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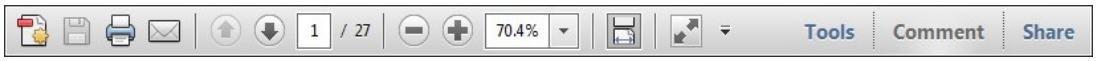

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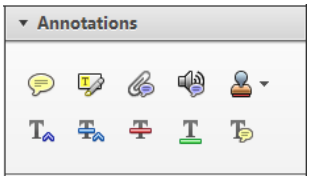

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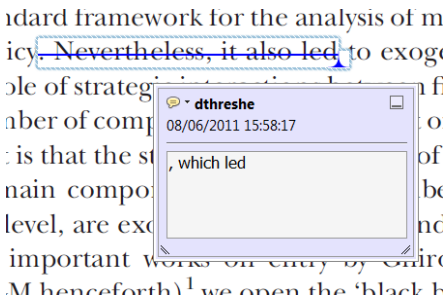

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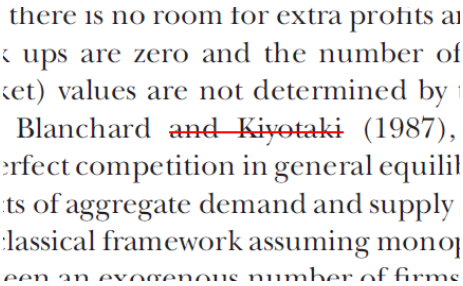

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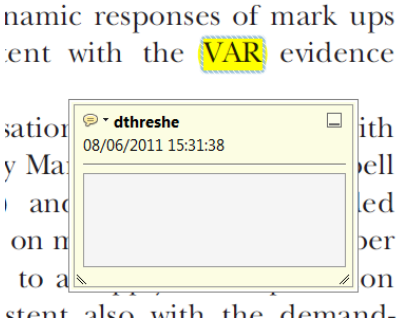

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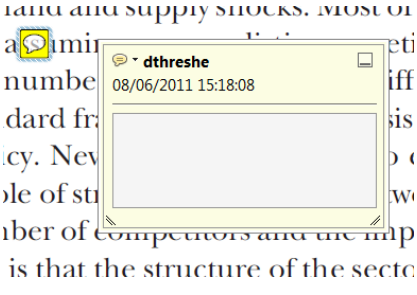

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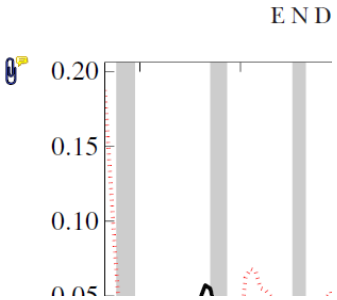

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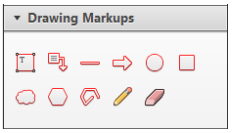

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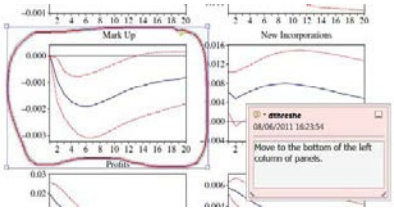

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ORIGINAL ARTICLE

Study on cortisol, cortisone and prednisolone presence in urine of Chianina cattle breed

L. Chiesa[1], S. Pavone[2], E. Pasquale[1], R. Pavlovic[1], S. Panseri[1], A. Valiani[2], F.Arioli[3] and E. Manuali[2]

1 Department of Veterinary Science and Public Health, Laboratory of Animal Food Inspection, University of Milan, Milan, Italy
2 Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy, and
3 Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy

Summary

The Chianina, one of the oldest and most important cattle breeds of Italy, is now reared all over the world. The Chianina has been known and appreciated since ancient times because, from a nutritional point of view, its meat has no proper rivals. To date, studies have been performed to evaluate the genetic profile of the breed, but knowledge about the chemical profile is generally lacking. Due to the increased interest from farmers regarding breeding of the Chianina, this study proposes a preliminary evaluation of main endogenous urinary corticosteroids (cortisol and cortisone) and most commonly used synthetic one (dexamethasone). Moreover, after recent findings regarding the presence of endogenous prednisolone in the urine of more popular breeds, particular attention was given to analysis of the presence of prednisolone and prednisone, as well. For this aim, the urine samples of 12 young cows and 30 young bulls was collected at the farms and analysed using a fit-for-purpose LC-MS/MS method. The preliminary results of this study show that prednisolone was found only in Chianina females (3 out of 12). Cortisol and cortisone were found at concentrations that showed a high inter-individual variability, and that were higher in female urine compared to that of males.

Keywords Chianina breed, cortisol, prednisolone, bovine urine, food safety

Correspondence R. Pavlovic, Laboratory of Animal Food Inspection, Department of Veterinary Sciences and Public Health, University of Milan, Via Celoria 10 20133 Milan, Italy. Tel: +39 0250317931/30; Fax: xxxxx; E-mail: radmilachem@yahoo.com

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Introduction

The Chianina is an ancient Italian breed of cattle, which originated from Val di Chiana in central Italy, a valley between the regions of Tuscany and Umbria; nowadays, it is present in different countries of the world as a cross-breed. The Chianina, once used as a working breed, is exclusively bred for meat production in modern animal husbandry. The milk, however, is only sufficient for suckling. The most striking features of the breed are the enormous resistance to severe environmental conditions, and large-scale and well-defined muscles that give excellent meat with a commercially superior aspect and good nutritional characteristics, as a result of multiple genetic combined factors (Associazione Nazionale Allevatori Bovini Italiani da Carne, 2014).

