Effects of low-dose dexamethasone and prednisolone long term administration in beef calf: chemical and morphological investigation

Abstract

An analytical, pharmacokinetic and histopathologic investigation was conducted by two experimental trials on beef cattle in order to determine fate and effects of dexamethasone and prednisolone, administered to distinct cattle groups at low dosage for long periods of time. In trial 1, eighteen Charolaise beef cattle, male, 17–22-months-old, were divided in three groups: to group A ($n = 6$) dexamethasone-21-sodium-phosphate 0.7 mg day^{-1} per *os* for 40 days was administered; group B ($n = 6$) was orally treated with prednisolone 15 mg day^{-1} for 30 days, while group C ($n = 6$) served as negative control. Urine was collected at days 0, 7, 15, 25 and 47 from groups A and C, and at days 0, 8, 18 and 42 from group B. In trial 2, sixteen Friesian cattle, male, 10–17-months-old, were randomly divided into two groups: group D ($n = 8$) was administered prednisolone 30 mg day^{-1} per *os* for 35 days, while group K ($n = 8$) served as control. In both trials, the animals were slaughtered after a 6-days drug withdrawal and thymus and livers were collected and properly stored until the analysis was performed. Quantitative determinations of dexamethasone, prednisolone and its main metabolite, prednisone, in urine and liver samples were conducted by HPLC–MS/MS, after the analytical procedure was optimized and fully validated. The method validation included the assessment of specificity, linearity, precision, trueness, robustness, CC_α and CC_β values. By a morphological point of view, severe atrophy of thymus parenchyma was observed in group A, together with a significant ($P < 0.005$) reduction of the mean thymus weight (217 ± 94 g), while group B (646 ± 215 g) presented normal thymus features and weights (group C, 415 ± 116 g). Accordingly, no differences were found in trial 2 for groups D (727 ± 275 g) and K (642 ± 173 g). Average dexamethasone concentrations in group A urine samples ranged from 1.4 to 3.0 μg L^{-1} during the treatment, while no residue was detected in the urine samples collected 6–7 days after the end of the treatment. Low amounts of dexamethasone (<1 μg L^{-1}) were detected in liver samples of group A. All average prednisolone concentrations in group B urine samples (sum of conjugate and free form) turned out to be below 1.0 μg L^{-1} during the treatment, despite the much higher concentration administered (15–30 mg day^{-1}) with respect to dexamethasone in group A (0.7 mg day^{-1}). No prednisolone residues were found in the urine and liver samples taken at the slaughterhouse. The absence of any prednisolone residue in the urine samples of control group animals supports the theory that the origin of this molecule is fundamentally exogenous, at least for this cattle category maintained under unstressing conditions. Remarkable findings are represented by the absence of thymus atrophy in the prednisolone treated animals and the extremely low residue concentrations found in urine during the treatment. Both findings reveal that the detection of illegal growth-promoting treatments with this drug is difficult.

Keywords

- Cattle; Dexamethasone; Prednisolone; Thymus; High performance liquid chromatography–tandem mass spectrometry; Urine

1. Introduction

Dexamethasone and prednisolone are synthetic glucocorticoids extensively used as therapeutic agents in veterinary practice for the treatment of a wide range of metabolic diseases, shock, stress and inflammatory disorders in farm animals. Although their administration in animals is primarily therapeutic, in some European Countries glucocorticoids are also utilized illegally as growth promoters, either alone or in association with anabolic steroids, to improve quality and quantity of meat in veal calves and beef production [1], [2] and [3].

The strong pharmacological activity of most synthetic corticosteroids makes the residues of these molecules potentially dangerous for meat consumers. As a consequence, their administration for growth-promoting purposes is banned in the EU by the Council Directive 96/23/EC, and their use in livestock is restricted to therapeutic indications requiring an official record of the treatment by a licensed veterinarian and the application of appropriate withdrawal periods in order to comply with maximum residue limits (MRLs) established for bovine edible tissues [4]. However, the illegal administration of low dosages of dexamethasone and prednisolone has considerably increased in the last years, with the purpose of enhancing the carcass and meat quality traits and reduce feed conversion ratio [5], [6], [7] and [8].

The assurance of safe and good quality food is a fundamental request of the consumers. Due to the risk of toxic effects on the human organism by glucocorticoid residues [9], the development and optimization of effective methods for detecting treated animals is of primary importance [10]. When dexamethasone is administered at low dosage, its rapid metabolism and excretion makes the residues determination difficult[11], even by using targeted GC/MS and LC/MS/MS methods [12], [13], [14], [15], [16], [17], [18] and [19], as the urinary drug concentration is very low during the treatment and completely absent a short time after the interruption of the treatment [20]. Slight thymus atrophy and presence of residues in urine ranging from 3 to 400 μg L^{-1} in veal calves treated with low doses of prednisolone have been described by Groot et al. [21], while little is known at the moment about the prednisolone urinary excretion and the possible presence of residues in the liver after low-dose, long-term treatment of beef cattle.

Aim of the present work was to determine under comparable experimental conditions, dexamethasone and prednisolone residues in urine and liver samples arising from long-term orally treated beef cattle, together with their possible gross and histopathological effects on the animals thymus. This study was also designed to investigate the kinetics of dexamethasone and prednisolone urinary excretion in beef cattle using a LC–MS/MS method, validated in agreement with the Commission Decision 2002/657/EC. To this aim, the animals were experimentally treated with dexamethasone and prednisolone, according to a growth promoting protocol, in order to provide useful chemical or morphological evidence that could possibly discriminate legal from illegal drug administration.

