

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

HPLC-ESI-MS/MS assessment of the tetrahydro-metabolites of cortisol and cortisone in bovine urine: promising markers of dexamethasone and prednisolone treatment

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1611522> since 2016-11-11T16:56:36Z

Published version:

DOI:10.1080/19440049.2016.1202453

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This is the author's final version of the contribution published as:

Chiesa, Luca; Panseri, Sara; Pavlovic, Radmila; Cannizzo, Francesca Tiziana; Biolatti, Bartolomeo; Divari, Sara; Villa, Roberto; Arioli, Francesco.
HPLC-ESI-MS/MS assessment of the tetrahydro-metabolites of cortisol and cortisone in bovine urine: promising markers of dexamethasone and prednisolone treatment. *FOOD ADDITIVES & CONTAMINANTS. PART A. CHEMISTRY, ANALYSIS, CONTROL, EXPOSURE & RISK ASSESSMENT*. 33 (7) pp: 1175-1189.
DOI: 10.1080/19440049.2016.1202453

The publisher's version is available at:

<http://www.tandfonline.com/doi/full/10.1080/19440049.2016.1202453>

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/>

HPLC-ESI-MS/MS assessment of the tetrahydro-metabolites of cortisol and cortisone in bovine urine: promising markers of dexamethasone and prednisolone treatment

Chiesa L¹, Panseri S¹, Pavlovic R², Cannizzo FT³, Biolatti B³, Divari S³, Villa R⁴, Arioli F⁴.

¹a Department of Veterinary Science and Public Health, University of Milan, Milan, Italy.

²d Department of Chemistry, Faculty of Medicine, University of Nis, Nis, Serbia.

³b Department of Veterinary Science, University of Turin, Grugliasco, Italy.

⁴c Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy.

Abstract

The effects of long-term administration of low doses of dexamethasone (DX) and prednisolone (PL) on the metabolism of endogenous corticosteroids were investigated in veal calves. In addition to cortisol (F) and cortisone (E), whose interconversion is regulated by 11 β -hydroxysteroid dehydrogenases (11 β HSDs), special attention was paid to tetrahydrocortisol (THF), allo-tetrahydrocortisol (aTHF), tetrahydrocortisone (THE) and allo-tetrahydrocortisone (aTHE), which are produced from F and E by catalytic activity of 5 α and 5 β -reductases. A specifically developed HPLC-ESI-MS/MS method achieved the complete chromatographic separation of two pairs of diastereoisomers (THF/aTHF and THE/aTHE), which, with appropriate mass fragmentation patterns, provided an unambiguous conformation. The method was linear ($r(2) > 0.9905$; 0.5-25 ng ml⁻¹), with LOQ of 0.5 ng ml⁻¹. Recoveries were in range 75-114%, while matrix effects were minimal. The experimental study was carried out on three groups of male Friesian veal calves: group PL (n = 6, PL acetate 15 mg day⁻¹ p.o. for 31 days); group DX (n = 5, 5 mg of estradiol (E2) i.m., weekly, and 0.4 mg day⁻¹ of DX p.o. for 31 days) and a control group (n = 8). Urine was collected before, during (twice) and at the end of treatment. During PL administration, the tetrahydro-metabolite levels decreased gradually and remained low after the suspension of treatment. DX reduced urinary THF that persisted after the treatment, while THE levels decreased during the experiment, but rebounded substantially after the DX was withdrawn. Both DX and PL significantly interfered with the production of F and E, leading to their complete depletion. Taken together, the results demonstrate the influence of DX and PL administration on 11 β HSD activity and their impact on dysfunction of the 5-reductase pathway. In conclusion, profiling tetrahydro-metabolites of F and E might serve as an alternative, indirect but reliable, non-invasive procedure for assessing the impact of synthetic glucocorticosteroids administration.

KEYWORDS:

HPLC-ESI-MS/MS; Tetrahydrocortisol; dexamethasone; food safety; prednisolone; tetrahydrocortisone

Introduction

Endogenous corticosteroids, mineralocorticoids and glucocorticoids, represent an essential physiological class of steroidal structures that are synthesised in the adrenal cortex. These hormones are involved in a wide range of pathophysiological processes such as the stress response, inflammation, immune function, hydro-electrolyte balance, reproduction and behaviour (Vincenti et al. 2012 Vincenti M, Leporati M, Capra P, Gatto S, Attucci A, Barbarino G, Nebbia C. 2012. A field survey on the presence of prednisolone and prednisone in urine samples from untreated cows. *Food Addit Contam Part A*. 29:1893–1900). The most important glucocorticosteroid is cortisol (F), which exhibits hormonal activity; whereas cortisone (E) is the inactive hormone produced from F by 11 β -hydroxysteroid dehydrogenase (11 β -HSD) (White 2001 White PC. 2001. Synthesis and metabolism of corticosteroids. In: Becker KL, editor. *Principles and practice of endocrinology and metabolism*. Philadelphia (PA): Lippincott Williams & Wilkins; p. 704–719.) (Figure 1). There are two 11 β -HSD isoforms: type I triggers the conversion of E into F, while type II regulates the conversion of F into E. F and E are metabolised to the corresponding A-ring-reduced metabolites: tetrahydrocortisol (THF), allo-tetrahydrocortisol (aTHF), tetrahydrocortisone (THE) and allo-tetrahydrocortisone (aTHE) by a two-step reduction, firstly of the delta 4 double bond, catalysed by 5 α - or 5 β -reductase, followed by the reduction of the 3-keto group, catalysed by 3-oxoreductase (Draper & Stewart 2005 Draper N, Stewart PM. 2005. 11beta-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. *J Endocrinol*. 186:251–271). Studies on human urine have shown that the F/E ratio can be used as a marker of inhibition of 11 β -HSD type II (Tomlinson et al. 2007 Tomlinson JW, Sherlock M, Hughes B, Hughes SV, Kilvington F, Barlett W, Courtney R, Rejto P, Carley W, Stewart PM. 2007. Inhibition of 11beta-hydroxysteroid dehydrogenase type 1 activity in vivo limits glucocorticoid exposure to human adipose tissue and decreases lipolysis. *J Clin Endocrinol Metab*. 92:857–864), while the (THF + aTHF)/THE ratio can be considered a marker of inhibition of 11 β -HSD type I (Raffaelli et al. 2006 Raffaelli A, Saba A, Vignali E, Marcocci C, Salvadori P. 2006. Direct determination of the ratio of tetrahydrocortisol + allo-tetrahydrocortisol to tetrahydrocortisone in urine by LC-MS–MS. *J Chromatogr B*. 830:278–285; Courtney et al. 2008; Courtney R, Stewart PM, Toh M, Ndongo MN, Calle RA, Hirshberg B. 2008. Modulation of 11 β -hydroxysteroid dehydrogenase (11 β HSD) activity biomarkers and pharmacokinetics of PF-00915275, a selective 11 β HSD1 inhibitor. *Clin Endocrinol Metab*. 93:550–556).

