding to increase muscle protein accretion; however, the use of such growth promoters is illegal in the European Union [1]. Control of illegal use of steroids is a difficult issue to achieve for many reasons; these drugs have a short persistence in serum and urine and they are generally converted into metabolites partially characterized [2–4]. In addition, difficulties have been found in distinguishing between the administered hormone

(exogenous hormone) and the endogenous one produced by the animal [4, 6, 7]. Different strategies have been used for the detection of hormones and its metabolites. The drug or its metabolites could be detected directly in urine, plasma and faeces [5, 8–10]; alternatively, it could be measured through the presence of specific proteins, whose expression is modulated by the exogenous hormone administration. Recent reports describe in liver [11] or in prostate gland and testis [12] the expression of specific protein as potentially biomarker of illicit treatment. In the last years, the advent of new MS-based molecular approaches allowed the discovery of novel biomarkers related to hormone exposure. The biomarker is one of the various biological effects induced in an organism by the drug treatment; it could be defined as a substance whose expression is modulated by the exposure to a toxicological foreign compound.

Boldenone (1,4-androstadiene-17β-ol-3-one, β-Bol) and boldione (1,4-androstadiene-3,17-dione, ADD) are chemically derivatives of testosterone and of 4-androstene-3,17-dione (AED), respectively, known as strong anabolics and low to moderately androgenic agents [12]. ADD the dione form of β-Bol, in calves is a direct precursor to that anabolic steroid [4, 13]. Indeed, it is activated by the same widely distributed enzyme, namely 17β-hydroxysteroid dehydrogenase, which converts AED to testosterone. Recently, its role as both precursor and metabolite of boldenone has been confirmed *in vitro* using subcellular fractions obtained from calf liver [14, 15]. Moreover, ADD is considered the direct precursor of 1,4-androstadiene-17α-ol-3-one (α-Bol), the inactive epimer of β-Bol. Despite the rate of *in vitro* formation of α-Bol is much lower than that of β-Bol [14], the former is the main metabolite detected *in vivo* in cattle urine.

Both β-Bol and ADD are illegally used in racehorses and athletes to improve sport performance [15]. Because of the known properties displayed by natural and synthetic androgens to react with specific receptors and to promote protein synthesis at muscular level, both compounds during the last decades were widely used also in cattle breeding for meat production, due to their favourable effects on the animal growth and feed conversion. Scientific evidences supported the presence β-Bol as physiological in horses and swine as well as the presence of α-Bol in bovine urine from other sources that illegal treatment. Among these, possible endogenous occurrence of the latter steroid was related to feed quality or phytosterols/hormone precursors in animal feed [16].

The results of several studies on β-Bol were reviewed to update the knowledge on the illegal use or the natural occurrence in cattle [7]. The decision taken by the experts on the surveillance strategy of boldenone misuse in veal calves was that the presence of α-Bol conjugates should be considered as a sign of suspected treatment only at concentrations higher that 2 ng/mL; similarly, the presence in urine of β-Bol conjugates at any level was considered as a proof of illegal treatment. Conversely, the recommended minimum performance level required to the analytical methods adopted for surveillance for α-Bol and β-Bol conjugates in urine of bovine was set at 1 ng/mL [17]. Few methods for LC-MS quantitative determination of ADD, β-Bol and α-Bol in bovine urine have been described in literature [9, 10]. Draisci *et al.* [8] validated an LC-MS/MS methodology to analyse α-Bol, β-Bol and ADD in bovine urine. The method was successfully applied to the Italian Official Residue Control program. Recently, this method was validated to quantify α-Bol, β-Bol and ADD in bovine urine, faeces, feed and skin swab sample, according to the latest EU guidelines [9]. However, no reports have been reported on qualitative and quantitative LC-MS determination of α-Bol, β-Bol and ADD in bovine plasma.

In 2002, an illegal preparation for farm use containing a cocktail of boldenone, ADD and a boldenone ester, discovered in Italy by the Carabinieri – Nucleo Antisofisticazioni e Sanità of Rome [18], was used as model for reproduction in veal calves of an illegal cocktail [13]. To check kinetic behaviour and traceability of steroid compounds from this preparation in cattle urine, a preliminary experiment was conducted administering once to the calves the overall low dosage of 10 mg/head (1 mg of ADD plus 9 mg of β-Bol). In this work, the results of a study on steroid disposition and elimination rates are presented. To this purpose, a dedicated LC-MS/MS procedure was developed to detect α-Bol, β-Bol and ADD in plasma samples. Thus, plasma and urinary pharmacokinetics of these exogenous hormones were determined after oral drug administration to different bovine individuals. In parallel, the proteomic profile of bovine plasma samples collected at various times after hormone treatment was comparatively evaluated. This analysis lead to the detection of a specific protein with a highly increased expression, further characterized for its structure by combined MS mapping procedures. Its altered concentration persisted even at times after drug administration when normal steroids level was observed in plasma and urine samples. Accordingly, this protein may be proposed for a more extensive screening to evaluate its performance as candidate biomarker for androgenic steroid treatment of breeding animals.

2 Materials and methods

2.1 Chemicals

All solvents were of HPLC or analytical grade and purchased from Riedel-de Haen (Seelze, Germany). Water was purified by Milli-Q System (Millipore, Bedford, MA, USA). β-Glucuronidase/arylsulphatase (*Helix pomatia*) from Merck (Darmstadt, Germany) was used as supplied. α-Bol and 17β-testosterone-d_2 (β-T-d_2) were provided by RIVM (Bilthoven, The Netherlands). β-Bol was purchased from Riedel-de Haen and ADD from Steraloids (Newport, RI, USA).