2. Materials and methods

2.1. Animal and experimental design

Two trials, 1 and 2, were carried out on beef cattle of different breeds. In trial 1, eighteen Charolaise beef cattle, male, 17–22-month-old, were divided into three groups: dexamethasone-21-sodium-phosphate 0.7 mg day^{-1} was administered per *os* for 40 days to group A ($n = 6$), while group B ($n = 6$) was orally treated with prednisolone acetate 15 mg day^{-1} for 30 days. Group C ($n = 6$) served as negative control. In trial 2, sixteen Friesian cattle, male, 10–17-months-old, were randomly divided into two groups: group D ($n = 8$) was administered prednisolone acetate 30 mg day^{-1} per *os* for 35 days, while group K ($n = 8$) served as control. In both trials, the animals were slaughtered after a 6-days drug withdrawal. All groups of experimental animals were kept in separate boxes, 10 m × 15 m, and fed with a diet consisting of corn silage, corn, hay and a commercial protein supplement; water was supplied *ad libitum*. The experiment was authorised by the Italian Ministry of Health and the Ethics Committee of the University of Turin. Carcases of treated animals were destroyed. All animals treated orally, before the distribution of the feed each morning, were tied to the feeding trough, where two trained technicians gave one capsule containing the compound per application, using a drenching gun. Control animals were treated with a placebo.

2.2. Processing of thymic tissue

Both cervical and thoracic portions of thymus of each animal were collected soon after slaughter and weighed. The thymus relative weight was calculated as the ratio between the weight of organ (g) and the body weight of beef cattle (kg). Tissue samples were fixed in 10% neutral buffered formalin and paraffin embedded according to routine histological procedures. Representative sections of each sample were stained with haematoxylin and eosin for histological examination.

2.3. Sample collection

Trial 1: urine samples were collected at days 0, 7, 15, 25 and 47 from groups A and C, and at days 0, 8, 18 and 42 from group B. Trial 2: urine were collected at days 0, 10, 31, 41. Urine samples were collected at early morning after spontaneous micturition, divided in aliquots and stored at −80 °C until the analysis was performed. The last sampling was carried out at the slaughter house for all groups.

In both trials livers were collected at slaughter and stored at −80 °C until analysis.

2.4. Chemicals, reagents, standard solutions and calibration curves

Sodium hydroxide and hydrochloric acid were supplied by Carlo Erba Reagenti (Milan, Italy). Dexamethasone, prednisone, prednisolone, diethylether, acetonitrile, and *t*-butylmethylether were supplied by Sigma–Aldrich (St. Louis, MO, USA). Sodium acetate was from Riedel-de-Haën (Seelze Germany). Beta-glucuronidase/aryl-sulfatase was supplied by Roche Diagnostics (Mannheim, Germany). Triamcinolone acetonide-d_6 (Internal Standard) was supplied by RIVM (Bilthoven, The Netherlands). Acetate buffers at different pH values were prepared by adding hydrochloric acid to a 0.1 M sodium acetate solution.

Stock standard solutions of prednisone, prednisolone and dexamethasone were prepared in acetonitrile at a concentration of 1000 mg L^{-1} and stored at −20 °C in the dark.

Calibration curves were obtained by spiking blank bovine urine with 0.1 μg mL^{-1} dexamethasone, prednisone and prednisolone working solutions, at five concentration levels (0, 0.5, 1.0, 2.0 and 5.0 μg L^{-1} for each analyte). Blank bovine liver samples were similarly spiked (0, 0.5, 2.0, 5.0 and 10.0 μg kg^{-1} for each analyte).

Most validation experiments were performed using negative reference urine and liver samples (5.0 mL and 2.5 g, respectively), obtained from strictly non-treated animals, and spiked with 0.1 μg mL^{-1} working dexamethasone, prednisone and prednisolone solutions, yielding 3 validation levels at concentrations of 0.5, 1.0, and 1.5 μg L^{-1} in urine and 0.5, 1.0 and 2.0 μg kg^{-1} (dexamethasone and prednisone) or 0.5, 2.0 and 5.0 μg kg^{-1} (prednisolone) in liver. The analysis of each concentration level was replicated six times and the whole set of experiments (three concentration levels replicated six times) was repeated three times. Working solutions of dexamethasone, prednisone, prednisolone and triamcinolone acetonide d_6, stored at–20 °C and analyzed regularly up to three months after the preparation, did not exhibit any degradation of the analytes.

2.5. Sample preparation

2.5.1. Urine

5.0 mL of urine or spiked urine sample was transferred into 30 mL glass tubes and 100 μL of the internal standard (IS) solution (0.1 ng mm μL^{-1} = mg L^{-1}) was added. The pH was adjusted to approximately 5.0 and then 20 μL glucuronidase/arylsulfatase solution was added. Enzymatic deconjugation was carried out for 2 h in a water bath at 37 °C. The pH was adjusted to 8.5–9.5 by means of NaOH 1 M and HCl 1 M. The hydrolysed sample was subjected to liquid/liquid extraction by adding 10 mL of diethylether. The centrifuge tube was shaken vigorously for 5 min by means of a vortex multimixer (Tecnovetro, Monza, Italy) and then centrifuged at 3500 rpm for 5 min (model Megafuge 1.0 Heraeus from ASHI, Milan, Italy). The supernatant organic phase was transferred

into a 10 mL glass tube and evaporated to dryness under a gentle stream of nitrogen and mild heating (40 °C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was redissolved in 50 µL of H_2O/CH_3CN (70/30) solution and transferred into the analytical vials for the LC–MS/MS analysis.

2.5.2. Liver

Liver samples (2.5 g) were homogenated and then 50 µL of the internal standard solution (0.1 ng μL^{-1} = mg L^{-1}) was added. 10 mL of 0.1 M acetate buffer was added and extraction (5 min of shaking plus 5 min of ultrasonic bath) was carried out. After centrifugation (3500 rpm for 5 min), the aqueous phase was extracted with 10 mL of *t*-butylmethylether. The organic phase was transferred into a 10 mL glass tube and evaporated to dryness under a gentle stream of nitrogen and mild heating (50 °C). The residue was redissolved in 50 µL of H_2O/CH_3CN (70/30) solution and transferred into the analytical vials.

