LC-MS/MS analyses of bile and histological analyses of thymus as diagnostic tools to detect low dose dexamethasone illicit treatment in beef cattle at slaughterhouse

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ABSTRACT

Dexamethasone (DXM) is a synthetic adrenal corticosteroid with anti-inflammatory properties used for therapeutic purposes in a wide range of pathologies and of the most common corticosteroids used for anabolic purposes in beef cattle. It is proven that DXM induces histological changes, traceable as increasing fatty infiltration of the thymus associated with a concurrent decrease of the cortex-medulla ratio, so the histological examination of the thymus gland has been established as an indirect morphological biomarker. The aim of the present study is to compare thymus histology and DXM concentrations in biological fluids collected at slaughterhouse after 1 month of DXM treatment. Our findings demonstrate that a low dosage of DXM administered to 12 months-old-Chianina beef cattle induces severe thymic atrophy with concurrent reduction of the cortex/medulla ratio, demonstrable even when DXM residues are not found in serum and urine samples. It is worth to note that, at the slaughterhouse, DXM residues are detectable in bile samples, indicating the ability of this biological fluid to bio-concentrate the administered drug if compared to serum and urine. Therefore, bile could be candidates as new liquid matrix for the screening programs planned to contrast the illegal use of anabolic substances.

1. Introduction

The illicit use in long-term low-dose administration of Dexamethasone (DXM) has been described to improve beef tenderness and increase live weight via water retention[1]. European regulations [2] banned the administration of growth promoters, including corticosteroids, allowing their use only for therapeutic purposes. However their administration continues illicitly[1]. For all these reasons, maximum residue limits[3] have been fixed in various matrices to ensure consumer safety and adequate withdrawal times have been established for each authorized molecule. As part of this legislative framework, routine monitoring is compulsory within Member States[1] and the

national program for residue surveillance (NRP), based on risk analysis and local productions features, is drawn up every year by EU countries. The detection of drug residues in biological samples is generally complex. At present, the "gold standard" technique for the targeted approaches used in official control analysis is liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which can detect directly the drug substance or its metabolites. However, considering the low concentrations used for illicit treatments, the large number of molecules potentially used (alone or in cocktail) and their rapid metabolism and excretion, these compounds cannot be easily and consistently detected by targeted chemical methods (LC-MS/MS)[4,5]. Bile has been proved to act as bio-accumulator of administered drugs (and/

or their metabolites) for a longer period than the "traditional" ones[6]. Indeed, Moretti et al.[7] recently validated a method for DXM and its metabolites detection in bile samples within a more complex multi-screening analyses aiming at improving the screening activities carried out in NRP. The plan is nowadays well settled, but more data are needed to better define differences among cattle breed, especially considering the autochthonous slow-growing ones often reared in rural areas. Chianina is an ancient Italian cattle breed, raised in Central Italy for over twenty-two centuries, appreciated for its extremely large size (bulls stand up to 1,700 kg and 1.8 m), but rarely cited in international literature [8,9]. Chianina plays an important role in the Italian meat industry: worldwide it's a recognized producer of top quality beef, specifically the famous "Florentine steak", and has also been accredited with the Protected Geographical Indication "White Bullock of the Central Appenines"[8]. According to its slow-growing parameters, Chianina bullocks can be slaughtered between 12 and 24 months.

The aim of the present study is to compare DXM concentrations in different fluid samples from Chianina bullock at slaughterhouse to provide new tools for illicit treatment detection including bile as new matrix for chemical analyses, applying a method previously developed and validated by the same chemical team[7] and support the recorded results by histological and morphometrical analyses of cortex/medulla ratio in thymus.

2. Experimental

2.1. Study design

A total of sixteen 12-months-old-males-Chianina beef cattle were randomly divided in two groups of eight animals each, homogenous for body weight and age, and farmed in the same conditions in two separated boxes (10 × 15 m). Free access to water and feed (silage, soy, maize, straw and mineral supplements) was guaranteed throughout the study. Clinical examinations were performed daily by a veterinarian to monitor animal health status and welfare. After a month of acclimatization, the treated group (n = 8) received 0.7 mg of DXM (RAPISON®, Fatro S. p. A. Ozzano Emilia, Bologna, Italy) once a day for 28 days, while the control group (n = 8) received a placebo (the same composition except DXM). All animals were treated orally each morning, during feed distribution. During the period spent at the farm, all animals resulted healthy and none received any antimicrobial treatment. At day 0, 7, 14, 21, 28 and 33 urine and serum were sampled and stored at −20 °C up to analysis.