At present, the scientific literature regarding the Chianina has been concentrated on the assessment and improvement of the quality of the meat and studies concerning the genetic variability of the breed (Forabosco et al., 2004). Several groups have evaluated the duration of the Chianina productive life, which affects the costs for farmers, thus increasing profits. An average productive life for the Italian Chianina of approximately 5 years has been reported; however, some cows have reached a productive life of more than 15 years (Forabosco et al., 2004, 2010).

Timperio et al. (2009) described the genetic and phenotypic profile of Chianina to estimate the phylogenetic distance of Chianina from other breeds. As the musculature is particularly developed with well-defined structures, it is reasonable to speculate that the steroid metabolic pathways might be different from other breeds, e.g. depending on a superior feed efficiency associated with increased levels of glucocorticoids (Montanholi et al., 2009), rather than on the quantity of glucocorticoid receptors (Sauerwein et al., 1991). The studies on corticosteroid profiles in bovines have principally been performed in different common breeds of cattle, such as the Holstein-Friesian, Limousine and Charolaise (Doornenbal,

1977; Ferranti et al., 2011; Pegolo et al., 2012; Bertocchi et al., 2013; Pavlovic et al., 2013). However, there is a lack of knowledge about the steroid profile in particular breeds of cattle, including the ancient Italian Chianina.

The corticosteroids, peculiarly glucocorticoids, are a class of steroid hormones that are synthesized and secreted by the *zona fasciculata* of the adrenal cortex. Cortisol (CL) is the most important glucocorticoid, exhibiting important immunological, cardiovascular, homeostatic and metabolic functions, and is converted into the inactive metabolite, cortisone (CN), mainly in the liver (Osamu, 2001; Shimada et al., 2001). Besides this, CL, measured in different matrices and in various species, is frequently considered a stress-sensitive marker (Carlsson et al., 2007; Mongillo et al., 2014; Ott et al., 2014). In the European Union (UE), corticosteroid use in cattle is permitted only for therapeutic aims. However, their illegal use as growth promoters, either alone or in association with anabolic steroids, to improve the quality and quantity of meat in young bull and beef, cannot be excluded. Uncontrolled administration of these substances could be a risk for meat consumers due to the intense pharmacological activities of these compounds (Courtheyn et al., 2002).

Therefore, the use of these compounds is strictly regulated by European legislation: the Commission Regulation (EEC) No 37/2010 establishes maximum residue limits (MRLs) for betamethasone, dexamethasone (DX), methylprednisolone and prednisolone (PL) in milk and tissues intended for human consumption.

Corticosteroids are allocated in the group B2f (other pharmacologically active substances) by the EU Council Directive 96/23/EC as amended. Monitoring of corticosteroids in livestock, as with other pharmacologically substances, is usually carried out both in the farm and at the slaughterhouse, as stated e.g. by the Italian National Residue Control Plan (Italian Ministry of Health; Dept. of Food Safety and Veterinary Public Health, 2014). On farms, the controls should be performed through the analysis of urine, while the matrices sampled at the slaughterhouse are urine and liver, a tissue for which MRLs are set.

Over the past 5 years, a strong debate has arisen regarding the origin of PL. As a matter of fact, a significant increase of PL positive cases has been observed in cattle (European Commission Staff Working Document, 2010), and its endogenous presence has been shown in horses (Fidani et al., 2012); also, it was recently reported in the urine of pigs (mainly sampled at the slaughterhouse) by different Member States of the European Union (De Rijke et al., 2014) .

On the basis of these results, the unique exogenous origin of PL in urine has been questioned. PL could naturally occur in the urine as a result of microbial contamination from faecal or environmental origin due to poor collection and storage operations (Arioli et al., 2010) or due to stressful conditions to which bovines are exposed during transport and slaughtering (Pompa et al., 2011). Those authors indubitably demonstrated the favourable influence of stress to the presence of urine PL by an *in vivo* experiment where the samples were collected taking into consideration all the safety precautions to have the stress as single variable. Particularly, the production of PL was examined after its pharmacological induction given by the treatment with a synthetic analogue of adrenocorticotropic hormone, and taking into account the stress related to transport and slaughter procedures. All urine samples collected after the treatment and at the slaughter presented PL. The presence of PL was also evaluated in the adrenal glands, responsible for the production of the corticosteroids, but contrary with expectations, the glands seem to have a secondary role in the production of PL (Bertocchi et al., 2013). In the same article, author brought a hypothesis regarding the PL production in the gut by the intestinal microflora activities. Nevertheless, a satisfied explanation on the metabolic pathway implicated in the mechanism of synthesis of PL has not yet been provided (Nebbia et al., 2014).

Highly significant data arises from the study recently published by De Rijke et al. (2014). The authors demonstrated that CL could be *in vitro* transformed into PL by action of S9 fraction of liver enzymes. This fraction of liver homogenate contains: the microsomal compartment with enzymes that are implied in phase I metabolism (i.e. cytochrome P450 isoforms) and the cytosolic portion that contains transferases, enzymes involved in phase II metabolism. It remains to be established which of those enzymes is responsible for introduction of double bond into C_1-C_2 position of CL A ring.