2.6. Instrumentation

Chromatographic separations were performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), including a vacuum degasser, a binary pump, an autosampler and a column thermostat. The liquid chromatograph was equipped with a Merck LiChroCART – C18 (5 µm) 150 mm × 4.6 mm column and a Phenomenex SecurityGuard 4.0 mm × 2.0 mm pre-column. The chromatographic run was carried out by a binary mobile phase of water and acetonitrile, using the following program: isocratic with 28% acetonitrile for 8 min; linear gradient from 28% to 35% in 2 min; isocratic with 35% acetonitrile for 8 min; linear gradient from 35% to 50% in 4 min; isocratic with 50% acetonitrile for 7 min; total run time 29 min. The injection volume was 20 µL for liver extracts and 10 µL for urine, while the flow-rate was 0.5 mL min^{-1}. The LC was interfaced to an Applied Biosystems API 4000 triple–quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in atmospheric pressure chemical ionization (APCI) – positive ion mode. The other MS parameters were set as follows: curtain gas: 10 psi; nebulizer gas: 50 psi; probe temperature: 300 °C; gas for collisional activation: N_2 at 2 psi; nebulizer current: 3 µA; entrance potential: 10 V. Ion acquisition was operated at unit mass resolution in the selected reaction monitoring (SRM) mode, using the transitions from the protonated molecular ion of each analyte to the fragment ions indicated in Table 1.

Table 1. Mass spectrometric acquisition parameters for selected reaction monitoring operating mode.

Analyte	Precursor ion *m/z*	Declustering potential (V)	Product ions (**Q** = quantifier transition)	Collision energy (V)	Collision cell exit potential (V)
Prednisone	359.1	70	359.0 → 313.2 **Q**	19	9
			359.1 → 295.2	20	9
			359.1 → 267.2	21	7
Prednisolone	361.3	55	361.3 → 265.2	24	9
			361.3 → 279.2 **Q**	18	9
			361.3 → 223.2	29	9
Dexamethasone	393.3	55	393.3 → 373.2 **Q**	15	11
			393.3 → 355.4	19	11
			393.3 → 337.3	18	10
Triamcinolone acetonide D_6	441.4	65	441.4 → 421.3 **Q**	15	14
			441.4 → 403.4	21	13

2.7. Analytical method validation

The guiding principles expressed in the Commission Decision 2002/657/CE were followed to validate our analytical method on both urine and liver samples. Positive identification of the analytes was expressed by the recognition of 4.5 identification points, namely the SRM transitions listed in Table 1. Specificity, linearity, precision (in terms of repeatability and within-laboratory reproducibility), trueness, CC_α, CC_β and ruggedness were estimated from data collected at 3 different concentration levels (see below).

2.7.1. Selectivity

Twenty different blank urine and liver samples were deconjugated, extracted and analyzed as described above. The occurrence of possible interferences from endogenous substances was tested by monitoring the SRM profiles characteristic for each investigated compound, at the retention time interval expected for their elution.

2.7.2. Linearity

Instrumental linearity is not prescribed as a validation parameter by the Decision 2002/657/CE. Notwithstanding, this parameter was studied in order to estimate if the quantification range of the method lied within the instrumental dynamic linear range. Six increasing concentrations of each analyte pure standard solutions (0.2, 0.5, 1.0, 5.0, 10.0 and 20.0 μg L^{-1}, two replicates for each

level) were injected, to set up the linearity testing curves. Slope and intercept were determined by the squares regression method and the fit was verified using squared correlation coefficients (R^2).

2.7.3. Matrix effects

Matrix effects possibly due to ion-enhancement or ion-suppression were evaluated by comparing the slopes of the calibration curves obtained by spiking the blank urine extracts with those arising from water standard solutions. A *t*-test at 95% confidence level was used to compare each couple of slopes.

2.7.4. Precision–trueness

Validation levels for urine were set at 0.5, 1.0 and 1.5 μg L^{-1} for each analyte, while for liver samples the spiked concentration levels were 0.5, 1.0 and 2.0 μg kg^{-1} for dexamethasone and prednisone and 0.5, 2.0 and 5.0 μg kg^{-1} for prednisolone. Trueness, repeatability and intra-laboratory reproducibility were estimated for both matrices by quantifying the analytes from 18 validation replicates at the three validation levels cited, using the IS correction factor. An ANOVA test was set for each validation level, by means of which repeatability CV% and within-laboratory reproducibility were calculated.

2.7.5. CC_α and CC_β

The lowest concentration level used for the method validation on dexamethasone prednisone and prednisolone was 0.5 μg L^{-1} (μg kg^{-1} for liver). The decision limit (CC_α) at $\alpha = 1\%$ was calculated from the within-laboratory reproducibility standard deviation (WR) value at the lowest validation level:

$$CC\alpha = 0.5 + 2.33 \times WR_{\text{lowest validation level}}$$

Similarly, the detection capability (CC_β) at $\beta = 5\%$ was calculated from the value obtained by pooling the standard deviations (WR_{pooled}) from the first and second validation levels:

$$CC\beta = CC\alpha + 1.64 \times WR_{pooled}$$

2.7.6. Extraction recovery

The extraction recoveries were estimated for each analyte in the urine matrix by quantifying (by external calibration) the 18 validation replicates for each validation level. For each level, the average recovery and its respective CV% were calculated. The recovery factor, for liver extraction was evaluated by means of an identical procedure and calculated on the lowest validation level.

2.7.7. Ruggedness

Ruggedness test was conducted by introducing slight variations (±10% maximum) to previously selected analytical parameters and observing the resulting changes in term of quantitative response on blank liver and urine samples spiked at the lowest validation level. A Youden approach was used, in order to minimize the number of experiments required.

Parameters selected for the liver procedure were: extraction buffer pH (4.5–5.5), weight of blank matrix (2.2–2.8 g) used to set each point of the calibration curve, centrifugation speed (3000–4000 rpm), extract drying temperature (45–55 °C for liver; 35–45 °C for urine) and composition (65/35–75/25 water/acetonitrile) of the solution used to redissolve the extract prior of the LC–MS injection.