2.2 Animals and treatment

Five veal calves at the age of 30 days were purchased, and kept in an experimental authorized farm under controlled

conditions. Calves were fed a commercial milk replacer, until the age of four months (150 ± 15 kg m.b.w.). At day 0, calves received 200 mL of reconstituted milk containing a combination of β-Bol and ADD (9 and 1 mg, respectively) dissolved in ethanol (5 mL). The residual milk daily intake was administered immediately after.

2.3 Sampling

Since blood plasma may contain some residual and potentially informative molecules in regards to almost any disease state and can be easily collected from an individual animal, it would appear ideal to investigate the presence of exogenous hormones, metabolites, or biomarker discovery [19]. Blood samples were collected from the jugular vein using a needle holder and K_3EDTA Vacutainer tubes for three days before, just before (0 h) and 0.25, 0.5, 1, 2, 4, 8, 24 and 36 h after the drug administration. The blood samples were centrifuged (4000 rpm) at 4°C for 10 min and added with a protease inhibitors cocktail (Sigma). Aliquots (20 μL) of plasma were stored at −20°C pending the analysis. Each aliquot was used only once and no experiments were performed with samples thawed and frozen again. Each analysis was performed in triplicate after different times of storage; no differences were observed in proteomic or immunochemical analysis for samples with different storage times. Urine samples were collected from each animal using a clean kettle, at the first urinary output of the day (within 2–4 h, after feeding) and 9 and 24 h later.

2.4 Metabolite extraction

Urinary concentration of β-Bol, α-Bol and ADD was determined, before and after deconjugation with *H. pomatia* extracts, using an LC-MS/MS method developed and validated by ISS laboratory as reported by Draisci *et al.* [8]. Plasma concentrations were determined by a modification of the method mentioned above. Briefly, 2.0 mL of plasma was fortified with 5 ng of β-T-d_2 (internal standard) and 6.0 mL of 0.15 M acetate buffer solution (ABS), pH 5.0, were added. The sample was purified by SPE using C_{18} cartridge (Baker C_{18}, 500 mg, 3 mL) previously conditioned with 2.5 mL of methanol and 5 mL of water. After sample loading, cartridges were washed with 5 mL of ABS, 10 mL of water and 3 mL of methanol–water (70:30 v/v). Finally, analytes were eluted with 3 mL of methanol; this solvent was removed using an evaporation block at 40°C, under nitrogen, and the residue dissolved in 100 μL of methanol. Five microliters of this solution were injected into the LC-APCI (atmospheric pressure chemical ionization)-Q-MS/MS device.

2.5 Steroid LC-APCI-Q-MS/MS analysis

Analyses were carried out on an LC binary pump and an auto sampler series 200 (Perkin Elmer, USA) coupled to a triple quadrupole mass spectrometer API 3000 (Applied Biosystem, Foster City, CA, USA) equipped with an APCI source and heated nebulizer interface operating in positive ion mode at 350°C. Chromatographic separation was obtained under isocratic conditions using an RP-HPLC column (250 × 2.1 mm, 5 μm) Allure C_{18} (Restek, Bellefonte, PA, USA) with a mobile phase of ACN–water (65:35 v/v) containing 5 mM ammonium acetate, using a flow of 140 μL/min. Ultrahigh-purity nitrogen was used as the curtain gas and air was used as the nebulizer and auxiliary gas. Discharge current was set at 2 μA. Full-scan mass spectra were acquired in positive ion mode from *m/z* 200 to 400. The $[M + H]^+$ molecule at *m/z* 287 (for α-Bol and β-Bol), *m/z* 285 (for ADD) and *m/z* 291 (for β-T-d_2) was the precursor ion used for CID experiments and two product ions for each anabolic hormone were identified to carry out multiple reaction monitoring LC-APCI-Q-MS/MS analyses. Precursor product ion combinations of (i) *m/z* 287 → 121, *m/z* 287 → 135 and *m/z* 287 → 173 were used for both α-Bol and β-Bol; (ii) *m/z* 285 → 121, *m/z* 285 → 147 and *m/z* 285 → 151 were used for ADD (iii) *m/z* 291 → 99 and *m/z* 291 → 111 were used for β-T-d_2. Peak area ratios of analyte to internal standard were calculated using Analyst 1.4.1 software from Applied Biosystems. Two milliliters of blank urine and plasma samples were fortified with analyte and internal standard (5 ng/mL), resulting in three analytical series, each with six hormone concentrations, *i.e.* urine (i) 0.2–20.0 ng/mL of α-Bol and β-Bol; (ii) 0.3–20.0 ng/mL of ADD; plasma (iii) 0.4–5.0 ng/mL of α-Bol and β-Bol; (iv) 0.3–5.0 ng/mL of ADD. Linear plots were observed in all cases. Analyte concentrations were interpolated from these calibration curves, constructed by plotting peak area ratio of the analyte to internal standard.