Furthermore, treatment with synthetic glucocorticoids could interfere with the production and release of endogenous ones (Mazzarino et al., 2006). On the other hand, limited information is available regarding the endogenous glucocorticoid profile in bovine urine samples, and only a few studies have been undertaken to analyse natural corticosteroids in the urine of untreated bovines or in those treated with synthetic corticosteroids (Savu et al., 1996; Capolongo et al., 2007; Ferranti et al.,

2011; Vincenti et al., 2012; Leporati et al., 2013; Nebbia et al., 2014). The inhibitory effects of PL and dexamethasone could eventually be evaluated by analysis of the ratio of CL to CN. Therefore, the evaluation of natural corticosteroids may be considered a tool for defining a condition regarding the abuse of therapeutic agents (Pavlovic et al., 2012).

Because the literature on the characterisation of corticosteroids in Chianina urine is not yet available, the main objective of our study was to obtain a preliminary evaluation of the physiological levels of CL and CN in the urine of Chianina young bulls and young cows. To this aim, a fit-for-purpose LC-MS/MS method was developed and applied. Moreover, PL was examined to check the possibility of its endogenous presence. Additionally, eventual illicit treatments either with prednisone (PN), the oxidised PL metabolite, or with DX (the most frequently used synthetic corticosteroid), could also be accessed by our LC-MS/MS method.

Materials and methods

Chemicals and reagents

CL, CN, PL, PN and DX were purchased from Sigma-Aldrich (St. Louis, MO, USA). The internal standard prednisolone-d6 was from CDN Isotopes (Pointe-Claire, Quebec, Canada). All other chemicals were from Fluka Chemie GmbH (Buchs, Switzerland). Ultrapure water was obtained through a Milli-Q system (Millipore, Molsheim, France). Standard stock solutions were prepared in methanol (1 mg/ml) and stored at −40 °C. Working solutions were prepared daily by diluting the stock solutions with methanol/water (50:50, v/v).

Sample preparation

The following extraction procedure was optimized: 4 mL of organic mixture (*tert*-butyl methyl ether and ethyl acetate, 4:1) was added to a 2 ml urine sample which had been previously spiked with prednisolone-d6 (PD6, final concentration 2 ng/ml) used as internal standard (IS). After shaking in a vertical rotary shaker for 20 min, the sample was centrifuged at 1300 *g* for 15 min. The upper organic layer was collected with a Pasteur pipette, transferred to a 10 ml glass tube and dried under vacuum in a centrifugal evaporator at a temperature of 30 °C. The residue was dissolved in 200 μl of the mobile phase (50% methanol and 50% formic acid in a 0.1% aqueous solution) and transferred to vials for HPLC–MS/MS analysis. The injection volume was 20 μl.

Urine collection

Forty-two healthy Chianina beef (12 young cows and 30 young bulls) aged between 11 and 23 months were reared in two selected farms in the Perugia province, and submitted to periodic unplanned checks for residues of corticosteroid molecules included in the National Residues Control Plans (Italian Ministry of Health; Dept. of Food Safety and Veterinary Public Health, 2014).

Regarding the housing, animals were kept outside pens with overhead shelter with deep litter. All the animals received the same feed, which was *ad libitum*. Feed ingredients were consisted of corn meal, field beans, middling of durum wheat, straw and hay ground.

Urine samples were collected at the farm into long-handled sterile container during the spontaneous micturition. The sampling was done in the morning between 8.00 and 11.00 AM.

A visual inspection was made to check the turbidity or the presence of raw materials. Only clean urine was sampled, frozen and taken to the laboratory for the storage at −40 °C, until extraction and analysis.

Instrumentation

LC MS/MS analysis was carried out by a Thermo Finnigan HPLC system (Thermo Fisher, San Josè, CA, USA), consisting in a Surveyor MS quaternary pump with a degasser, and a Surveyor AS autosampler, a column oven and a Rheodyne valve with 20 μl sample loop. The mass spectrometer system was a TSQ Quantum triple quadrupole (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray interface (ESI) set in the negative polarity ionisation mode. The analysis was performed in multiple reaction monitoring (MRM). Acquisition data were recorded and elaborated using XCALIBUR™ software from Thermo.

LC Conditions

Analytical separations were achieved using a Synergi Hydro RP reverse-phase HPLC column 150 × 2.0 mm, with an internal diameter of 4 μm, and a C18 guard column measuring 4 × 3.0 mm (Phenomenex, Torrance, CA, USA), at a column oven temperature of 30 °C. Binary gradient profiles were developed using a water solution of 0.1% formic acid (A) and methanol (B) at a flow rate of 250 μl/min. The chromatographic conditions regarding phase B were as follows: from 25 to 70% 0–20 min; achieving 95% in the 21st min, holding for 3 min, returning to

starting values in the 26th min, and finally equilibrating up to the 31st min.

MS/MS conditions

The LC instrument was coupled to a TSQ Quantum, triple quadrupole mass spectrometer with an electrospray ionisation source (ESI) set in the negative mode.

Acquisition parameters were optimized in the ion spray mode with direct continuous pump-syringe infusion of standard solutions of the analytes at the concentration of 1 μg/ml at a flow rate of the syringe of 10 μl/min and a pump flow rate of 100 μl/min in the ion source of the mass spectrometer.