For urine analysis, the following parameters were tested: temperature and duration of the deconjugation step, extract drying temperature and composition of the solution used to redissolve the extract prior of the LC–MS injection.

3. Results

3.1. Gross and microscopic findings in the thymus

The absolute and relative thymus weights for the animals included in the trials are reported in Table 2. From the data relative to trial 1, it is evident that the thymus weight and volume of the animals treated with dexamethasone (group A) were significantly ($P = 0.0047$) reduced with respect to the ones of control animals (group C). In fact, the thymus mean weight of groups A and C was respectively of 217 ± 94 g and 415 ± 116 g (Table 2). Even more significant ($P = 0.0021$) differences are found when the relative thymus weights are compared, as the confidence intervals for groups A (0.16–0.42 g kg^{-1}) and group C (0.46–0.92 g kg^{-1}) do not overlap at all.

Table 2. Absolute and relative thymus weight of treated (A, B, D) and control (C, K) animals.

Absolute weight (g)	Trial 1			Trial 2	
Groups	A*	B	C*	D	K
Individual values (g)	247	552	358	612	508
	157	395	249	878	869
	194	976	600	555	641
	202	754	393	1323	535
	115	466	438	488	833
	387	732	451	569	464

Absolute weight (g)	Trial 1			Trial 2	
Groups	A*	B	C*	D	K
				809	§
				580	§
Average	217	646	415	727	642
Standard deviation	94	215	116	275	173
Confidence intervals (c.l. 95%)	118–316	420–872	293–536	497–957	460–823
Experimental (tabulated)	A vs. C	B vs. C	A vs. B	D vs K	
t-Student value (c.l. 95%)	3.24	2.31	4.46	0.71	
	(2.31)	(2.31)	(2.31)	(2.15)	
P-value for null-hypothesis	0.0047	0.0509	0.0015	0.4930	

Relative weight (g kg^{-1})	Trial 1			Trial 2	
Groups	A	B	C	D	K
Individual values (g kg^{-1})	0.31	0.84	0.62	1.212	0.968
	0.20	0.56	0.41	1.557	1.640
	0.28	1.38	1.07	0.925	0.960
	0.27	1.03	0.61	2.224	0.843
	0.15	0.65	0.69	0.683	1.488
	0.51	0.98	0.75	0.834	0.810
				1.172	§
				0.824	§
Average	0.29	0.91	0.69	1.18	1.12
Standard deviation	0.12	0.30	0.22	0.51	0.35
Confidence intervals (c.l. 95%)	0.16–0.42	0.60–1.22	0.46–0.92	0.76–1.60	0.75–1.49
Experimental (tabulated)	A vs. C	B vs. C	A Vs. B	D vs. K	
t-Student value (c.l. 95%)	3.95	1.44	4.75	0.26	
	(2.31)	(2.31)	(2.31)	(2.15)	
P-value for null-hypothesis	0.0021	0.1842	0.0012	0.7963	

* Data partially published by Cannizzo et al. [22].

§ Two control animals of the K group were not slaughtered six days after drug withdrawal.

Both cervical and thoracic portions of the thymuses of group B animals appeared normal. Accordingly, their mean absolute and relative weight were 646 ± 215 g and 0.91 ± 0.30, apparently even higher than in control group C. This slightly higher weight of group B thymuses with respect to control group C was nonetheless not significant, particularly as their relative weights are compared by a Student *t*-test at 95% confidence level, yielding an experimental value of 1.44 vs. a tabulated

value of 2.31. Application of non-parametric Mann–Whitney statistic test to groups A, B and C data yielded the same conclusions drawn from parametric *t*-test.

By a morphological point of view, group A showed severe thymus atrophy, characterized by a serious volume reduction of the organ, which in some animals almost disappeared and was replaced by fat tissue. Histologically, group A showed severe thymic cortical atrophy, while the medullary framework was still present though reduced, showing a pronounced rarefaction of lymphocytes. The thymic cortex was almost completely replaced by fat tissue leaving only scattered remnants localised close to the medulla. In contrast, group B thymuses appeared both macroscopically and histologically normal. The histology of the thymus of the control beef cattle (group C) was similar to that of young calves. The parenchyma had normal features characterised by a cortex filled with proliferating T cells with a smaller proportion of associated epithelial cells. More mature T cells were found in the medulla, where epithelial cells, dendritic cells and macrophages were more abundant. Almost none, or very few, adipose cells were observed in the peripheral layers of the cortex.

The results of trial 2 confirmed the outcome of trial 1 for group B animals, even though the dose of prednisolone administered to group D animals was doubled with respect to group B. In fact, the thymus weight and volume of the control (group K: 642 ± 173 g) and treated (group D: 727 ± 275 g) animals were not significantly different ($t = 0.71$; $P = 0.4930$). The mean relative thymus weight of groups K and D turned out even closer (1.12 ± 0.35 vs. 1.18 ± 0.51 g kg^{-1}; $t = 0.26$ vs. $t_{0.95} = 2.15$). Macroscopically and histologically thymuses of group D were as normal like controls, showing similar findings of groups B and C of trial 1.

3.2. Validation and interpretation of results

An overview of the results obtained for the analytical method validation is given in Table 3.

Table 3. Intra-laboratory precision (CV%), recovery (%), bias (%), CC_α and CC_β values (μg L^{-1}) for the three analytes at three concentration values.