2.6 2-DE gel analysis

Twelve microliters of plasma were added to 12 μL of solution containing 10% w/v SDS and 2.3% w/v DTT, heated at 95°C for 5 min and then diluted in 480 μL of buffer containing 7.0 M urea, 2 M thiourea, 2% w/v CHAPS, 10 mM DTT, 1% v/v pH 3–10 IPG buffer (Amersham Biosciences, Piscataway, NJ, USA) and 40 mM Tris-HCl. Each sample was loaded onto NL IPG strips (24 cm, pH 3–10) and incubated at room temperature to obtain a perfect strip rehydratation. SDS (0.25% w/v) within rehydratation buffer blocked unwanted enzymatic reactions, such as proteolysis, and did not alter protein focalization as result of the other denaturants presence [20]. IEF was conducted using an IGP Phor II system (Amersham Biosciences) for the first dimension analysis, and an Ettan Dalt-six apparatus for SDS-PAGE (Amersham Biosciences), according to the manufacturer's instructions. Focused strips were equilibrated with 6 M urea, 26 mM DTT, 4% w/v SDS, 30% v/v glycerol in 0.1 M Tris-HCl (pH 6.8) for 15 min, followed by 6 M urea, 0.38 M iodoacetamide, 4% w/v SDS, 30% v/v glycerol and 0.01% w/v bromophenol blue in 0.1 M Tris-HCl, pH 6.8, for 10 min and then applied directly to 12% SDS-polyacrylamide gels and separated at 150 V.

After Colloidal CBB (Sigma) staining, gels were scanned with an Image Scanner 2-D Apparatus (Amersham Biosciences). 2-DE was performed in triplicate for each plasma sample. p*I* of proteins was estimated by mixing a plasma sample with a p*I* protein standard (BioRad).

2.7 *In silico* analysis of 2-DE maps

Comparative analysis of gels was performed with the Image Master 2-D Platinum software, which allowed estimation of the normalized intensity volume for each protein within the same gel, matching of spots present within the set of gels belonging to a single calf having different volumes (quantitative differences) and identification of spots present in some samples but not in others (qualitative differences) [21]. 2-DE maps allowed detection of almost 700 different spots that under the adopted scanning parameters exhibited a relative volume higher than 0.02%. *In silico* analysis was performed by cropping the region below 100 kDa where the best spots reproducibility was observed; accordingly, the analysis was limited to almost 100 different spots from each sample. Normalized volume values were visualized as histograms representing the vol% of the matched spots from plasma samples obtained different time after the treatment *versus* the control plasma (*i.e.* samples obtained before the hormone administration). Mean and median of logarithmic ratios of the intensity values for each pair of matched spots was determined. Latter values were clustered around 0, showing that the mean error of our analysis was normally distributed. Normal distribution was further demonstrated by the Kolmogorov–Smirnov test, performed using GraphPad supplier computer software. Since the Kolmogorov–Smirnov parameter was closed to 0.09, SDs of logarithmic ratio distribution were a valid parameter to assess the variability of our analysis and the significance of the quantitative differences measured. A logarithmic ratio >0.27 (three times above the maximal value of logarithmic ratio SD) was used as a cut-off criterion to identify differentially expressed proteins with a p value <0.05. The differences in protein expressions between plasma samples collected before and after the treatment were reported in a bar graph illustrating (mean $\pm$ SD) the time-related changes in vol% in matched spots.

2.8 MALDI-TOF-MS and μLC-ESI-IT-MS/MS analysis

Spots from 2-DE were excised from the gel, *S*-alkylated and digested with trypsin, as previously reported [21]. Samples were desalted using μZipTipC18 tips (Millipore) before MALDI-TOF-MS analysis or directly analysed by μLC-ESI-IT-MS/MS. Peptide mixtures were loaded on the MALDI target together with CHCA as matrix, using the dried droplet technique [22]. Samples were analysed with a Voyager-DE PRO spectrometer (Applera, Foster City). Peptide mass spectra for protein identification experiments were acquired in reflectron mode and in linear mode for extensive mass mapping experiments. Internal mass calibration was performed with peptides derived from protease autoproteolysis. Data were elaborated using the DataExplorer 5.1 software (Applera). Peptide mixtures were also analysed by using an LCQ Deca Xp Plus mass spectrometer (ThermoFinnigan, San Josè, USA) equipped with an electrospray source connected to a Phoenix 40 pump (ThermoFinnigan) [23]. Peptide mixtures were separated on a capillary Hypersil-Keystone Aquasil C_{18} Kappa column (100×0.32 mm, 5 μm) using a linear gradient from 10 to 60% of ACN in 0.1% formic acid, over 60 min, at flow rate of 5 μL/min. Spectra were acquired in the range of m/z 200–2000. Data were elaborated using the BioWorks 3.1 software provided by the manufacturer.

ProFound software was used to identify spots from NCBI nonredundant database by PMF experiments [24]. Candidates with ProFound Est'd Z scores >2 were further evaluated by the comparison with M_r and p*I* experimental values obtained from 2-DE. SEQUEST software was used to identify proteins with data deriving from μLC-ESI-IT-MS/MS experiments [25]. Candidates from NCBI nonredundant database with more than three identified CID spectra of peptides belonging to the same protein and SEQUEST X_{corr} values >2.5 were further evaluated by the comparison with experimental M_r values obtained from 2-DE. Assignment of peptide signals to specific protein modifications was achieved by GPMAW 4.23 software (Lighthouse Data, Odense, Denmark), which generated a mass database output based on protein sequence, protease specificity and dynamic modification of cysteine residues.