The optimized parameters were the following: capillary voltage 3200V; ion transfer capillary temperature 340 °C; and sheath and auxiliary gas (nitrogen) were fixed at 30 and 10 (arbitrary units), respectively. The collision gas was argon at 1.5 mTorr and the peak resolution of 0.70 Da FWHM was used. The precursor ions, i.e. the formiate adducts of the studied compounds ($[M+HCOO]^-$), are shown in Table 1, together with the product ions, tube lens and collision energies.

Method validation

A standard stock solution of 1 mg/ml of corticosteroids was prepared in methanol. Standard spiking solutions at concentrations of 1 μg/ml were prepared by diluting the stock standard solution. The appropriate amount of standard spiking solution was added to 2 ml of urine samples. The validation calibration curves were constructed by adding scalar amounts of the spiking solutions to a urine sample (in triplicate for each point) to obtain concentrations of 0, 0.25, 0.5, 1.0, 2, 5 and 10 ng/ml, following the entire sample processing. The equations of calibration curves ($Y = m(\pm sm) \times X + b(\pm sb)$) were obtained by plotting the ratio of the peak area of the analyte/IS against the analyte concentration. The ratios of the peak area of the analyte/IS were corrected, for the presence of endogenous compounds (CL and CN) in the matrix, by subtracting the corresponding ratios of the blank. The presence of PL, PN and DX was not revealed in urine matrix used for validation purposes. The fitting of the linearity was verified by squared correlation coefficients (R^2). The limit of detection (LOD) and limit of quantification (LOQ) were calculated with the equations LOD = 10 × SDR/m and LOQ = 10 × SDR/m, respectively (Miller and Miller, 2000; FDA Guidance, 2001), where SDR is residual standard deviation of a Y-intercept.

Whenever the concentrations found in the real samples were above the highest point of validation calibration curve the construction of quantification (working) calibration curve is mandatory. Therefore, in the few case of CN and CL the levels were calculated from working calibration curve built specifically of 8 points (0–100 ng/ml) that covers the whole range of concentration.

Method precision (as within-day repeatability) expressed as a relative standard deviation (RSD) was determined by replicate analyses of blank urine samples ($n = 6$) fortified with 0.25, 1 and 10 ng/mL of each compound. The same data were used in evaluation of trueness. The spiked samples (0.25, 1 and 10 ng/ml) were also used for the determination of the intermediate method precision (inter-day repeatability), which was obtained from analysis over five consecutive days. Instrumental precision was evaluated by 10 injections of the standard solution at concentration of 10 ng/ml for each compound according to the optimal operative conditions. In this way, the repeatability of the instrumental system, expressed as relative standard deviations (RSD), was acquired.

The criteria for the identification of corticosteroids as a result of LC-MS/MS analysis were evaluated according to European Union guidelines (Commission Decision 2002/657/EC). The presence of the analytes being investigated was assessed by comparing the ratio of the chromatographic retention time of the analyte

Table 1 Precursor ions and specific diagnostic ions with Tube Lens values and CE (collision energies) of the targeted corticosteroids. Ions for quantification are in bold

Analyte	Precursor ion (m/z)	Product ions (m/z)	Tube lens	CE
Cortisol	407	282	74	37
		297	74	33
		331[a]	74	20
Cortisone	405	**301**	67	21
		329[a]	67	20
		359	67	12
Prednisolone	405	187	71	30
		280	71	35
		329[a]	71	19
Prednisone	403	**299**	71	20
		327[a]	71	19
		357	71	12
Dexamethasone	437	307	81	33
		361[a]	81	20
		391	81	14
Prednisolone-d6	411	284	77	37
		299	77	32
		333[a]	77	19

[a]Most abundant product ion.

to that of the internal standard; the relative retention time of the analyte should correspond to that of the calibration solution at a tolerance of ±2.5%. Three transitions were monitored for each analyte with a signal-to-noise ratio greater than 3. All ion ratios of compounds from real samples were within the recommended tolerances when compared with the standards. The quantifier ion was the one with the highest signal-to-noise value of the three diagnostic ions.

Statistical analysis

All calculations regarding the method validation were performed in EXCEL software (Microsoft Corp., USA) using the multiple linear regression analysis.

The Shapiro-Wilk test used to check the normality of results of two datasets (young cows' and young bulls' results). Based on the response of the normality test, non-parametric Mann–Whitney Rank Sum Test was used to check the differences between the median values of two datasets. The statistical analysis was performed using SIGMA STAT (Statistical Analysis System, version 2.03) statistical software package (Jandel Scientific GmbH, Herckrath, Germany). A p-value of <0.05 was defined as the level of statistical significance.

Results and discussion

Method validation

A fit-for-purpose LC–MS/MS method was developed to analyse Chianina urine samples for different corticosteroids. Urine sampling was strictly performed as recommended by the Italian National Residue Control Plan; particular care was taken to exclude any possible role played by faecal bacteria in the neo-formation of PL that could give false positive results, as previously reported (Arioli et al., 2010; Italian Ministry of Health; Dept. of Food Safety and Veterinary Public Health, 2014). The instrumental precision, expressed as the relative standard deviation (RSD), was in the range 2.8–7.2% for all of the analysed corticosteroids, which is considered as a very good repeatability of the instrumental system with stabile LC-MS/MS response. Good linearity was obtained for matrix calibration curves with correlation coefficients higher than 0.998 for all compounds in the observed concentration range. The within-day precision (also the trueness) was evaluated for three spiking levels (0.25, 1 and 10 ng/ml). In particular, the lowest spiked level gave the highest RSD values in range 14–19.3%, while other two were below 13.2%. The inter-day precision was between 13% and 20.3%. These precision values were lower than 22%, which was the value proposed by Thompson (2000).