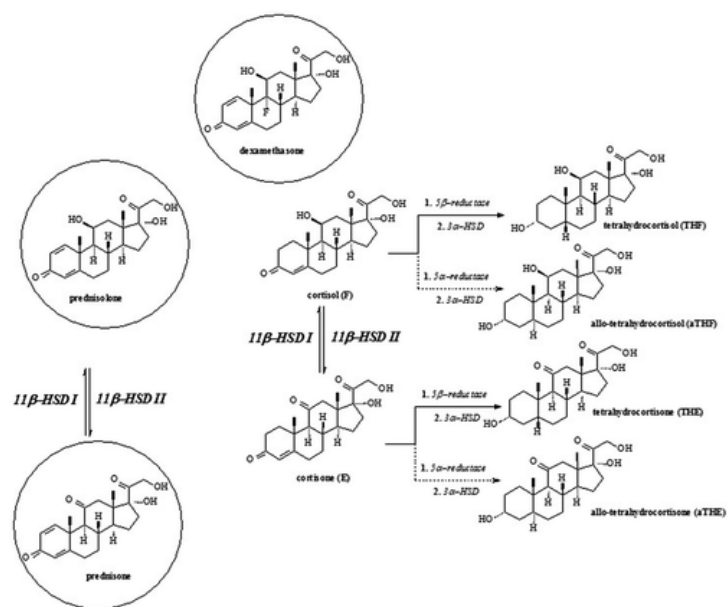


Figure 1. Interconversion of cortisol/cortisone by 11β -hydroxysteroid dehydrogenases (11β -HSDs) and formation of their main A ring-reduced metabolites: tetrahydrocortisol and tetrahydrocortisone. Dashed arrows denote the probable metabolic route that would lead to corresponding diastereoisomers allo-tetrahydrocortisol and allo-tetrahydrocortisone formation. The structures of syntactic glucocorticosteroids are presented as well.

However, due to the important role of natural corticosteroids in several bodily functions, they also have been exogenously administered to animals and humans in order to obtain health benefits and also to improve physical and growth performance. This has led to the chemical synthesis of corticosteroids with increased activity, for example, prednisolone (PL) and dexamethasone (DX). Introduction of a double bond in position C1-C2 of F and the formation of a conjugated π -electronic system of prednisolone results in significantly increased pharmacological activity extending the duration of glucocorticosteroid therapeutic effects. DX is a C9-fluorinated and C16-methylated PL derivative, characterised by pronounced glucocorticoid efficacy associated with a nearly complete loss of mineralocorticoid activity.

Data from the Italian National Residues Plan (PNR) showed an increase in illegal use of DX, while particular attention was drawn to PL, as this corticosteroid may, in certain metabolic conditions, be produced endogenously (Arioli et al. [2010](#) Arioli F, Fidani M, Casati A, Fracchiolla ML, Pompa G. 2010. Investigation on possible transformations of cortisol, cortisone and cortisol glucuronide in bovine faecal matter using liquid chromatography–mass spectrometry. *Steroids*. 75:350–354.; Pompa et al. [2011](#) Pompa G, Arioli F, Cassati A, Fidani M, Bertocchi L, Dusi G. 2011. Investigation of the origin of prednisolone in cow urine. *Steroids*. 76:104–110). However, the persistent use of these substances requires a permanent commitment of public veterinary services for their prevention and control. For this purpose, different analytical methods are commonly used, but the only method that is legally recognised is direct chemical analysis of the administered substances, which, although specific and sensitive, has some limitations. Furthermore, the very quick biotransformation of PL and DX and very low dosage makes their detection particularly