Urine								
	Intralaboratory reproducibility (CV %)			Recovery (%) Mean ± St. dev.			CC_α (μg L^{-1})	CC_β (μg L^{-1})
Analyte concentration	0.5 (μg L^{-1})	1.0 (μg L^{-1})	1.5 (μg L^{-1})	0.5 (μg L^{-1})	1.0 (μg L^{-1})	1.5 (μg L^{-1})		
Dexamethasone	7.3	4.8	2.5	88 ± 11	64 ± 14	57 ± 16	0.59	0.65
Prednisone	13.5	10.7	4.8	57 ± 5	39 ± 4	30 ± 3	0.66	0.79
Prednisolone	14.4	9.1	7.1	39 ± 6	30 ± 9	26 ± 9	0.67	0.80

Liver						
	Intralaboratory reproducibility (CV%)			Recovery (%) Mean ± St. dev.	CC_α (µg L^{-1})	CC_β (µg L^{-1})
Analyte concentration	0.5 (µg kg^{-1})	1.0*–2.0** (µg kg^{-1})	2.0*–5.0** (µg kg^{-1})	0.5 (µg kg^{-1})		
Dexamethasone*	17.5	13.3	8.1	88 ± 24	0.70	0.88
Prednisone*	17.4	15.7	10.7	93 ± 6	0.70	0.90
Prednisolone**	9.2	12.7	7.9	45 ± 7	0.61	0.75

	Urine			Liver		
	Bias (%)			Bias (%)		
Analyte concentration	0.5 (µg L^{-1})	1.0 (µg L^{-1})	1.5 (µg L^{-1})	0.5 (µg kg^{-1})	1.0*–2.0** (µg kg^{-1})	2.0*–5.0** (µg kg^{-1})
Dexamethasone*	+1%	−1%	0%	+18%	−6%	−9%
Prednisone*	−8%	+8%	−3%	−12%	+10%	−2%
Prednisolone**	+3%	0%	0%	+10%	+14%	0%

3.2.1. Selectivity

The SRM chromatographic profiles obtained from 20 blank matrix samples (urine and liver) did not show the presence of any significant signal (S/N < 3) at the relative retention time typical of the studied compounds and the internal standard, indicating that the method is selective and no interfering substances are present in the biological matrices.

3.2.2. Linearity

Calibration curves built from standard solution were linear in the range 0.2–20 µg L^{-1}. All analytes were visually checked for linear fit. The calibration fit showed squared correlation coefficients (R^2) of 0.9999, 0.9993 and 0.9993 for dexamethasone, prednisone and prednisolone respectively. The quantification range lies, for both liver and urine method, within the instrumental dynamic linear range. Calibration curves, based on spiked blank urine and liver samples and created before each analytical session, also proved linear without exception.

3.2.3. Matrix effects

The slopes of the calibration curves built by spiking the extracts from either water or blank urine samples were compared and the percent differences were used to test the matrix effect, which appeared modest (≤4%) and statistically not significant for dexamethasone, prednisolone and

prednisone. Accordingly, the differences are shared between positive (dexamethasone +4.0%) and negative (prednisolone −2.2% and prednisone −3.3%). Uncertainties in slope values ranged from 1% to 3%.

3.2.4. Precision and trueness

Intra-laboratory reproducibility was expressed by the experimental coefficients of variation, ranging between 2.5% and 14.4% for the spiked urine samples and between 7.9% and 17.5% for the spiked liver samples. Although the Decision 2002/657/CE does not set a fixed intra-laboratory CV limit at the concentration range used in this study, precision values below 20% are considered entirely satisfactory by any validation protocol.

Quite similarly, limited bias from true values were recorded for both urine (0–8%) and liver (0–18%) samples. Taking into account that the IS correction factor compensated for extraction yields variability, very reasonable and repeatable concentration assessment could be obtained.

3.2.5. Sensitivity, CC_α and CC_β

The present method was optimized with the purpose of detecting considerably small concentrations of the analytes: although the quantification range yielding acceptable accuracy was not expanded below 0.5 μg L^{-1}, limit of detection (LOD) values were estimated around 0.05 μg L^{-1}, one order-of-magnitude lower. Fig. 1presents the SRM chromatograms obtained from a blank urine sample spiked with the three analytes at 0.5 μg L^{-1} concentration; only the quantifier transition is shown for each analyte. S/N ratios exceeding 35 were observed for all substances, from which the cited LOD values were calculated. Accordingly, concentrations in the range 0.05–0.5 μg L^{-1}, occasionally detected in real samples from pharmacokinetic experiments (see below), have to be considered as reasonable estimations, not accurate determinations. CC_α and CC_β values, calculated from the lowest validation concentration, homogeneously exceeded this limit of 0.5 μg L^{-1}.