All performance parameters for the corticosteroids analysed are summarized in Table 2. The LODs and LOQs were ranged from 0.07 to 0.20 ng/ml and from 0.23 to 0.67, respectively (Table 2), which compared with other studies resulted in comparable or better values (Ferranti et al., 2011; Pompa et al., 2011; Fidani et al., 2012; Vincenti et al., 2012; Leporati et al., 2013) Fig. 1.

Representative chromatograms and mass spectra of a spiked urine sample at the concentration of 10 ng/ml are reported in Fig. 2. The analyte peaks were completely resolved, which proves a satisfactory chromatographic separation. Furthermore, no interfering signals were present in the corresponding retention times of each analyte of interest. It has to be noted, however, that through LC-MS/MS analysis, it is possible to see ions at 187 and 280 m/z, which are considered to be characteristic of PL (Fidani et al., 2012).

Sample size evaluation

Before the evaluation of the presence of corticosteroids in urine samples from Chianina, a correct sample size was well-considered to obtain a statistically significant number of animals. In particular, the sample collection was made ensuring the detection of the predicted prevalence, i.e. the expected frequency of endogenous PL detection in urine according to

Table 2 Results of method validation for targeted corticosteroids

	Calibration curve (n = 3) $Y = b(\pm sb)X + m(sm)$	R^2	LOD (ng/ml)	LOQ (ng/ml)
CL	Y = 0.1494(0.0020)X + 0.1816(±0.0169)	0.9971	0.19	0.63
CN	Y = 0.249(±0.0036)X + 0.1688(±0.0169)	0.9967	0.20	0.67
PL	Y = 0.1099(±0,0009)X + 0.0338(±0.0032)	0.9988	0.09	0.29
PN	Y = 0,9367(±0.0047)X + 0,2890(±0.0219)	0.9996	0.07	0.23
DX	Y = 2.122X(±0.0.238)X + 0.5248(±0.1110)	0.9980	0.16	0.52

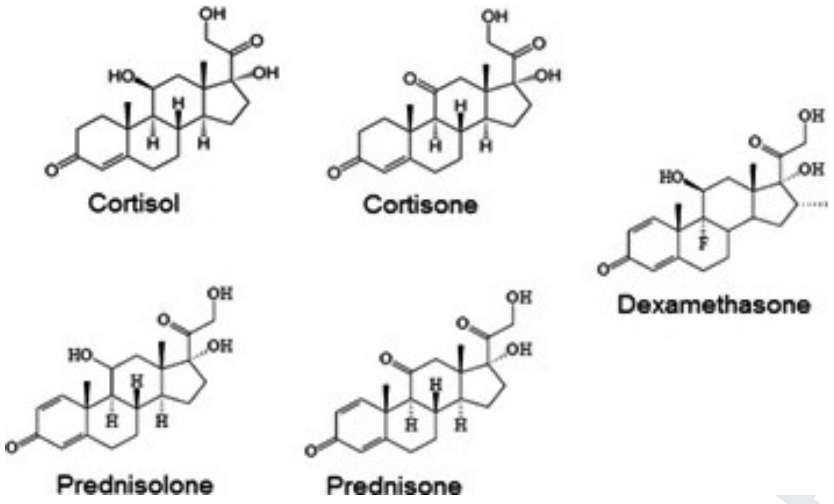

Fig. 1 Chemical structure of corticosteroids [20] analysed.

Bottarelli and Ostianello (2011), the sample size is calculated trough the following formula:

$$n = \frac{1.96^2 \times P \times (1 - P)}{(D)^2}$$

1.96 - the *Z* constant or deviation from the mean value accepted to get the desired confidence level. When the level of significance is 5% and the confidence level is 95%, the *Z* constant should be 1.96;

P - the expected or frequency of sample positive for the presence of PL;

D - the required precision.

Existing data on the frequency of PL detection suitable were mainly from urine sample of race horses (78.5%; Fidani et al., 2012) and of bovine taken from the slaughterhouse (cows at slaughter = 71% positive; Bertocchi et al., 2013). Less data on sample urine of bovine collected at the farm were available. A percentage of urine samples positive for PL at the farm of six was calculated by Bertocchi et al. (2013), while a 5% of urine bovine sampled at the farm was reported by Vincenti et al. (2012). The author, however, evidenced that all these samples had a concentration value of PL lower than reported detection limits. Ferranti et al. (2011) reported a frequency of PL detection for urine sampled collected at the farm of 7% and, recently, De Rijke et al. (2014) showed a frequency of detection of the corticosteroid of 40%, but in this work, the place of urine collection was not mentioned.

Based on the frequencies reported regarding the samples collected at the farm, it might be supposed a prevalence for PL detection in Chianina urine of 6% ($P = 0.06$); and a precision of $\pm 10\%$ ($D = 0.1$). Hence, applying the above-mentioned formula the required sample size would be 21 animals. Finally, sample size was extended to 42 considering the variability of the samples in term of gender of the animals, age and different farm origin.