difficult. Therefore, new methodological approaches to reinforce veterinary controls are urgently needed (Cannizzo et al. [2011](#) Cannizzo FT, Capra P, Divari S, Ciccotelli V, Biolatti B, Vincenti M. 2011. Effects of low-dose dexamethasone and prednisolone long-term administration in beef calf: chemical and morphological investigation. *Anal Chim Acta*. 700:95–104; Divari et al. [2011](#) Divari S, Cannizzo FT, Uslenghi F, Pregel P, Mulasso C, Spada F, De Maria R, Biolatti B. 2011. Corticosteroid hormone receptors and prereceptors as new biomarkers of the illegal use of glucocorticoids in meat production. *J Agric Food Chem*. 59:2120–2125; Vascellari et al. [2012](#) Vascellari M, Capello K, Stefani A, Biancotto G, Moro L, Stella R, Pozza G, Mutinelli F. 2012. Evaluation of thymus morphology and serum cortisol concentration as indirect biomarkers to detect low-dose dexamethasone illegal treatment in beef cattle. *BMC Vet Res*. 8:129; Pegolo et al. [2015](#) Pegolo S, Di Camillo B, Montesissa C, Cannizzo FT, Biolatti B, Bargelloni L. 2015. Toxicogenomic markers for corticosteroid treatment in beef cattle: integrated analysis of transcriptomic data. *Food Chem Toxicol*. 77:1–11; Pirro et al. [2015](#) Pirro V, Girolami F, Spalenza V, Gardini G, Badino P, Nebbia C. 2015. Set-up of a multivariate approach based on serum biomarkers as an alternative strategy for the screening evaluation of the potential abuse of growth promoters in veal calves. *Food Addit Contam Part A*. 32:702–711).

Focusing on the veterinary field, the most desirable action of hormones has always been to increase the economic benefit by reducing the costs and obtaining more products of animal origin with shorter production times. Synthetic corticosteroids administered to bovines, results in an improved feed conversion ratio and produce an increase in live weight. This effect probably depends on their direct action as glucocorticoids and on their interference with endogenous F synthesis and metabolism (Möstl et al. 1999 Möstl E, Messmann S, Bagu E, Robia C, Palme R. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. *Zentralbl Veterinarmed A*. 46:621–631. Quinkler and Stewart ([2003](#) Quinkler M, Stewart PM. 2003. Hypertension and the cortisol-cortisone shuttle. *J Clin Endocrinol Metab*. 88:2384–2392) showed that activity of the ‘cortisol-cortisone shuttle’ system is influenced by the presence of exogenous glucocorticoids, like DX and PL.

The effects of administration of synthetic corticosteroids on the urinary profile of F has been investigated in humans in detail (Mazzarino et al. 2006 Mazzarino M, Rossi F, Giacomelli L, Botre F. 2006. Effect of the systemic versus inhalatory administration of synthetic glucocorticoids on the urinary steroid profile as studied by gas chromatography–mass spectrometry. *Anal Chim Acta*. 559:30–36). On the other hand, few studies have been devoted to investigate the correlation between natural and administered synthetic corticosteroids in order to identify indirect biomarkers for eventual treatments (Savu et al. 1996 Savu SR, Silvestro L, Haag A, Sörgel F. 1996. A confirmatory HPLC-MS/MS method for ten synthetic corticosteroids in bovine urines. *J Mass Spectrom*. 31:1651–1363.; Capolongo et al. [2007](#) Capolongo F, Tapparo M, Merlanti R, Ravarotto L, Tealdo E, Gallina G, Montesissa C, Dacasto M. 2007. Illicit treatments in cattle and urinary 6beta-hydroxycortisol/cortisol ratio. *Anal Chim Acta*. 586:228–232; Vincenti et al. [2009](#) Vincenti M, Girolami F, Capra P, Pazzi M, Carletti M, Gardini G, Nebbia C. 2009. Dexamethasone urinary excretion in cattle. *J Agric Food Chem*. 57:1299–1306, [2012](#) Vincenti M, Leporati M, Capra P, Gatto S, Attucci A, Barbarino G, Nebbia C. 2012. A field survey on the presence of prednisolone and prednisone in urine samples from untreated cows. *Food Addit Contam Part A*. 29:1893–1900; Ferranti et al. [2011](#) Ferranti C, delli Quadri F, Palleschi L, Marchiafava C, Pezzolati M, Bozzeta E, Carmelli M, Draisci R. 2011. Studies on the presence of natural and synthetic corticosteroids in bovine urine. *Steroids*. 76:616–625, [2013](#) Ferranti C, Famele M, Palleschi L, Bozzetta E, Pezzolati M, Draisci R. 2013. Excretion profile of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-promoting administration of dexamethasone. *Steroids*. 78:803–812; Nebbia et al. [2014](#) Nebbia C, Capra P, Leporati M, Girolami F, Barbarino G, Gatto S, Vincenti M. 2014. Profile of the urinary excretion of prednisolone and its metabolites in finishing

bulls and cows treated with a therapeutic schedule. BMC Vet Res. 10:237). Therefore, a method to identify natural corticosteroid levels in bovines could be an important tool for the control of illicit corticosteroid treatment in livestock production. Recently, we have published (Pavlovic et al. [2013](#) Pavlovic R, Cannizzo FT, Panseri S, Biolatti B, Trutic N, Biondi PA, Chiesa L. 2013. Tetrahydro-metabolites of cortisol and cortisone in bovine urine evaluated by HPLC-ESI-mass spectrometry. J Steroid Biochem Mol Biol. 135:30–35) that PL treatment influenced 11 β -HSD, which brought up questions about the impact of PL treatment on the THF and THE metabolic rates. Accordingly, the role and importance of reduced forms of F and E and their metabolic rates during treatment with exogenous glucocorticoids are lacking and remain to be established.