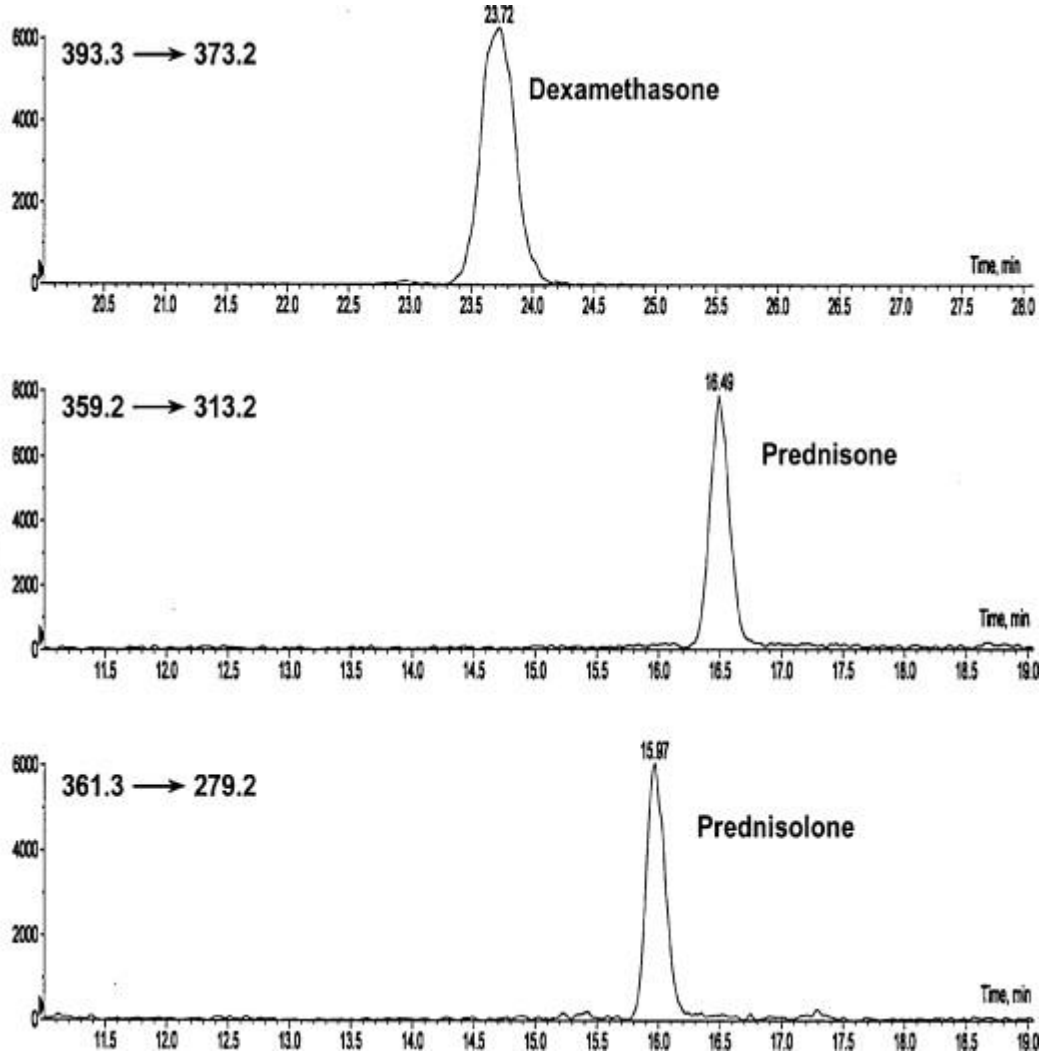

Fig. 1. Selected reaction monitoring chromatograms, obtained from a blank urine sample spiked with dexamethasone, prednisone and prednisolone at 0.5 μg L^{-1} concentration. The main (quantifier) transition is shown for each analyte.

3.2.6. Extraction recovery

The extraction recoveries reported in Table 3 give evidence that dexamethasone is more extensively extracted than prednisone and prednisolone, possibly due to the hydrophobic character imparted by its fluorine atom. Moreover, recoveries appear to be more complete from liver than from urine. Although the absolute recoveries of prednisone and prednisolone from urine are not entirely satisfactory (ranging around 30–40%), the extraction yields are rather constant. This repeatability, together with the use of an internal standard (i.e. triamcinolone acetonide-d_6) in real sample analysis, largely compensate for incomplete recoveries, resulting in accurate quantitation of the analytes.

3.2.7. Ruggedness

By using the Youden approach, eight experiments for each matrix were performed to test the method robustness. No statistically significant variation of the detected concentrations were observed by changing by 10% the extraction buffer, pH, weight of blank matrix, extract drying temperature and composition of the residue dissolving solution (liver), nor from temperature and duration of the deconjugation step, extract drying temperature and composition of the residue dissolving solution (urine).

3.3. Dexamethasone and prednisolone excretion kinetics

Quantitative determination results for the real urine samples arising from the experimental trials are reported in Table 4. It is evident that dexamethasone was detected in all group A samples collected during the period of drug administration, although at low concentration level, and was not detectable anymore six days after the end of the treatment. Average dexamethasone concentration (μg L^{-1}) in group A samples was 0 (day 0), 1.27 (day 7), 1.94 (day 15), 1.05 (day 25) and 0 (day 47) respectively. Fig. 2a reports the SRM chromatograms of the three transitions characteristic for dexamethasone, obtained from animal 7A at day 25.

Table 4. Quantitative determinations of dexamethasone, prednisolone and prednisone on real urine samples from the experimental trials. All concentrations are expressed as μg L^{-1}. "n.d." stands for "not detected".

Animal ID	Dexamethasone					Prednisone	Prednisolone
	Day 0	Day 7	Day 15	Day 25	Day 47	All samples	All samples
7 A	n.d.	2.19	2.54	2.51	n.d.	n.d.	n.d.
8 A	n.d.	2.03	3.07	2.38	n.d.	n.d.	n.d.
9 A	n.d.	1.19	2.37	0.10	n.d.	n.d.	n.d.
10 A	n.d.	1.03	0.11	0.06	n.d.	n.d.	n.d.
11 A	n.d.	0.10	3.43	0.13	n.d.	n.d.	n.d.
12 A	n.d.	1.08	0.13	1.15	n.d.	n.d.	n.d.

Animal ID	Dexamethasone	Prednisone				Prednisolone			
	All samples	Day 0	Day 8	Day 18	Day 42	Day 0	Day 8	Day 18	Day 42
13 B	n.d.	n.d.	0.10	0.12	n.d.	n.d.	0.27	0.60	0.10
14 B	n.d.	n.d.	0.16	n.d.	n.d.	n.d.	1.16	0.73	n.d.
15 B	n.d.	n.d.	0.10	0.07	n.d.	n.d.	0.91	0.96	n.d.
16 B	n.d.	n.d.	n.d.	0.09	n.d.	n.d.	n.d.	1.18	0.09
17 B	n.d.	n.d.	0.06	0.15	n.d.	n.d.	1.04	1.32	n.d.
18 B	n.d.	n.d.	0.11	0.13	n.d.	n.d.	0.59	0.92	n.d.

ID	Dexamethasone	Prednisone	Prednisolone
19–24 C	n.d.	n.d.	n.d.