Analysis of Chianina urine samples

Based on previous researches and our own experience, we studied only free steroids in Chianina urine samples. In particular, Antignac et al. (2002) revealed that enzymatic hydrolysis proved to be unnecessary for the cleavage of conjugated CL glucuronide and sulphate forms, as almost all CL in bovines is in its free form. To some extent, this disagrees with the results shown by Arioli et al. (2012), who found a significant increase in CL concentration after enzymatic deconjugation. However, with regard to PL, Arioli et al. (2012) showed that utilization of *beta-glucuronidase* is not indispensable, as this corticosteroid is present almost exclusively in bovine urine in its free form. In addition, the deconjugation process itself can negatively influence the accuracy of analyte measurements (Pozo et al., 2008). Taking all of this into account, we chose to perform our analysis on the free form of the corticosteroids of interest.

The evaluation of corticosteroid profile in Chianina urine was performed on 12 young cows and 32 young bulls. The data obtained in the current study are presented in Tables 3A and 3B. All of the samples analysed were below the LOD regarding PN and DX. PL was absent in all of the male urine samples analysed. With regard to females, PL was detected in 3 of the 12 urine samples at concentrations of 1.5, 0.6 and

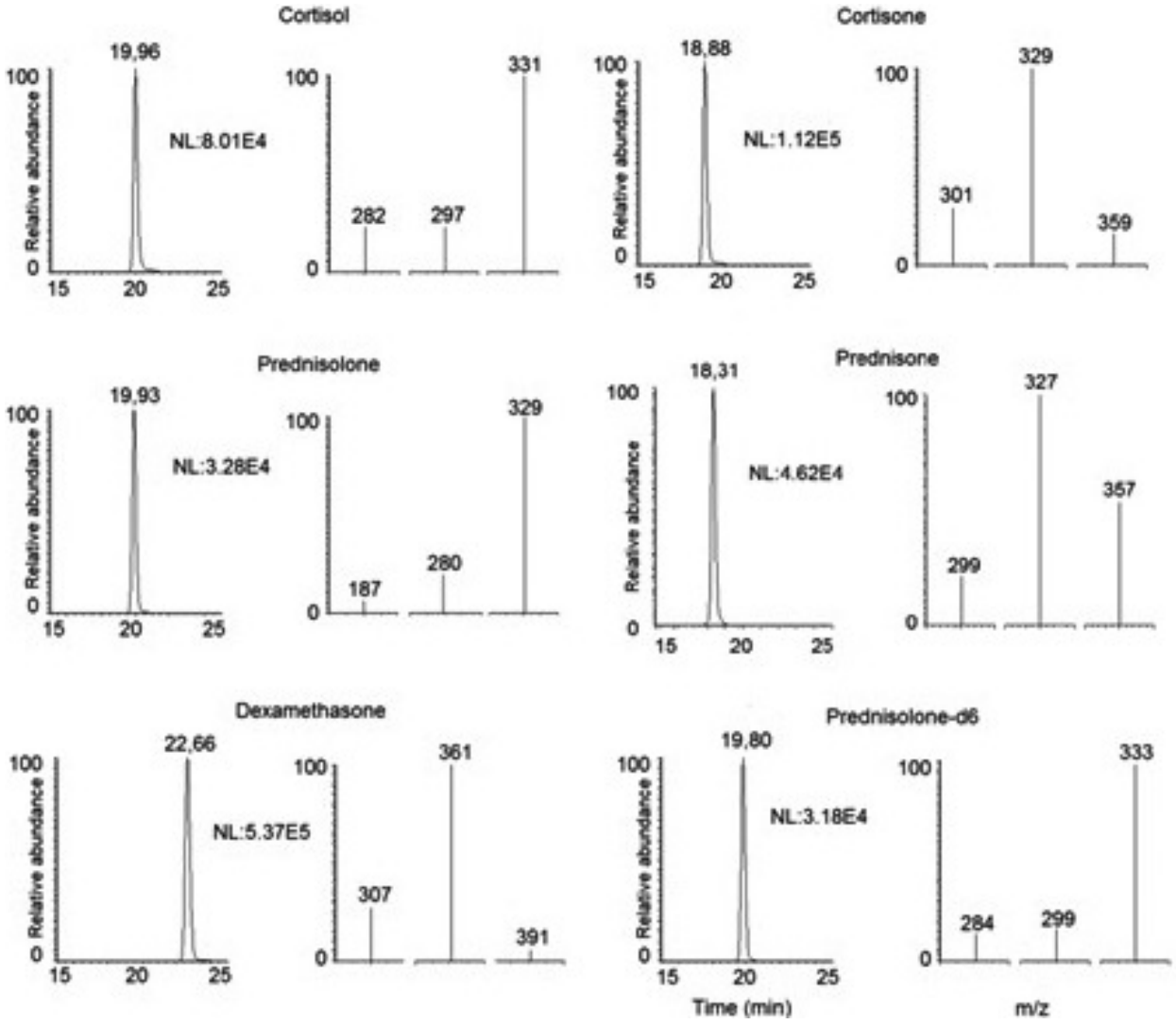

Fig. 2 Reconstructed LC–MS/MS chromatograms of a spiked urine sample with the respective ion spectra at the concentration of 10 ng/ml. 21

0.3 ng/ml. Figure 3 shows the reconstructed LC-MS/MS chromatograms and respective ion spectra of three corticosteroids detected in a urine sample from a Chianina young cow. The comparison between PL chromatograms and ion mass spectra reported in Figs 2 and 3 confirms the positivity for the presence of the analyte in the urine sample analysed (Fig. 3), which was demonstrated by the respect to the Commission Decision 2002/657/EC criteria, including retention time and the respect of ion ratio tolerances.