In order to understand better the biological relevance of cattle's 11 β -HSDs and their activity in response to treatment with synthetic corticosteroids, it would be necessary to establish baseline levels of the endogenous corticosteroids. Remarkably, not many studies about the concentrations of F and E in bovine urine are present in the literature (Antignac et al. [2002](#) Antignac JP, Le Bizec B, Monteau F, André F. 2002. Study of natural and artificial corticosteroid phase II metabolites in bovine urine using HPLC-MS/MS. Steroids. 67:873–882; Pavlovic et al. [2012](#) Pavlovic R, Chiesa LM, Soncin S, Panseri S, Cannizzo FT, Biolatti B, Biondi PA. 2012. Determination of cortisol, cortisone, prednisolone and prednisone in bovine urine by liquid chromatography–electrospray ionisation single quadrupole mass spectrometry. J Liq Chromatogr RT. 35:444–457; Ferranti et al. [2013](#) Ferranti C, Famele M, Palleschi L, Bozzetta E, Pezzolato M, Draisci R. 2013. Excretion profile of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-promoting administration of dexamethasone. Steroids. 78:803–812). In addition, due to their low concentrations, synthetic and/or (pseudo)natural corticosteroids are often not detectable in biological matrices by official methods. For both purposes, a highly sensitive and selective analysis is required. Our study was focused on the development of a quantitative HPLC-ESI-MS/MS method for simultaneous measurement of F, E, THF, aTHF, THE, aTHE, PL, prednisone (PN) and DX in bovine urine. Our analytical procedure could then be used to make a preliminary estimate of the effects of PL and DX administration on the amounts and corresponding ratios of targeted natural corticosteroids. This would consequently allow an evaluation of the utility of their quantification in studying either the activity of the enzymes responsible for their interconversion or any possible application to revealing the abuse of synthetic corticosteroids in bovine agriculture. This might be considered an important tool, which can be used for exposing illegal use of corticosteroids.

Materials and methods

Chemicals and reagents

F, E, PL, PN and DX were purchased from Sigma-Aldrich (St. Louis, MO, USA) while THF, aTHF, THE and aTHE were obtained from Steraloids, Inc. (Newport, RI, USA). The internal standard PL-d6 (PL-d6) was from CDN Isotopes (Pointe-Claire, QC, 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⁻¹) and stored at -40°C. Working solutions were prepared daily by diluting the stock solutions with a methanol/water (50:50, v/v) mixture. Roche Diagnostics GmbH (Boehringer Mannheim, Germany) supplied beta-glucuronidase from *Escherichia coli* K 12 (EC 3.2.1.31) and β -glucuronidase/arylsulfatase from *Helix pomatia* (EC 3.2.1.31/EC 3.1.6.1).

Sample preparation

The sample purification was analogous to that previously established for human urine by Cuzzola et al. ([2009](#) Cuzzola A, Petri A, Mazzini F, Salvadori P. 2009. Application of hyphenated mass

spectrometry techniques for the analysis of urinary free glucocorticoids. Rapid Commun Mass Spectrom. 23:2975–2982) with slight modifications regarding bovine urine (Pavlovic et al. [2012](#) Pavlovic R, Chiesa LM, Soncin S, Panseri S, Cannizzo FT, Biolatti B, Biondi PA. 2012. Determination of cortisol, cortisone, prednisolone and prednisone in bovine urine by liquid chromatography–electrospray ionisation single quadrupole mass spectrometry. J Liq Chromatogr RT. 35:444–457). Briefly, a 1 ml aliquot of filtered urine was diluted with 2 ml phosphate buffer (0.2 M, pH 6.2) and incubated with 40 μl β -glucuronidase from *E. coli* at 55°C for 4 h. Alternatively, during preliminary experiment we performed deconjugation with 20 μl of β -glucuronidase/sulphatase from *H. pomatia* at 45°C for 4 h. After cooling to RT, 20 μl of 0.1 $\mu\text{g ml}^{-1}$ internal standard was added for a final concentration of 2 ppb. Each sample was extracted using an Oasis HLB cartridge (3 ml, 60 mg, Waters) with Supelco SPE vacuum manifold. The HLB columns were conditioned by passage of 2 ml methanol followed by 2 ml water. The sample was deposited on the column and washed first with 2 ml of 10% methanol and then with 1 ml of 2% ammonia in 50% methanol. The elution to recover the corticosteroids of interest was performed with 2 ml of methanol. The eluate was evaporated under a stream of nitrogen and reconstituted in 1 ml of initial mobile phase.

LC-MS/MS instrumentation and analytical conditions

LC-MS/MS analysis was carried out on a Thermo Finnigan HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), consisting of a Surveyor MS quaternary pump with a degasser, 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 equipped with an electrospray interface (ESI) set in the negative polarity ionisation mode. The analysis was performed in MRM mode. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo.

Chromatographic separation was achieved using a Raptor (Restek) biphenyl column (150 \times 2.1 mm, 2.7 μm) with a Raptor (Restek) biphenyl 2.7 (5 \times 2.1 mm) guard column, which was kept at 30°C. The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents A (aqueous formic acid, 0.1%), and B (100% MeOH) at a flow rate of 200 $\mu\text{l min}^{-1}$. The gradient programme began at 75% A changing to 40% A in the 20th min. Subsequently, the mobile phase A rapidly decreased to 10% in next 2 min, which remained constant up to the 25th min. The mobile phase was returned to initial conditions at 28th min, with the equilibration time that included the interval from the 28th to the 35th min.

Acquisition parameters were optimised in the ion spray mode with direct continuous pump-syringe infusion of standard solutions of the analytes at the concentration of 1 $\mu\text{g ml}^{-1}$ at a flow rate of the syringe of 10 $\mu\text{l min}^{-1}$ and a pump flow rate of 100 $\mu\text{l min}^{-1}$ in the ion source of the mass spectrometer. The optimised parameters were the following: capillary voltage 4000 V; 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.

Assay validation

The proposed procedure was validated in order to evaluate the method in terms of specificity, linearity, sensitivity, precision, matrix effects and recovery.