Animal ID	Dexamethasone	Prednisone				Prednisolone			
	All samples	Day 0	Day 10	Day 31	Day 41	Day 0	Day 10	Day 31	Day 41
25 D	n.d.	n.d.	<0.10	<0.10	n.d.	n.d.	0.51	<0.10	n.d.
26 D	n.d.	n.d.	<0.10	n.d.	<0.1	n.d.	<0.10	n.d.	n.d.
27 D	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.10	n.d.
28 D	n.d.	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	0.68	n.d.
29 D	n.d.	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	0.67	n.d.
30 D	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
31 D	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
32 D	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

ID	Dexamethasone	Prednisone	Prednisolone
All K	n.d.	n.d.	n.d.

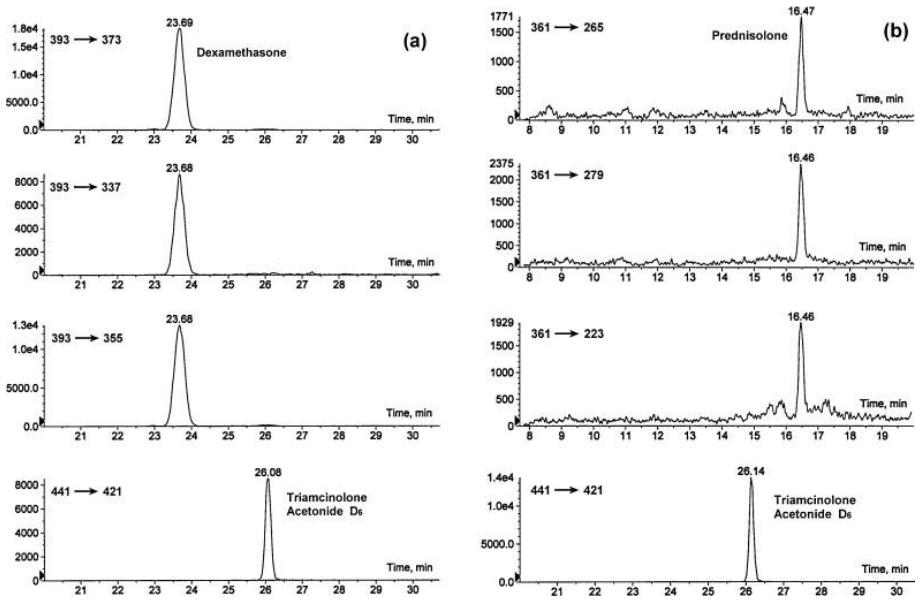

Fig. 2. (a) Selected reaction monitoring chromatograms, obtained from the urine sample collected from animal 7A, at day 25, after the beginning of the treatment with dexamethasone. (b) SRM chromatograms, from animal 13B sample collected at day 8 after the beginning of the treatment with prednisolone. A single trace for the internal standard is included at the bottom of the figures.

In contrast, prednisolone was detected in most, but not all, the urine samples collected when the drug treatment was going on (groups B and D), despite the significantly higher dose administered with respect to dexamethasone (20–40 times higher). Apparently, the detected concentrations do not depend on the administered dose, as they turned out lower for the higher dose (group D vs. group B). Average prednisolone concentration in group B urines, representing the sum of both

conjugate and free form of the analyte, was 0 (day 0), 0.66 (day 8), 0.95 (day 18) and 0 (day 42) μg L^{-1}, respectively. Again, no detectable residues were found in the samples taken at the slaughterhouse. An example of prednisolone excretion determination is reported in Fig. 2b, showing the three SRM profiles for prednisolone, obtained from animal 13B at day 8 with approximate concentration of 0.27 μg L^{-1}. Prednisone was found at even lower concentrations, as it is formed as a metabolite from the interconversion of prednisolone. Its average concentration was 0.09 μg L^{-1}both at day 8 and day 18 samples.

It is worth noting that prednisolone and prednisone were not detected in any urine of control animals nor in the ones of group A animals and at day 0 (i.e. before the beginning of the drug treatments), supporting the theory that they are unlikely to be produced as endogenous substances, at least not for Charolaise and Friesian healthy male cattle 10–22 months old, or are perhaps produced at extremely low and barely detectable concentrations.

All liver samples collected from the animals treated with dexamethasone showed the presence of the drug at trace level, i.e. largely below the CC_{α} value (0.70 μg L^{-1}), whereas all liver samples yielded negative results for the presence of both prednisolone and prednisone.

4. Discussion

Gross and microscopic evidences noticed during the present study in the thymus of beef cattle treated with synthetic corticosteroids are quite clear. On one hand, thymus atrophy following long term administration of low doses of dexamethasone, already observed in previous studies [23] and [24], has been fully confirmed. Extensive thymus modifications induced by dexamethasone administration are testified by both highly significant volume and weight reductions and severe histological modifications. In particular, massive lymphoid depletion was observed, together with important fat infiltration into the parenchyma.

On the other hand, similar long term treatment of beef cattle with prednisolone appears to have no effects on the thymus tissue, also when increasing doses are administered to different breeds, as demonstrated by trials 1 and 2. Even more surprising is the absence of any macroscopic reduction of the thymuses volume and weight: the average thymus weight of the cattle treated with prednisolone is even higher than that of the control groups, although the differences are not statistically significant. The direct consequence of the lack of any macroscopic and microscopic thymus change in the animals treated with prednisolone, following two different growth-promoting protocols, is that this fundamental and highly suggestive biological marker cannot be used anymore to unequivocally detect the illegal treatments with this corticosteroid molecule in beef cattle. The thymus atrophy observed by Groot et al. [21] in veal calves treated with prednisolone may be related to the different age and metabolism of animals still having an inactive rumen, due

to the particular liquid alimentation lasting for the life-long fattening cycle. Moreover, this preliminary study cited [21] did not involve a statistically significant number of animals (only 3), sufficient to draw ultimate conclusions.