2.9 Immunochemical analysis

A Western blot analysis was performed on serum samples collected different times after the hormone treatment. Briefly, SDS-PAGE of plasma proteins was performed according to Laemmli [26]. Thirty micrograms of serum proteins were fractionated on a 10–20% acrylamide gradient gel, under reducing condition and then electrotransferred onto NC (Hybond C extra, Amersham, UK). Transferred proteins were treated with 4% v/v milk in PBS for 1 h at 4°C, incubated with a rabbit anti-apolipoprotein A1 (ApoA1) polyclonal antibody (Biogenesis, UK), overnight at room temperature, or with a nonimmune rabbit serum, as negative control [27]. This antibody was generated against human ApoA1, but it crossreacted with ApoA1 from other species, including *Bos taurus*, as reported from manufacturer. Its reactivity was evaluated on human plasma and on purified human ApoA1 (Sigma) (data not shown). A buffer containing 10 mM Tris-HCl pH 8, 150 mM NaCl, 0.005% w/v Tween-20 was used for washes, and as solvent for peroxidase-labelled secondary antibody reaction. Peroxidase was revealed by enhanced fluorescence (ECL, Amersham) detected by autoradiography (Hyperfilm, Amersham).

3 Results and discussion

3.1 Kinetic profiles of boldenone, boldione and related metabolites after drug administration

Calves were treated with β-Bol and ADD, as described in the experimental section. Aliquots of plasma and urine samples were taken at different times and analysed for steroids occurrence. A sensitive LC-APCI-Q-MS/MS procedure was developed detecting β-Bol, α-Bol and ADD in plasma samples; similarly, excretion profile of these exogenous hormones was assayed as previously reported [8]. Figure 1 shows representative multiple reaction monitoring LC-APCI-Q-MS/MS profiles of calf plasma spiked with α-Bol, β-Bol, ADD and β-T-d_2 (5 ng/mL). A good separation of β-Bol (t_r = 7.6 min), α-Bol (t_r = 9.8 min), ADD (t_r = 9.7 min) and

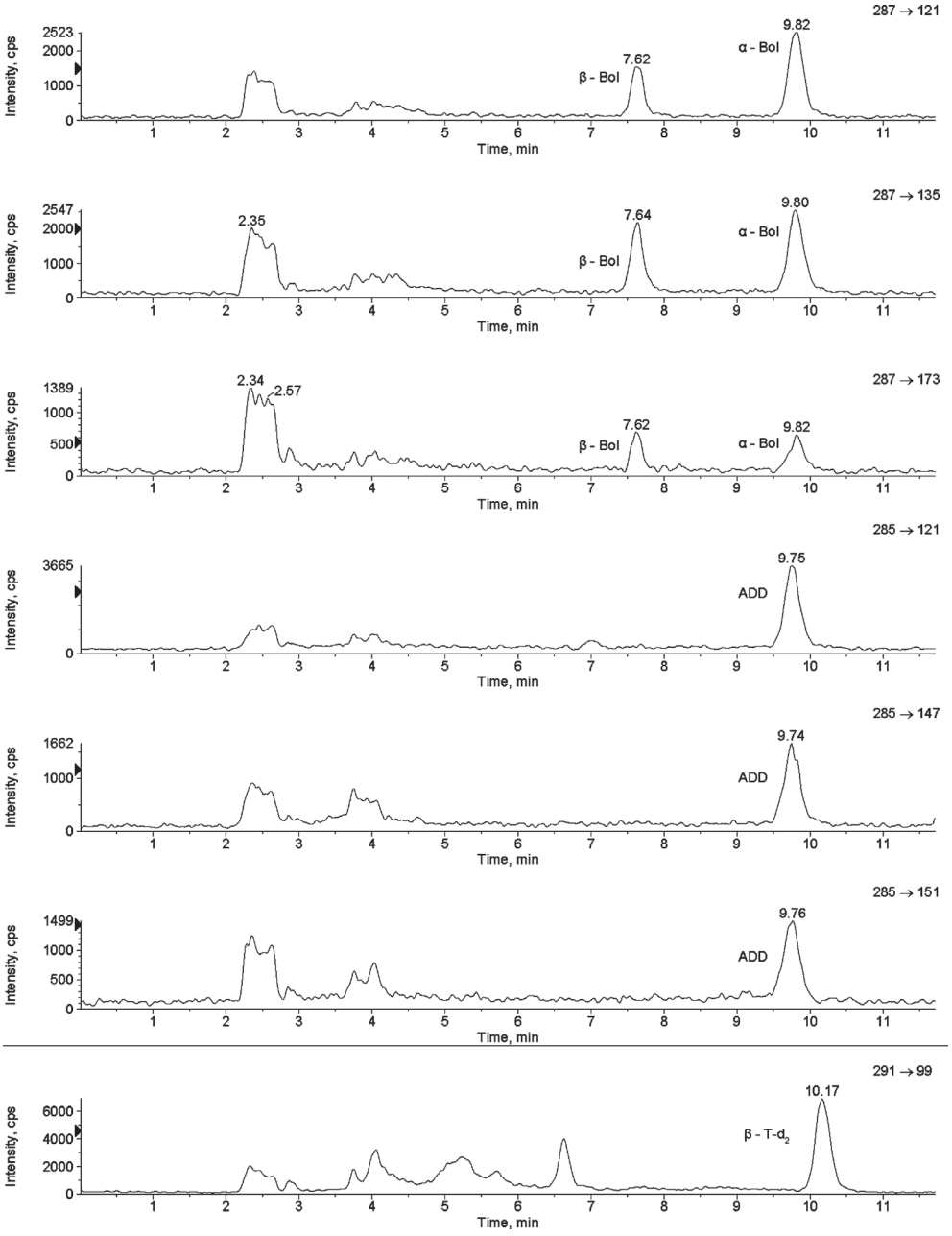

Figure 1. LC-APCI-Q-MS/MS analysis of bovine biological fluid samples after boldenone/boldione administration. Representative multiple reaction monitoring LC-APCI-Q-MS/MS profiles of calves plasma spiked with α-Bol, β-Bol, ADD and β-T-d_2 (5 ng/mL). Steroids detected as well as the fragmentation transitions used for steroid quantization are reported in each profile.