Specificity

During preliminary experiments, urine from bovines that were variable in age, gender and breed were randomly analysed. During those initial surveys, we found considerable number of samples with very low amounts (traces) of the endogenous compounds of interest: THF, THE, F and E. Furthermore, in some samples it was not possible to detect any of them. Those samples were considered as blank samples for the specificity evaluation. We analysed an appropriate number of representative blank samples ($n = 10$) and checked for any interference (signals, peaks, ion traces) in the region of interest where the target analyte was expected to elute. The occurrence of possible interference from closely related substances (isomers, metabolites, degradation products, endogenous substances, matrix constituents) was tested by monitoring the MRM profiles of investigated compounds at the retention time intervals expected for their elution. The identification of corticosteroids performed by means of LC-MS/MS was done according to European Union guidelines (Commission Decision [2002](#) European Commission. 2002. Decision No. 657/2002. Off J Eur Comm Legis. 221:8–36./657/EC). The presence of the analytes being investigated was assessed by comparing the ratio of the chromatographic retention time of the analyte 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 $\pm 2.5\%$. Three/four transitions were monitored for each analyte with a signal-to-noise (S/N) ratio greater than 3. All ion ratios of compounds from real samples were within the recommended tolerances when compared with the standards. A S/N ratio greater than 3 was considered satisfactory in order to confirm the diagnostic ions. The quantifier ion was the one with the highest S/N value, which was not always the most abundant ion.

Calibration curve preparation, linearity and sensitivity

A standard stock solution (1 mg ml^{-1}) of each corticosteroid was prepared in methanol. Standard spiking solutions at concentrations of $1 \text{ } \mu\text{g ml}^{-1}$ were prepared by dilution of the stock standard solution. Bovine urine samples that in preliminary experiments did not revealed the presence of THF, THE, F and E were diluted with water (1:2) and exploited as blank matrices. Equal aliquots (2 ml) of a blank urine sample were fortified with seven different amounts of corticosteroids (0.5, 1, 2.5, 5, 10, 25 and 50 ng ml^{-1}) and with a constant quantity of internal standard (2 ng ml^{-1}) was subjected to the above described procedure. The analysis was performed in triplicate. The linear calibration curves were established by employing the IS calibration method: the ratio between the peak area of the analytes and the peak area of IS was plotted on the y-axis with scalar concentration levels of standards plotted on the x-axis, leading to the equation $y = m \cdot x + b$. The linear fit was verified by squared correlation coefficients (R^2). Sensitivity was determined as the lower limit of quantification (LLOQ). This parameter was calculated from the lowest point on the calibration curve under the following conditions: (1) the analyte response at the LLOQ should be at least five times the response compared with blank response; and (2) the analyte peak should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120% (USFDA: Guidance for Industry Bioanalytical methods validation). The matrix-match calibration curves that served as quality control samples (QCs) were repeated and were verified at the beginning of each working session involving the analysis of urinary excretion samples.

Precision and repeatability

The overall precision was broken down into two factors: instrumental and method precision. *Instrumental precision* was evaluated by six injections of each analyte at concentrations of 10 ng ml^{-1} according to the optimal operative conditions. In this way, the repeatability of the instrumental system, expressed as RSDs, was acquired. *Method precision* defined as intra-day repeatability of the assay, presented also as RSD, was determined by replicate analyses of blank

urine samples ($n = 6$) fortified with 2.5 and 10 ng ml⁻¹ of each compound. The intermediate (inter-day) method repeatability was determined on six consecutive days by processing the same samples used for intraday precision.

Matrix effects

The matrix effect (ME) was examined by comparing the mean peak areas of the analytes and the IS between two different series. The first series consisted of blank urine samples spiked after the extraction with 5 ng ml⁻¹ of each corticosteroid, while the second series consisted of reference standards. The ME was defined as follows: $ME\% = 100 \times \text{series 1}/\text{series 2}$, and it would indicate the possibility of ionisation suppression or enhancement for analytes and IS. An endogenous matrix effect is implied if the ratio is less than 85% or more than 115% (Matuszewski et al. 2003 Matuszewski BK, Constanzer ML, Chavez-Eng CM. 2003. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 75:3019–3030.).

Recovery

Recovery data for all compounds were obtained in triplicate analyses of urine samples before and after adding known amounts of standards at lower (2.5 ng ml⁻¹) and higher (10 ng ml⁻¹) spiked concentrations. Recovery was calculated from the percentage difference between the quantitation results according to the following equation:

$$\text{Recovery (\%)} = 100 \times \frac{[\text{Conc}_{(\text{urine} + \text{spike})} - \text{Conc}_{(\text{urine})}]}{\text{Conc}_{(\text{spike})}}$$

(1)

Animal study

The study was carried out on 19 male Friesian veal calves randomly assigned to three experimental groups. Group PL ($n = 6$) was administered PL acetate, 15 mg day⁻¹ p.o. for 31 days; group DX ($n = 5$) was administered 5 mg of estradiol (E2) i.m., weekly for 6 weeks, and 0.4 mg day⁻¹ of DX p.o., for 31 days starting from the second injection of E2. Group C ($n = 8$) was untreated. Urine was collected at days 0 (1 week before treatment), 15, 29 and 40 in the early morning after spontaneous micturition by licensed veterinarians, taking care to prevent faecal contamination. The last sampling was carried out 3 days after treatment suspension. The samples were divided into aliquots and stored at -80°C until the analysis was performed.