Our results indicated that PL was present only in urine samples from female bovines. The results obtained with PL positive female bovine urine samples could be explained by different hypotheses. First, the activity of the enzymes involved in the transformation of CL in PL could be higher in females than in males. Recently, Bertocchi et al. (2013) published a study on a large number of lactating bovines where a comparison was made of the results obtained following the analysis of urine collected at the farm and immediately after slaughter. The data obtained in urine samples confirmed that PL can be endogenously produced in dairy bovines. The second hypothesis regards a stress factor that induces the production of PL. Stress, even if minimal, could be caused by the sampling operation itself. Pompa et al. (2011) showed the neoformation of PL in the urine of stressed cows. Moreover, it has been confirmed that stress, caused by transport and slaughter, could induce increased levels of PL or CL in urine. In fact, those authors found PL sporadically in non-treated dairy bovines, but when the dairy bovines were pharmacologically stressed, PL

Table 3 Overall analytical results on corticosteroids in urine of Chianina young cows (A) and young bulls (B)

	CL	CN	PL	PN	DX
(A) Young cows (12 animals)					
Positive (%)	7 (58%)	6 (50%)	3 (25%)	0%	0%
Median (ng ml)	14.2	9.95	0.562	nd	nd
25^{th}–75^{th} percentile	6.1–35.9	3.9–21.3	0.3–1.5		
(B) Young bulls (30 animals)					
Positive (%)	25 (83%)	25 (83%)	0%	0%	0%
Median (ng ml)	5.4	5.3	nd	nd	Nd
25th–75th percentile	2.8–9.5*	2.7–8.6†			

Data are reported as median (ng ml) with corresponding 25th–75th percentile with number an percentage of positive findings in parenthesis.
nd = not detected.
*Not different from the corresponding corticosteroid in young cows urine (Mann-Whitney Test, p = 0.068).
†Not different from the corresponding corticosteroid in young cows urine (Mann-Whitney Test, p = 0.221).

was constantly detected until 7 h after the first treatment. Furthermore, the presence of endogenous PL in bovine urine seems to be strongly related to a state of stress of the animals during transport and at the slaughterhouse. In our study, the samples were collected at the farm under a minimum stress condition for the animals. However, to date, there has not been an objectively evaluated relationship between stress and PL status with gender of animal. It appears reasonable to assume that Chianina females are more sensitive than males to stress due to sampling. Thirdly, contamination of the sample is not to be excluded, as previously reported by Arioli et al. (2012), who demonstrated that the storage of non-contaminated urine at a relatively high temperature and contamination by faecal or environmental microflora can induce the neo-formation of PL.

It should be noted that high concentrations of CL and CN were found in both female and male Chianina bovines. CL was found in 7 out of 12 young cows' urine, while CN was detected in 6 out of 12 samples. The minimum concentrations of CL and CN detected in Chianina young cow urine were 3.4 and 0.75 ng/ml, while the maximum were 49.7 and 22.6 ng/ml, respectively. In young bull urine, CL and CL were detected in 25 out of 30 samples. The minimum levels of CL and CN were 0.95 and 0.82 ng/ml, while the maximum were 93.7 and 46.2 ng/ml, respectively. Those last remarkably elevated values were registered in a sample that does not have any particular differences comparing with rest of the group. In addition,