The most traditional tool used to detect illegal drugs administration in animal breeding is the chemical analysis of urine and/or liver samples. The detection and quantification of undeclared drugs in these biological matrices represent a clear proof of illegal breeder behaviour and is generally sufficient to produce his judiciary conviction. However, decreased and minute doses of corticosteroids are nowadays administered to the animals for quite long periods of time to produce unnatural weight increase, making the analytical identification of the drug progressively more difficult. In particular, the inadequacy of most immunochemical methods for detecting illegal dexamethasone administrations has been recently underlined[20]. Consequently, LC–MS/MS techniques are increasingly developed and utilized also for screening purposes, allowing direct, rapid, selective and sensitive detection of corticosteroids in a variety of biological matrices [12], [13], [14], [15], [16], [17], [18] and [19], thanks to the continuous improvement of instrumentation technology.

The LC–MS/MS method described in the present study was optimized and validated for the objectives of this research, but can be used for a broader range of corticosteroids or their metabolites, as long as the parameters required by the Commission Decision 2002/657/EC are positively verified. In fact, the results obtained in the validation process assure that the protocol used is concurrently selective, precise, sensitive, robust and provides reasonably accurate determination of dexamethasone, prednisolone and prednisone in the low μg L^{-1} concentration range for both urine and liver, but the sample extraction and deconjugation treatments are sufficiently unspecific to be profitably applicable to other corticosteroids. Besides, the chromatographic conditions were designed and successfully tested for the separation of dexamethasone and betamethasone, as this appears to be necessary whenever the identity of one specific epimer has to be unquestionably ascertained.

The quantitative analysis of target corticosteroids in real trials samples produced quite surprising results, particularly for the groups B and D, arising from prednisolone treatments. In a previous study [20], we already observed that very low concentrations (below 2 μg L^{-1}) of dexamethasone were observed in the urine of cattle, during the second half of a 60-days long treatment with 0.7 mg day^{-1} dexamethasone sodium phosphate. The results presented in Table 4 for group A fully confirm this observation, as dexamethasone urinary concentrations around 2 μg L^{-1} were determined, as in the first 30 days of the previous study.

Much more unexpected were the extremely low concentrations (0.5–1.0 μg L^{-1}) of prednisolone found in the urine of cattle treated with either 15 or 30 mg day^{-1} of prednisolone acetate, despite the higher administered dose with respect to dexamethasone (20–40 times higher). Although much higher drug concentrations were observed by Groot et al. [21] in the urine of prednisolone treated

veal calves, again the difference with respect to our study may be related to the different age and metabolism of animals, not yet ruminant, as well as on the higher dose, relative to the animal mass (120 μg kg day^{-1} vs. 30–60 μg kg day^{-1}). The partial interconversion to prednisone does not compensate for the missing parent drug excretion, but no other metabolite could be clearly identified in these urine samples. Extensive and targeted pharmacokinetic studies have to be undertaken in the future to fully understand the fate of administered prednisolone and to clarify whether its metabolites have to be looked for among the exogenous or the endogenous steroids. At the moment, very little is known from the scientific literature about prednisolone metabolites excretion in cattle, as well as on the possible illegal use of exogenous prednisolone.

The evidence that prednisolone and dexamethasone tend to decrease from urine during the drug administration and disappear both in urine and liver samples at the end of the treatment, makes the detection of treated animals at the slaughterhouse more difficult. On the other hand, the absence of any prednisolone residue in control samples, as well as in the urines collected before the beginning of the treatments and in group A samples, apparently confirms the exogenous origin of this molecule, at least when it is detected in urine and/or liver samples of beef cattle. As a matter of fact, urine samples collected from cows at slaughterhouse are frequently found positive to prednisolone (Italian National Residue Plan), raising the question if the use of such illegal treatments is widespread or if other possible origins besides drug administration, should also be considered, for example microbial transformation of cortisol to prednisolone in urine samples contaminated by faecal matter [25] or stressing conditions [26]. However, the low level of prednisolone residues found in urine of treated beef during the drug administration, and their complete absence at the abattoir after 6 days withdrawal, suggests to consider the low positivity, possibly found during the in field control activity, as a significant indication of prednisolone administration, at least for the beef cattle category.

5. Conclusions

The present study clearly demonstrates that long term administration of low doses of prednisolone to beef cattle does not cause thymus atrophy, while dexamethasone induces thymus atrophy in beef cattle which is clearly detectable both macroscopically and histologically at the slaughterhouse after a withdrawal time of one week. Consequently the gross and histopathological investigations do not appear to be useful in detecting illegal treatment with prednisolone at the dosages used in our growth-promoting protocols.

On the other hand, long-term low dosage administration of dexamethasone or prednisolone to beef cattle resulted in extremely low drug residue concentrations in the urine of treated animals. Therefore, a selective, sensitive, accurate robust and fully validated analytical method based on LC–MS/MS (SRM) has to be used to detect these residues, as in the present study. Corticosteroids

determination in the urine samples of 34 animals collected at regular time intervals before, during and after experimental treatments with dexamethasone or prednisolone, as well as in their liver after slaughter, surprisingly revealed that prednisolone residual concentrations in urine samples were even lower that those of dexamethasone, despite the much higher dose administered to obtain a comparable pharmacological effect. Neither presence of prednisolone was detected in the urine samples collected from 14 control animals, nor in the samples collected before the beginning of the treatments (106 negative samples overall).

Since at present the endogenous origin of prednisolone in beef cattle breaded under regular conditions has not been demonstrated yet, even modest positivity in the urine and liver chemical analysis should be treated as a strong evidence of recent suspiciously fraudulent prednisolone administration in this category, even if a few alternative hypotheses for possible endogenous production in cows under anomalous breeding situations have recently been suggested. Conversely, further studies are needed to identify reliable biomarkers in order to detect illegally treated beef with prednisolone, either at the farm or at the slaughter. In this respect, metabolic transformation, drug kinetics and genomic studies appear to be necessary to establish the most trustworthy markers and to investigate the ultimate fate of prednisolone.

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