Statistical analysis

According to the Shapiro–Wilk test, the data were not normally distributed in the majority of the groups tested, therefore non-parametric statistical evaluations were used. The Friedman repeated measures analysis of variance on ranks, followed by all pairwise multiple comparison procedures (Tukey's test), was used to check the differences between the medians of the three datasets. The statistical analyses were performed using Microsoft Excel spreadsheets and Sigma Stat (Statistical Analysis System, version 12.5) statistical software package (Jandel Scientific GmbH, Erkrath, Germany). A p -value < 0.05 was defined as the level of statistical significance.

Results and discussion

Corticosteroids, when administered to promote the growth of food production animals, usually are given in small doses over a relatively long time period. Because of their low concentrations and the fact that there is only limited understanding of their metabolism, the determination of exogenously administered corticosteroids remains difficult and rather problematic. The question that arises is whether the absence of these substances actually means that animals were not subjected to illicit treatment during breeding. An answer on this question cannot be given with certainty; studies on the impact of synthetic analogues on metabolism of endogenous glucocorticosteroids would perhaps provide some explanations. Therefore, the accurate and precise measurement of endogenous corticosteroids in bovine urine can be considered a potentially powerful tool in the identification of cattle that received illegal glucocorticoid treatments.

For this purpose, we employed an HPLC-ESI-MS/MS technique, which was preceded by convenient sample preparation methodology (the hydrolysis and SPE extraction). Sample preparation was generally conducted analogously to our previously published procedure (Pavlovic et al. 2012 Pavlovic R, Chiesa LM, Soncin S, Panseri S, Cannizzo FT, Biolatti B, Biondi PA. 2012. Determination of cortisol, cortisone, prednisolone and prednisone in bovine urine by liquid chromatography–electrospray ionisation single quadrupole mass spectrometry. *J Liq Chromatogr* RT. 35:444–457) with some aspects that were studied in more detail.

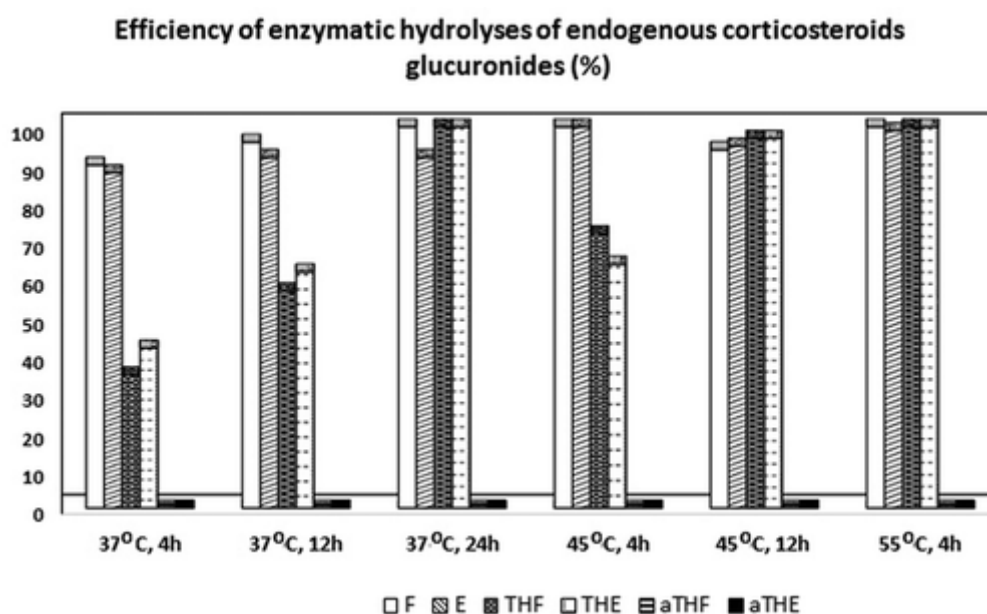
Enzymatic hydrolysis

Based on previous research and our own experience, we first studied the necessity of the deconjugation process of eventually present glucuronide/sulphate conjugates. As far as bovine urine is concerned, the greater part of tetrahydro-metabolites are excreted predominantly as glucuronide conjugates, while F and E are most prevalent in their free form (Antignac et al. 2002 Antignac JP, Le Bizec B, Monteau F, André F. 2002. Study of natural and artificial corticosteroid phase II metabolites in bovine urine using HPLC-MS/MS. *Steroids*. 67:873–882). Arioli et al. (2012 Arioli F, Casati A, Fidani M, Silvestri M, Pompa G. 2012. Prednisolone and prednisone neo-formation in bovine urine after sampling. *Animal*. 6:1023–1029) showed that, as far as PL is concerned, hydrolysis is not indispensable as this corticosteroid is present almost exclusively in its free form. Taking all of this into account, we chose to perform our analysis using the most specific enzyme (*E. coli* K 12 β -glucuronidase) to liberate the tetrahydro-corticosteroids. Therefore, the levels reported herein represent the sum of free and glucuronidated forms. This is an important point of clarification, as it can be speculated that alternative methods that hydrolyse both glucuronide and sulphate conjugates may cause an elevation in final concentrations. To exclude this type of doubt, during preliminary experiments, we performed the simultaneous cleavage of glucuronides and sulphate conjugates by applying *H. pomatia* juice as the enzyme source. The concentration did not vary significantly (less 5% for all compounds), while heterogeneous properties of the *H. pomatia* mixture provoked substantial matrix effects, pronounced background noise and a lower *S/N* ratio. We concluded that the sulphatase activity in addition to glucuronidase activity was not essential in this case.