Cattle were all slaughtered on day 33 (14 months of age), five days after the last drug administration. At the slaughterhouse, accurate *ante mortem* and *post mortem* inspections were conducted and serum, urine, bile and thymus were collected. Bile samples were taken after gall-bladder removal and immediately stored at −20 °C. Three bile samples of the treated groups were unfortunately lost during the slaughtering procedure. The experiment was authorized by the Italian Ministry of Health (number 775/2015-P) and carried out according to European Council Directive n.63/2010 adopted by Italian Government (D.lsg n. 26/2014). The carcasses of the treated animals were destroyed in accordance with European Council Directive 2003/74/EC.

2.2. Chemical analysis

2.2.1. Materials and reagents

Desametasone (DXM) was purchased from Sigma-Aldrich (Saint Louis, MO, USA) and DXM-d4 was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). LC-MS grade acetonitrile, acetic acid, hydrochloric acid, ethylacetate, methanol and sodium acetate were supplied by Sigma-Aldrich, as well as β-glucuronidase (*Helix pomatia* Type H-2 - code G0876). SPE OASIS HLB cartridges (60 mg/3mL) were purchased from Waters Corporation (Milford, MA, USA), while NH2 cartridges (100 mg/6mL) from Biotage (Uppsala, Sweden).SPE Strata-

X-C 33u (200 mg, 6 mL) were from Phenomenex (Torrance, CA, USA).

2.2.2. Sample treatment

Urine and serum sample treatments were developed on the basis of the protocols described by Biancotto et al.[10]and McWhinney et al. [14], respectively, bringing some modifications. Briefly, 1.5 mL of urine were added with internal standard (DXM-d4) and 10.5 mL of acetate buffer (pH = 5.0). After enzymatic hydrolysis, 6 mL of sample were purified through OASIS HLB and NH2 cartridges. After elution, the dried residue was re-suspended in 500 μL of a mixture acetonitrile/water/acetic acid (25:75:0.1 v:v:v) and injected in the LC system. After the addition of internal standard, the serum sample (300 μL) was treated with 500 μL of a solution 0.1 M of HCl and after about 30 min, 1 mL of water was added[11]. The centrifuged supernatant was then purified by OASIS HLB and NH2 cartridges. The dried residue was re-suspended in 200 μL and injected. Finally bile samples were prepared following exactly the procedure conceived by Moretti et al.[7] for the multiclass screening of fifty-three veterinary drugs among which DXM.

2.2.3. LC-MS/MS conditions

The determination of DXM was performed by liquid chromatography (Ultimate 3000 - Thermo Scientific, Waltham, MA, USA) coupled to a quadrupole-orbitrap hybrid mass analyzer (Q-Exactive, Thermo Scientific). For urine and serum samples, chromatographic separation was achieved on a Kinetex XB - C18 analytical column (100 × 3.0 mm, 2.6 μm, Phenomenex, Torrance, CA, USA) connected to a guard cartridge (Gemini C18, 4 × 2.0 mm, Phenomenex). The mobile phase was composed by (A) water with 0.1% acetic acid and (B) acetonitrile with 0.1% acetic acid. Elution was accomplished with the following solvents gradient: 0–0.5 min 0% B, 30% B at 10.5 min and kept unchanged until 13 min, 95% B at 20 min and kept unchanged until 24 min. Finally, the system returned to 0% B in 0.5 min and was re-equilibrated for 3.5 min. The flow rate and the injection volume were 0.4 mL/min and 10 μL, respectively. The acquisition was carried out in positive ionization mode (ESI +). The ESI temperature was set at 350 °C, the capillary temperature at 300 °C and the electrospray voltage at 3.5 kV. Sheath and auxiliary gas were 50 and 15 arbitrary units, respectively. The acquisition was performed in full scan/ddMS^2 mode. The parameters were optimized as follows: i) full scan acquisition: resolution 70,000 FWHM (at m/z 200), AGC target 1.0 × 10^6 and injection time = 300 ms; ii) dd-MS^2: 17,500 FWHM (at m/z 200), AGC target 5 × 10^5 and injection time = 80 ms. The Normalised Collision Energy (NCE) was set at 10. The precursor ion ([M + H]$^\pm$) was m/z 393.2066 and the fragment ions m/z 373.2000 and m/z 355.1896. Both procedures were successfully validated according to Commission Decision 2002/657/EC [12]. For bile samples the separation was achieved on a Kinetex XB C18 (100 × 3 mm, 2.6 μm, 100 Å, Phenomenex, Torrance, CA, USA) following the instrumental conditions detailed in Moretti et al. [9].