β-T-d_2 (t_r = 10.1 min) was observed. Specificity of the multiple reactions monitoring LC-MS/MS method was also demonstrated by the preparation and analysis of 20 blank plasma samples with and without internal standard (data not shown). No interference was observed around the hormone retention time in plasma samples. According to 2002/657/EC Commission Decision [1], detection capability (CCβ) and decision limit (CCα) values were assayed. In calf urine, a CCα value of 0.2, 0.2 and 0.3 ng/mL was determined for β-Bol, α-Bol and ADD, respectively; a CCβ value of 0.4, 0.4 and 0.5 ng/mL was similarly determined. In calf plasma, a CCα value of 0.4, 0.4 and 0.3 ng/mL was determined for β-Bol, α-Bol and ADD, respectively; a CCβ value of 0.5, 0.5 and 0.4 ng/mL was similarly determined.

A careful analysis of all chromatographic profiles demonstrated that ADD was never found either in urine or in plasma samples, differently from what reported for calves treated intramuscularly with high steroids dosages [7]. This result was interpreted on the basis of the different administration procedure and dosage of drugs used in our case. α-Bol and β-Bol concentration determined in plasma and urine samples are reported in Tables 1 and 2, respectively. In plasma, the latter compounds were detected at their highest levels in first sampling (15 min after administration). Their concentration decreased with a similar rate and both molecules were undetectable within 2 h after administration (Table 2). In urine, both compounds were present in samples collected 3 h after administration; in particular, α-Bol was the main metabolite excreted. β-Bol level decreased more slowly than that of α-Bol; however, after 9 h its concentration was close to the CCβ value, whereas α-Bol was detectable up to 24 h after administration (Table 1). The amount of α-Bol in blood and urine 15 min and 3 h after treatment, respectively, confirmed that α-Bol is the main *in vivo* metabolite [13]. Thus, it is tempting to speculate that β-Bol may undergo an oxidation by 17β-hydroxysteroid oxidoreductase leading to ADD, similarly to what observed for testosterone and nortestostreone [28]. Then, ADD is reduced by the very active 17α-hydroxysteroid oxidoreductase to yield α-Bol. The latter product is excreted *via* urine as glucoronide and/or sulphate derivatives.

Table 1. Mean concentration of α-boldenone, β-boldenone and ADD in plasma samples taken from five calves before and after treatment with β-boldenone and ADD

Sampling time (h)	α-Boldenone (ng/mL)	β-Boldenone (ng/mL)	ADD (ng/mL)
0	n.d.	n.d.	n.d.
0.25	0.94 ± 0.39	0.71 ± 0.16	n.d.
0.5	0.40 ± 0.21	0.62 ± 0.13	n.d.
1	0.57 ± 0.55	0.71 ± 0.50	n.d.
2	0.25 ± 0.01	0.27 ± 0.06	n.d.
4	n.d.	n.d.	n.d.
6	n.d.	n.d.	n.d.

n.d., not detected.

Table 2. Mean concentration of α-/β-boldenone conjugates and ADD in urine samples taken from five calves before and after treatment with β-boldenone and ADD

Sampling time (h)	α-Boldenone (ng/mL)	β-Boldenone (ng/mL)	ADD (ng/mL)
0	n.d.	n.d.	n.d.
3	349.01 ± 176.22	4.15 ± 0.78	n.d.
9	5.10 ± 0.01	0.22 ± 0.01	n.d.
24	5.37 ± 5.23	0.25 ± 0.06	n.d.
36	n.d.	n.d.	n.d.

n.d., not detected.

3.2 Proteomic analysis of plasma samples after boldenone/boldione administration

To set up a new analytical approach aimed extending time intervals suitable for detection of markers of exogenous hormones treatment in veal calves, a comparative proteomic investigation on plasma samples already assayed for β-Bol, α-Bol and ADD (see above) was undertaken. This analysis was based on the assumption that hormone administration may induce a direct or indirect effect on synthesis, suppression or postsynthetic modification of proteins present in plasma. Thus, the comparison of the proteomic profiles of plasma specimens before and after androgenic steroids treatment, should allow identification of specific proteins associated to illicit treatment of animals. These polypeptide species may be selected as candidate biomarkers to be screened during routine veterinary controls [11].

Thus, plasma aliquots were analysed by 2-DE, as described in the experimental section. Figure 2A shows a representative proteomic profile of the plasma samples collected; protein detection was achieved by Colloidal Blue Coomassie staining, which allowed visualization of different hundreds of proteins. Best resolution was obtained by loading samples containing 400 μg of plasma proteins. Attempts to increase the amount of loaded material resulted in gels poorly resolved, probably due to the high proportion of albumin present. No significant differences were observed between gels of samples from untreated animals in the cropped region below 100 kDa.