Special attention was dedicated to aTHF as it has been reported that its glucuronides require more extreme conditions to be cleaved (Cuzzola et al. 2014 Cuzzola A, Mazzini F, Petri A. 2014. A comprehensive study for the validation of a LC-MS/MS method for the determination of free and total forms of urinary cortisol and its metabolites. *J Pharm Biomed Anal*. 94:203–209) and because in our previous study it was not found (Pavlovic et al. 2012 Pavlovic R, Chiesa LM, Soncin S, Panseri S, Cannizzo FT, Biolatti B, Biondi PA. 2012. Determination of cortisol, cortisone, prednisolone and prednisone in bovine urine by liquid chromatography–electrospray ionisation

single quadrupole mass spectrometry. *J Liq Chromatogr RT.* 35:444–457). Hydrolysis experiments of urine samples were carried out varying the incubation time (4, 8 and 12 h) and temperature (37, 45 and 55°C), but once again this metabolite aTHF was not detected in our bovine samples. The same was noticed for aTHE. Finally, the temperature and duration of hydrolysis were optimised to 40 µl of enzyme solution at 55°C for 4 h (Figure 2). No substantial enhancement in yield of any corticosteroids was observed when any of these parameters was increased.

Figure 2. Influence of temperature and incubation time on the extent of enzymatic hydrolysis of conjugated corticosteroids in 1 ml of calves' urine at pH 6.2 with 40 µl β-glucuronidase from *Escherichia coli* K 12 (EC 3.2.1.31). The highest yield of hydrolysis for each compound is fixed as 100% and all other values are given as a percentage of this yield.



Sample SPE purification

Sample purification through an Oasis HLB cartridge was designed to quantitatively extract the corticosteroids of interest and minimise interferences present in the urine matrix. The SPE procedure that we applied was similar to that previously reported for human urine (Cuzzola et al. 2009 Cuzzola A, Petri A, Mazzini F, Salvadori P. 2009. Application of hyphenated mass spectrometry techniques for the analysis of urinary free glucocorticoids. *Rapid Commun Mass Spectrom.* 23:2975–2982), with necessary adjustments for bovine urine (Pavlovic et al. 2012 Pavlovic R, Chiesa LM, Soncin S, Panseri S, Cannizzo FT, Biolatti B, Biondi PA. 2012. Determination of cortisol, cortisone, prednisolone and prednisone in bovine urine by liquid chromatography–electrospray ionisation single quadrupole mass spectrometry. *J Liq Chromatogr RT.* 35:444–457). A particularly important modification was the employment of just 1 ml of bovine urine. Greater initial sample volumes, although they provided higher absolute responses, sometimes caused more pronounced background noise and (in a few cases) presented interfering peaks.

Optimisation of mass spectrometry acquisition and chromatographic separation

Fragmentation patterns of corticosteroids were studied in ESI(–) mode where pseudo-molecular ion species were present as formic acid adducts at $[M + HCOO]^-$. After preliminary trials, in full-scan