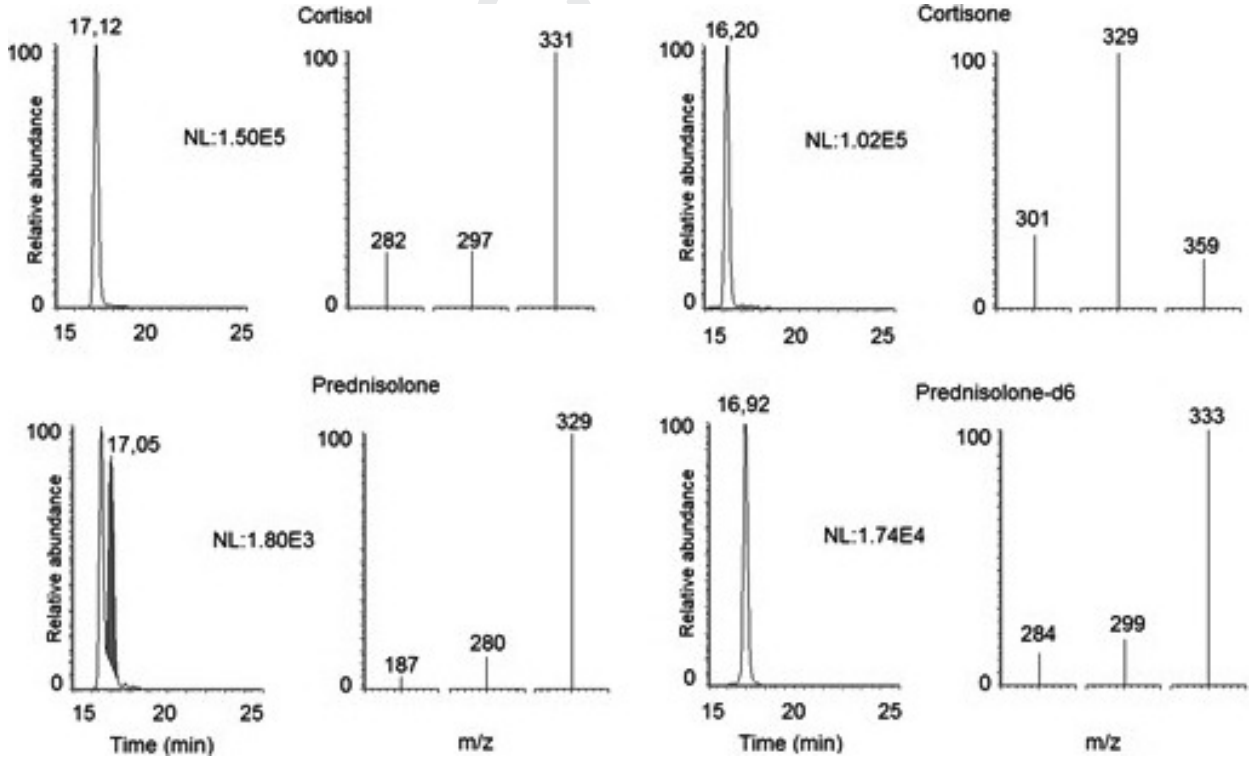

Fig. 3 Reconstructed LC-MS/MS chromatograms and respective ion spectra of the analytes detected in a urine sample from Chianina young cow 22 (49.7, 22.6 and 0.6 ng/ml for CL, CN and PL respectively).

what was the cause of complete disappearance of CL and CN in some Chianina urine samples, remains to be clarified. Ferranti et al. (2011) reported that it come about when the animals are treated with DX. This was the reason why we examined eventual presence of DX, and it was not reviled in any samples we dealt with. Consequently, total displacement of CN and CL should be searched in other metabolic direction, as far as Chianina breed is concerned.

Particular attention was paid to the three positive PL samples and eventual correlation with amount of CL and CN found in those matrices. One of them with highest amount of PL (1.5 ng/ml) revealed the presence of CL in amount of 8.2 ng/ml, while CN turned to be under detection limits. Only possible explanation would be that some individual metabolic particularities of this young Chianina cow redirect the transformation of CL towards PL, while the production of CN was somehow inhibited. Other two positive PL samples have revealed predominantly high CL and CN concentrations. In urine sample that expressed 0.3 ng/ml of PL, the amount of CL and CN were 36 ng/ml and 26 ng/ml, respectively. The last positive sample, besides having 0.6 ng/ml of the PL, had also maintained the same tendency: the CL quantity was 49 ng/ml while CN reached 22 ng/ml (Fig. 3). Unfortunately, the three positive PL values was not enough to perform reliable statistical evaluation regarding the correlation between the PL appearance and elevated CL and CN urinary concentration. Results presented by Bertocchi et al. (2013) demonstrated that PL could be endogenously produced in dairy cows and it was strongly related to elevated CN concentration in the state of extreme stress at the slaughterhouse. On the contrary, in this research, the young Chianina cows did not undergo any obvious stress; they were treated as all others animals enrolled in this study. Therefore, it can be assumed that some additional metabolic parameters and/or particular female physiological feature of Chianina breed influenced that the high CL and CN values were accompanied with PL presence.

Inter-individual variability of the results concerning the concentration of CL and CN is evident. Our limited data did not permit deeper statistical analysis on a relationship between the levels of CL and CN in young cows and young bulls, but there is an evident tendency that concentration of CL slightly higher in female ($P = 0.068$). We therefore concluded that there are no sex differences in the excretion of CL and CN in Chianina, which is in accordance with the results of Montanholi et al. (2009) and Sauerwein et al. (1991) (Table 3).

The CL/CN ratio is commonly used in human medicine to evaluate the activity of 11-beta-hydroxysteroid dehydrogenase II (11β-HSD II), the enzyme that converts CN to CL (Tomlinson et al., 2007). It is also useful to evaluate the CL/CN ratio in case of PL abuse, as previously suggested (Pavlovic et al., 2013), as a decline in this value is most probably due to inhibition of the 11β-HSD II activity, which is affected by PL treatment. The median (with corresponding 25th–75th percentile) of the CL/CN ratio in Chianina young cows urine was 1.81 (1.26–2.81), while in Chianina young bulls urine, it was of 1.17 (0.99–1.49). A different CL/CN ratio between young cows and bulls was significant ($p = 0.011$) when assessed with the nonparametric Mann–Witney test. Currently, there are few data concerning the differences between females and males in the 11β-HSD II activity. Our data could broaden the knowledge of the differences between young cows and young bulls because the analyses performed on urine from both genders of animals confirmed a significantly higher CL/CN ratio in young cows than in young bulls. Furthermore, our results could contribute to providing CL and CN baseline values in Chianina and, indirectly, an idea of the activity of 11β-HSD II. However, to better understand the activity of this enzyme in young Chianina cows and bulls, investigation of the presence not only of free CL and CN but also of the total amount of their tetrahydro-metabolites appears promising.

Conclusions

The study has proposed the first quantification of corticosteroids in Chianina urine collected at the farm. This preliminary research could confirm the presence of endogenous CL and CN in both males and females. On the other hand, it is notable that the levels of these corticosteroids in urine vary considerably. PL was detected only in female bovine and it probably shares the results of the latest studies on the endogenous nature of PL (Pompa et al., 2011; Bertocchi et al., 2013). It is necessary to extend the studies regarding corticosteroid levels in Chianina to a larger number of animals. It may also be helpful to examine the metabolic profile of these compounds in urine sampled at the farm and at the slaughterhouse, and investigate the presence of PL in other bovine matrices, such as the adrenal glands or liver.

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Conflict of interest

The authors declare that they have no conflict of interest.

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