To identify specific markers associated to illicit treatment, proteomic maps were obtained also for plasma samples collected from calves at different times after drug treatment. Resulting gels were compared to that obtained from samples collected before the hormone administration through a computer assisted analysis, which allowed visualization of a single protein spot, common to all animals, specific for treated veal calves (Fig. 2). This protein increased its expression in a time dependent manner; in fact, the spot emerged and acquired its identity, differentiating itself from an additional closely-migrating protein. The former compo-

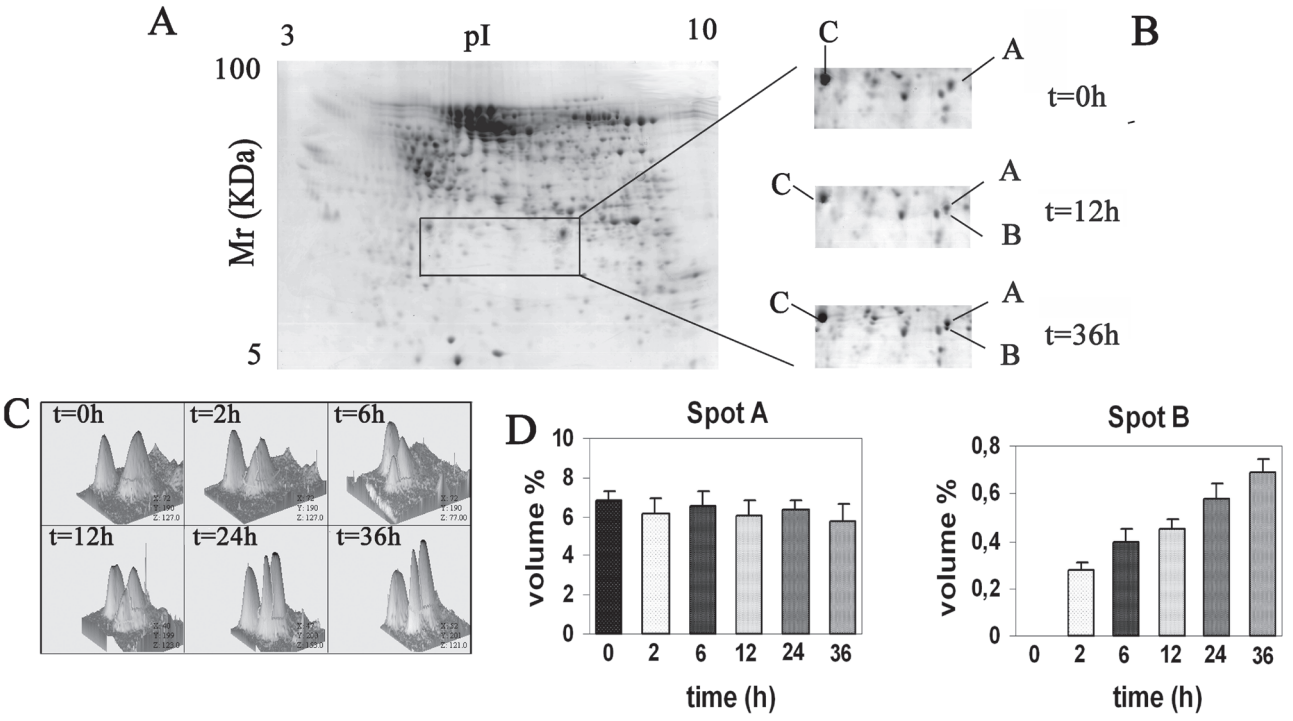

Figure 2. Proteomic analysis of bovine plasma samples after boldenone/boldione administration. A representative 2-DE proteome map of a bovine plasma sample is reported in panel A. Cropping represent the spot positions of truncated ApoA1 forms (spots A and B) and intact ApoA1 (spot C) in plasma samples taken before and after drug treatment (panel B). Sampling times are indicated. Spot B was proposed as candidate biomarker of illicit animal treatment. 3-D representation of the area of the gels where spots A and B were quantitatively evaluated (panel C). Peak baselines are showed; sampling times are also indicated in each subpanel. Quantitative profile of spots A and B with a statistical significance of $p<0.05$ (panel D). Quantification was made within individual gel cropped region and was calculated as normalized relative vol% of each spot. Data presented as histograms are means ± SD of three independent experiments performed on each of the five animals investigated.

nent migrated with p*I* 6.3 and M_r 22.0 kDa (spot B); as expected, the latter species showed close electrophoretic coordinates (p*I* 6.3 and M_r 22.7 kDa) (spot A). Statistical analysis of the spot intensity for these protein species, expressed as percentage volume of the total volume of matched spots in the surrounding region, confirmed that the emerging protein increased its volume in a time dependent manner, while the expression level of the closely-migrating protein did not change after the treatment (Fig. 2). Variation coefficients (expressed as vol%) were 0.10 ± 0.01 and 0.29 ± 0.02 for the spots A and B, respectively. A bar graph illustrating (mean ± SD) the time-related changes corresponding to the ApoA1 truncated forms, pertaining to all treated animals with respect to pretreatment values, is shown in Fig. 2. Both proteins were excised from the gel, digested with trypsin and analysed by MALDI-TOF-MS. PMF analysis and nonredundant sequence database searching allowed unambiguous identification of both spots as truncated forms of bovine ApoA1. The nature of the mass signals occurring in the spectra excluded the presence of other protein components in each analysed spot. Taking advantage of previous data published on bovine serum proteome database [22, 29], the spot corresponding to intact ApoA1 (pH 5.2 and M_r 26.0 kDa) (spot C) was similarly picked, digested and further confirmed for identification by PMF MALDI-TOF-MS experiments.

To characterize structural differences among the different ApoA1 forms identified in the proteomic maps of plasma from animals treated with exogenous hormones, the three spots associated to ApoA1 were also subjected to extensive mass mapping experiments. μLC-ESI-IT-MS/MS experiments were used to this purpose. The sequence coverage of entire ApoA1 lipoprotein and its truncated forms is shown in Fig. 3. These experiments demonstrated that a progressive removal of N-terminal amino acids characterized the truncated forms of bovine ApoA1 observed, with respect to non-proteolyzed ApoA1.