2.3. Thymus histology and morphometry

At the slaughterhouse the central area of the thoracic portion of the thymus was sampled, fixed in 10% neutral buffered formalin and paraffin-embedded with standard procedures. Four μm thick sections were stained with Haematoxylin and Eosin (H&E) for histological and morphometrical investigations. In order to evaluate DXM-related thymus atrophy, evidenced by adipose tissue infiltration of the parenchyma, the scoring system adopted by the Italian histological monitoring plan (histological NRP) was attributed. In detail, the amount of the assessment of fat infiltration, as indirect marker of thymus atrophy, was evaluated by light microscopy at low magnification (1x and 4x). This scoring system classifies the degrees of atrophy into 3 grade: grade 1 is attributed to minimal invasion of adipose tissue localized within the thymus septa; grade 2 is attributed to moderate invasion of adipose tissue in septa with minimal invasion of the cortex and grade 3,

Table 1
Chemical and histological analyses at slaughter expressed by single bullock.

| GROUP	ANIMAL ID	ANALYSES AT SLAUGHTER				
		Chemical Analyses			Histological Analyses	
		SERUM[a] (ng mL^{-1})	URINE[a] (ng mL^{-1})	BILE[a] (ng mL^{-1})	Thymus Atrophy Score	C/M ratio
CONTROL GROUP	1	< CCα	< CCα	< CCα	Grade 1	3.022
	2	< CCα	< CCα	< CCα	Grade 1	2.672
	3	< CCα	< CCα	< CCα	Grade 1	3.406
	4	< CCα	< CCα	< CCα	Grade 1	3.56
	5	< CCα	< CCα	< CCα	Grade 1	2.086
	6	< CCα	< CCα	< CCα	Grade 1	2.594
	7	< CCα	< CCα	< CCα	Grade 1	3.426
	8	< CCα	< CCα	< CCα	Grade 1	2.422
TREATED GROUP	1	< CCα	< CCα	N/A	Grade 3	0.570
	2	< CCα	< CCα	2	Grade 3	0.752
	3	< CCα	< CCα	1	Grade 3	1.252
	4	< CCα	< CCα	1	Grade 3	0.346
	5	< CCα	< CCα	2	Grade 3	0.862
	6	< CCα	< CCα	3	Grade 3	0.382
	7	< CCα	< CCα	N/A	Grade 3	0.388
	8	< CCα	< CCα	N/A	Grade 3	0.414

N/A: not analysed.
C/M ratio: Cortex/medulla ratio in Thymus.
CCα: limit of decision.
CCβ: detection capability.
 [a] The DXM concentrations were calculated using the isotopic dilution methodology.
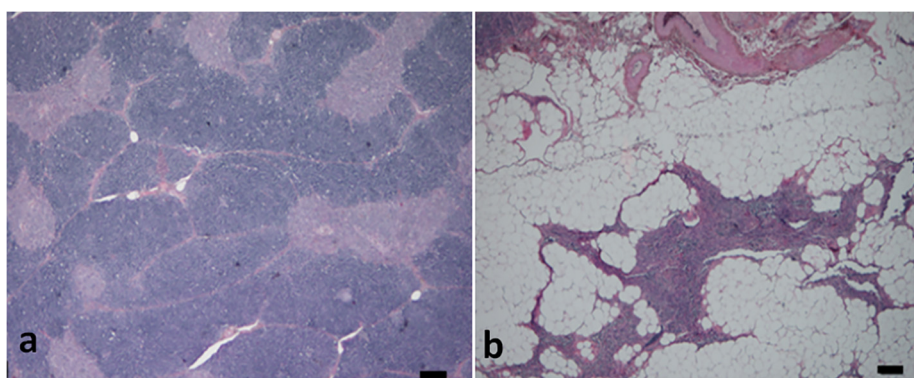
Fig. 1. (a) Slight physiological thymus atrophy due to mild infiltration of adipose tissue at the periphery of the lobules (score 1). (b) Severe adipose infiltration of the parenchyma and marked cortex atrophy that reduced the medullary framework, up to the complete replacement of the lymphoid tissue by fat (score 3). (H&E – Bars 200 μm).

ascribed to severe invasion of adipose tissue in the cortex with invasion of the medullar part. The histological examination was blindly performed by three independent observers. Interlaboratory concordance tests followed by K of Cohen test (IC 95%) was applied to evaluate the reproducibility of the diagnosis.

Morphometrical evaluation was performed at low magnification (5x) with the microscope Eclipse Ci-L (Nikon Corporation, Japan) using NIS-Elements Br-2 as software. For each slide, 5 functional lobules were randomly selected to evaluate cortex and medulla thickness and the cortex:medulla ratios (C/M) were calculated as reported by Bozzetta et al. [16] and Vascellari et al. [17]. Briefly, a graduated line was used to measure the extension of the lobule, starting and ending at the interlobular connective or adipose tissue. A parallel line was drawn to measure medulla thickness. The software converted the length of the lines into μm. The cortex thickness was obtained by subtracting the medulla thickness value from the first one and the cortex/medulla ratio (C/M) was calculated.

2.4. Statistical analysis

Data obtained and distributed in a normal way were analysed by ANOVA using the GLM procedure with the JMP-SAS software (2001), including the groups as fixed factor. Data in the results were reported as a quadratic mean ± mean standard error. The data distributed in a non-normal way were categorized by Kruskal-Wallis rank sum test using R software (R Development Core Team, 2013). Differences were determined by Unpaired *t* test or Tukey test and considered significant when $p < 0.05$.

3. Results and discussion

In vivo analyses revealed no DXM residues in the samples belonging to the control group. In the sera collected from treated animals, DXM residues were detected at 0.2 μg L^{-1} at the 28th day of treatment in 2 out of 8 of the analyzed samples. In the urine samples, DXM was detected during the overall treatment period, with maximum levels generally reached at the 14th day with a detection rate of 100%. The fluctuation trends of DXM residues in serum and urine samples observed in animals from the 1th day of administration to the slaughterhouse indicate high variability, which has to be taken into consideration within the National Residue Plan regulation. These data are in discordance with the findings of a previously published study[13] evaluating DXM urine excretion during the drug administration period and after the end of the treatment in beef cattle treated with low-dose of DXM.

At the slaughterhouse (5 days after the last administration), DXM residues were detected neither in serum nor in urine (Table 1), but it is worth to note that DXM was found in all the bile samples (five) belonging to the animals of the treated group, with estimated concentrations in the range of 1–3 μg L^{-1}. Obtained data confirm the main hypothesis that bile can be a valid matrix for detection of treatment at