3.3 Immunoblotting analysis of plasma samples after boldenone/boldione administration

Despite the limited number of inspected animals, the proteomic experiments reported above were suggestive for upregulation of a specific truncated form of ApoA1 in bovine plasma following boldenone/boldione treatment. Accordingly, specific analysis of ApoA1 expression in plasma speci-

>spot A. Truncated APOA1_BOVINE. Mr 22.7 kDa, pI 6.3
DDPQSSWDRVKDFATVYVEAIKDSGRDYVAQFEASALGKQLNLKLLDNWDTLASTLSKVREQLGPVT
EFWDNLEKETASLRQEMHKDLEEVKQKVQPYLDEFQKKWHEEVEIYRQKVAPLGEEFREGARQKVQE
QDKLSPLAQELRDRARAHVETLRQQLAPYSDDLRQRLTARLEALKEGGGSLAEYHAKASEQLKALGE
AKPVLEDLRQGLLPVLESLKVSILAAIDEASKKLNAQ

>spot B. Truncated APOA1_BOVINE. Mr 22.0 kDa, pI 6.3
DDPQSSWDRVKDFATVYVEAIKDSGRDYVAQFEASALGKQLNLKLLDNWDTLASTLSKVREQLGPVT
EFWDNLEKETASLRQEMHKDLEEVKQKVQPYLDEFQKKWHEEVEIYRQKVAPLGEEFREGARQKVQE
QDKLSPLAQELRDRARAHVETLRQQLAPYSDDLRQRLTARLEALKEGGGSLAEYHAKASEQLKALGE
AKPVLEDLRQGLLPVLESLKVSILAAIDEASKKLNAQ

>spot C. Intact APOA1_BOVINE. Mr 26.0 kDa, pI 5.2
DDPQSSWDRVKDFATVYVEAIKDSGRDYVAQFEASALGKQLNLKLLDNWDTLASTLSKVREQLGPVT
EFWDNLEKETASLRQEMHKDLEEVKQKVQPYLDEFQKKWHEEVEIYRQKVAPLGEEFREGARQKVQE
QDKLSPLAQELRDRARAHVETLRQQLAPYSDDLRQRLTARLEALKEGGGSLAEYHAKASEQLKALGE
AKPVLEDLRQGLLPVLESLKVSILAAIDEASKKLNAQ

Figure 3. Sequence coverage of the three ApoA1 forms reported in this work. Peptides detected by MS analysis are underlined.

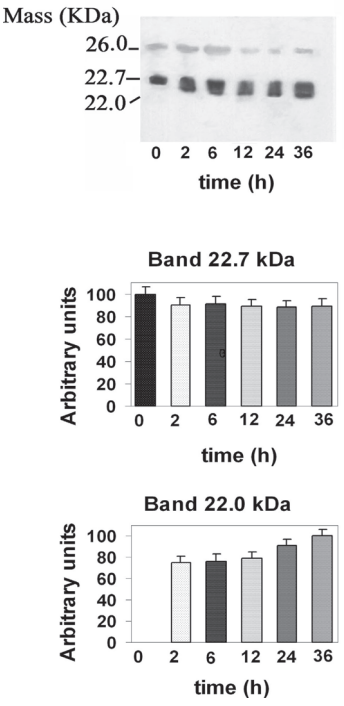

Figure 4. Western blotting analysis of bovine plasma samples following treatment with boldenone/boldione. Detection was obtained using a polyclonal anti-ApoA1 antibody. Plasma was sampled before treatment (0 h) and 2, 6, 12, 24, and 36 h after androgenic steroids administration. Protein level normalization was evaluated by a Red Ponceau staining of the NC filter after electrophoretic run and transfer (data not shown). Data presented as histograms are means ± SD of three independent experiments performed on each of the five animals investigated.

mens by immunoblotting techniques may have provided a simplified approach to reveal treatment of veal calves with androgenic steroid hormones.

This hypothesis found a positive confirm by Western blotting experiments using a polyclonal antibody anti-ApoA1, following SDS-PAGE separation of plasma samples. Immunoblotting analysis demonstrated that two forms of ApoA1 were detectable in plasma taken from animals not subjected to treatment with exogenous hormones (Fig. 4, upper panel). In contrast, an additional band, migrating with a lower molecular mass, became detectable after boldenone/boldione treatment. This molecular species became more intensively stained as a function of the time elapsed after drug administration (Fig. 4, upper panel). A densitometric evaluation of the time-related changes in ApoA1 truncated form bands intensity for all treated animals with respect to pretreatment is reported as a bar-graph (mean ± SD) (Fig. 4, middle and lower panels). These experiments confirmed the expression of an ApoA1 truncated form as result of androgenic steroid administration, similarly to what observed by proteomic analysis, and suggested that an immunochemical approach could represent a future simplified method to continue this research by large-scale screening and dose-dependent tests.