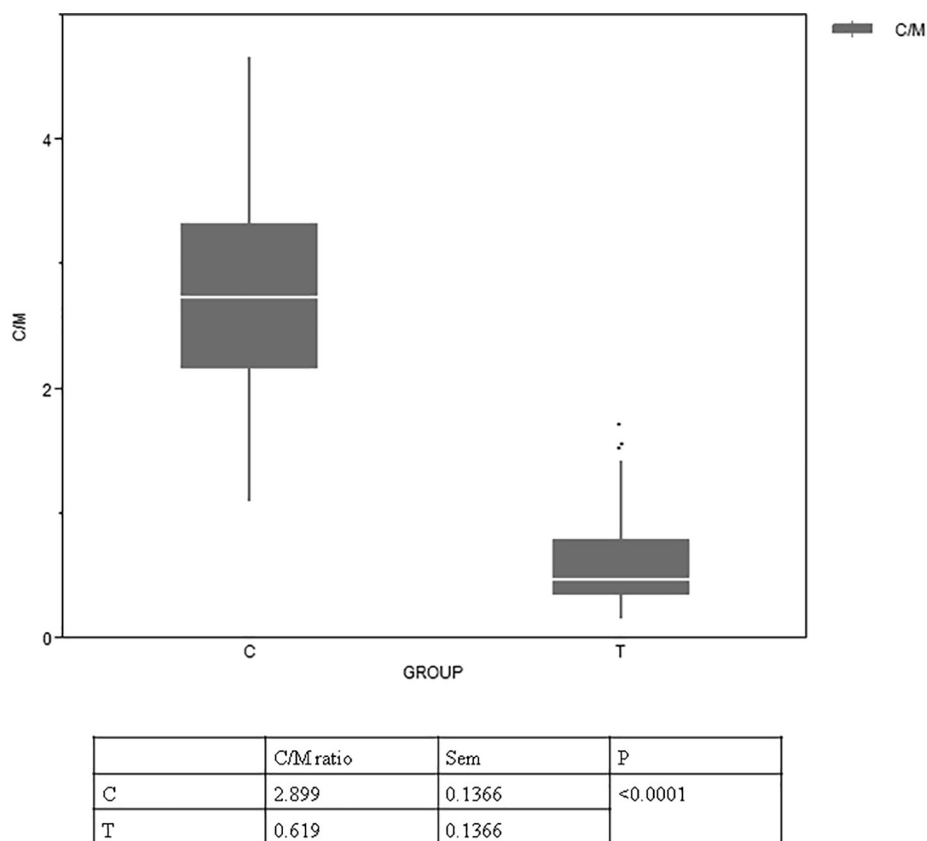|	C/M ratio	Sem	P
C	2.899	0.1366	<0.0001
T	0.619	0.1366	

Fig. 2. Average values of cortex:medulla ratio (C/M) and relative SEM and P.

slaughterhouse level. To support these findings, histological analyses have been conducted including both the routine analysis of atrophy score and a more accurate investigation of cortex/medulla ratio.

All thymus samples belonging to the control group showed mild thymic atrophy (score 1), while in all thymus glands from the treated group a severe adipose tissue infiltration with a severe cortex atrophy of the parenchyma (score 3) was detected (Fig. 1). The thymus scores' distributions resulted significantly different between groups (p < 0.0001). The inter-laboratory agreement was confirmed by K Cohen test with K value = 1 (95% confidence interval) resulting in an optimal concordance according to the Altman scale. Cortex/medulla ratio (C/M) data are shown in Fig. 2 and resulted different (p < 0.0001) between groups.

Severe thymus atrophy evidenced in the study confirm the validity of histological examination adopted in Italy within an official monitoring plan in order to identify farms at major risk of illicit treatment. It offers the advantage of detecting illicit treatments in a short time and at low cost through the effects that anabolic substances determine on target organs (thymus)[13–16] when urine residues are no more detectable. Indeed, results obtained with morphometry confirmed the findings of previous studies, indicating that the C/M ratio was reversed in the treated animals in comparison to the physiological 2:1 ratio[15] improving the approach to tackle the misuse of glucocorticoids in cattle. This is strongly associated with corticosteroids treatment, with an average value for treated group of 0.62. This value is slightly higher than that of Charolaise bulls aged 19–21 months treated with 0.75 mg/day DXM orally administered for 42 days, resulting in a C/M ratio of 0.53[16]. We believe that this small difference may be due not only to the older age of the Charolaise bulls, but mostly to the adoption of a DXM treatment protocol 12 days longer than ours, to the different suspension period and to the use of a slightly higher daily dosage. Our finding confirm that the C/M ratio can be examined as a simple and objective parameter, useful for the detection of illegal treatment with low doses of DXM, which could be applied independently from the thymus score[15,16] and could support chemical analyses.

4. Conclusions

Thymus atrophy and relating cortex:medulla ratio decreases with DXM treatment, which makes it potentially useful as a quantitative indirect marker for the screening histological assay. Furthermore, the methods developed for the chemical determinations of the molecules of interest in the trial presented are suitable for the purpose. DXM urinary excretion was no longer detected in slaughtered subjects 5 days after treatment suspension and these results confirm the high and rapid rate of urinary excretion of DXM, which could represent a weakness in monitoring plans all over Europe. Finally, considering that bile is able to bio-concentrate steroid metabolites, it can be strongly supported as complementary matrix for the improvement of the detection capacity of illicit use of DXM in cattle, since it increases the detection window in comparison to the traditional liquid matrices like serum and urine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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