3.4 Apolipoprotein A1

The experiments reported above prompted us to speculate on the possible role of ApoA1 proteolytic events after exogenous androgenic steroids assumption. ApoA1 is a major constituent of high density lipoproteins (HDL) [30], which is associated with reversed cholesterol transport, lipid/cholesterol binding, lecithin/cholesterol acyltransferase (LCAT) activation and specific receptors binding. A series of biochemical studies have been reported on human ApoA1, while poor data are available on the bovine counterpart. Human ApoA1 is 26 kDa protein presenting an N-terminal globular domain made of 43 amino acids and a 200 residues-long C-terminal portion responsible for lipid binding [31]. Recently, the crystal structure of lipid-free ApoA1 and a lipid-free truncated form of ApoA1, missing the N-terminal region 1–43, was published [32–34]; their structural comparison and recent biochemical studies on both protein forms allowed the formulation of structure–function relationships. On this basis, few models of ApoA1 bound to discoidal and spherical HDL were reported as well as dynamic simulations of protein lipid complexes [35]. These investigations suggested that protein N-terminus is an important structural element playing a dominant role in maintaining an apoA1 lipid-free conformational state [34]. Its removal induces a conformational variation of whole protein fold, differently positioning the C-terminal domain responsible for lipid binding [36–39]. This structural rearrangement may significantly affect protein interaction with lipids as well as the regulation of LCAT catalytic activity [40, 41].

Different proteases have been proved affecting HDL particles and, in particular, ApoA1 integrity by removing polypeptide fragments from protein N- and C-terminus. In

the case of chymase and other macrophage metalloproteases, ApoA1 proteolysis yielded functionally compromised HDL particles with a reduced ability to promote cellular cholesterol efflux [42–45]. Apart from elucidating complex structure–function relationships between HDL and ApoA1, our analysis led to the identification of a specific truncated form of ApoA1 in bovine plasma, formed as a consequence of androgenic hormone administration. Its appearance may be tentatively explained through different hypotheses: (i) occurrence of alternative splicing events on ApoA1 gene, similarly to what observed for a truncated ApoA1 like protein in human placental tissue [46]; (ii) a steroid-induced activation of various proteases in plasma [47]; (iii) a steroid-dependent conformational change of ApoA1 tertiary structure, leading to an increased susceptibility of the protein to post-translational proteolytic processing. Several papers reported that human and chum salmon ApoA1 [48, 49] become more susceptible to limited proteolysis in some conditions. Although the latter two hypotheses could tentatively explain the relationship between boldenone/boldione administration and expression of the ApoA1 truncated form in a very short time interval (2 h), none physiological mechanism can be proposed at present to explain the functional role associated to this truncated form of ApoA1. In our opinion, the latter hypothesis seems more probable and related to the observed sudden rise of steroids in plasma and to the effect of other steroids on cholesterol metabolism and HDL composition [50]. In fact, similarly to what observed for cholesterol during atherosclerosis, we may hypothesize that proteolytic events affecting protein N-terminus may control ApoA1 binding to steroids, limiting cellular efflux of anabolic drugs in plasma. A more detailed investigation on the structural and biochemical properties of this truncated bovine ApoA1 species, in comparison with intact ApoA1, are needed to fully elucidate this physiological process.

4 Concluding remarks

In this study, we reported an integrated analytical approach to detect treatment of veal calves with exogenous androgenic steroids. In particular, a specific, sensitive and reliable LC-APCI-Q-MS/MS method for the simultaneous analysis of β-Bol, α-Bol and ADD in bovine plasma was developed. Our *in vivo* experiments showed that both ADD and β-Bol were promptly absorbed when administered to fasted veal calves. Impossibility to detect ADD in plasma specimens supported its double identity as precursor and metabolite of β-Bol, confirming previous *in vitro* investigations on calf liver microsomes [3]. In contrast, boldenone epimers were detected in plasma and urine up to 2 and 24 h after drug administration, respectively. According to their CCβ, CCα values and measured levels, urine was proved as the best biological fluid to be sampled for direct illicit treatment detection. However, specimens for a positive analysis have to be taken within 24 h from drug administration. On the basis of their limited time of permanence in biological fluids, exogenous steroids treatment of veal calves was also investigated by detection of useful protein biomarkers in animal plasma. A proteomic approach was used to this purpose, revealing the occurrence of a truncated ApoA1 form, specific for drug treatment, clearly visible 2 h after boldenone/boldione administration and detectable even 36 h later. This molecular species has been here proposed as candidate biomarker of androgenic steroids treatment, easily detectable also by immunoblotting SDS-PAGE techniques, to be confirmed in massive screening analysis during veterinary controls. Taken all together, our data emphasize the importance of an integrated use of different analytical procedures to confront complex analytical challenges.

It has to be underlined how the combined approach reported in this work well illustrated the limits of each technique. LC-APCI-Q-MS/MS analysis allowed a sensitive detection of exogenous steroids in biological fluids, which have to be sampled up to 24 h after treatment. Extended times of sampling are compatible with the proteomic approach, whose sensitivity is not comparable to the pharmacological assay. Nevertheless, this study further supports preliminary reports on the potential impact of proteomic approaches for steroids misuse detection in breeding animals [11]. A recent study gave the guidelines for a correct application of biomarkers in the field of toxicology, pharmacology and drug development [51]. According to this report, the truncated form of ApoA1, here associated to hormonal administration, may be classified as 'biomarker of effect'. In fact, its expression can be considered as an effect arising from altered animal biochemical/physiological events. Future studies on a wider number of animals and based on prolonged protocols for animal treatment will validate the candidate biomarker proposed in this work; these investigations will also clarify the role of apoA1 and its truncated forms in the complex mechanisms of lipid transport mediated by HDL particles.

This work was partially supported by grants from the Italian National Research Council (AG-PO04-ISPAAM-C1), MIUR (FIRB2001 n. RBAU01PRLA), Progetto Strategico SARA – Fasc.:4AF/F2, Servizi Veterinari Regione Veneto and Fondazione Carige.

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