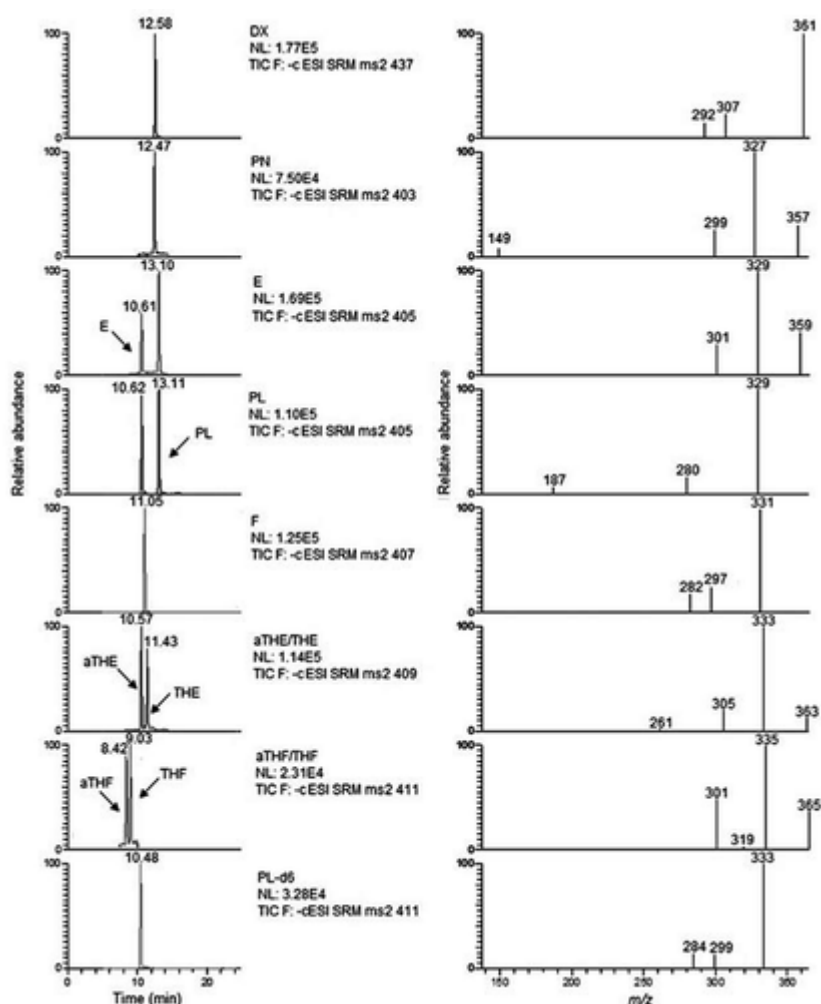
mode from 50 to 500 m/z , the minimum three product ions with the higher S/N ratio for each analyte and internal standard were chosen for identification. The diagnostic product ions (among which is the ion for quantification), the relative intensities, and the collision energies (CEs) are reported in Table 1 and Figure 3. The precise adjustment of CE for each MRM was performed in order to reach the highest sensitivity and specificity for each compound. However, the fragmentation of some analytes that had equal molecular mass (cortisone and PL, for example) was carefully studied in order to enhance the capabilities of the method, avoiding the common transition. The m/z values of the selected parent and daughter ions used for MS detection were generally in agreement with those previously reported by Chiesa et al. (2014 Chiesa L, Nobile M, Panseri S, Sgoifo Rossi CA, Pavlovic R, Arioli F. 2014. Detection of boldenone, its conjugates and androstadienedione, as well as five corticosteroids in bovine bile through a unique immunoaffinity column clean-up and two validated liquid chromatography–tandem mass spectrometry analyses. *Anal Chim Acta*. 852:137–145). Special consideration was focused on fragmentation of the tetrahydro-metabolites, as there is a limited number of publications that deal with the behaviour of their formic acid adducts in an ESI(–) source (Cuzzola et al. 2009 Cuzzola A, Petri A, Mazzini F, Salvadori P. 2009. Application of hyphenated mass spectrometry techniques for the analysis of urinary free glucocorticoids. *Rapid Commun Mass Spectrom*. 23:2975–2982). Our MS/MS conditions were tuned to achieve a mass-spectrometric profile with four characteristic ions, providing the specific product ion ratios that were used for confirmation purposes. The most abundant product ion that corresponds to the cleavage of the side-chain and loss of formaldehyde $[M - CH_2O-H]^-$ were m/z 333 and 335, respectively, for THF and THE, which is in agreement with Zhai et al. (2015 Zhai X, Chen F, Zhu C, Lu Y. 2015. A simple LC-MS/MS method for the determination of cortisol, cortisone and tetrahydro-metabolites in human urine: Assay development, validation and application in depression patients. *J Pharm Biomed Anal*. 107:450–455). The selection of the most abundant product ion was the best option in order to optimise the sensitivity and specificity of the tetrahydro-metabolite characterisations. Interestingly, it was not the case for F and E. The quantifiers that were chosen for them are other than the most abundant ions. The reason is simple: the most intense fragments from F and E are formed by cleavage of the C_{20} – C_{21} bond (ions m/z 329 and 331, respectively), and are also present in some interfering co-eluting compounds. The further fragment (319) of THF is most probably formed when $[M - CH_2O-H]^-$ loses the methane (16 Da) giving $[M - CH_2O-CH_4-H]^-$, which is similar to the finding reported for PL (Savu et al. 1996 Savu SR, Silvestro L, Haag A, Sörgel F. 1996. A confirmatory HPLC-MS/MS method for ten synthetic corticosteroids in bovine urines. *J Mass Spectrom*. 31:1651–1363.; Ferranti et al. 2011 Ferranti C, delli Quadri F, Palleschi L, Marchiafava C, Pezzolati M, Bozzeta E, Carmelli M, Draisi R. 2011. Studies on the presence of natural and synthetic corticosteroids in bovine urine. *Steroids*. 76:616–625). The ion m/z 301 is produced when H_2O subsequently is removed from the steroid moiety (probably via the hydroxyl group from C_{11}) leading to formation of the $[M - CH_2O-CH_4-H_2O-H]^-$ ion. These findings are analogous with those that Mitamura et al. (2014 Mitamura K, Satoh R, Kamibayashi M, Sato K, Iida T, Ikegawa S. 2014. Simultaneous determination of 18 tetrahydrocorticosteroid sulfates in human urine by liquid chromatography/electrospray ionization-tandem mass spectrometry. *Steroids*. 85:18–29) have recently reported describing transitions of different tetrahydrocorticosteroid sulphates. Regarding the THE, complete elimination of the side-chain (cleavage of C_{17} – C_{20} bond) provided a fragment at m/z 305, while for the product ion at m/z 261 structural assignments seem to be too speculative. The molecular $[M - H]^-$ ions (m/z 363 and 365) were identified in second transition, and their appearance was enabled by applying a low collision energy. Turpeinen et al. (2006 Turpeinen U, Markkanen H, Sane T, Hämäläinen E. 2006. Determination of free tetrahydrocortisol and tetrahydrocortisone ratio in urine by liquid chromatography-tandem mass spectrometry. *Scand J Clin Lab Invest*. 66:147–159), with methanol and water as the mobile phase, used those deprotonated molecular ions as a parent in the LC-MS/MS determination of tetrahydro-corticosteroids. Those authors also obtained a slightly different mass spectrum for two tetrahydro-isomers, which in our case was not possible.

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

Analyte	Precursor ion (<i>m/z</i>)	Product ions (<i>m/z</i>)	Tube lens
F	407	282 ₍₃₇₎ , 297 ₍₃₃₎ , 331 ^a ₍₂₀₎	74
E	405	137 ₍₄₅₎ , 301 ₍₂₂₎ , 329 ^a ₍₁₉₎ , 359 ₍₁₃₎	72
PL	405	187 ₍₃₀₎ , 280 ₍₃₅₎ , 329 ^a ₍₁₉₎	71
PN	403	149 ₍₄₅₎ , 299 ₍₂₁₎ , 327 ^a ₍₁₉₎ , 357 ₍₁₂₎	52
aTHE/THE	409	261 ₍₃₇₎ , 305 ₍₂₅₎ , 333 ₍₂₀₎ , 363 ₍₁₅₎	77
aTHF/THF	411	301 ₍₃₇₎ , 319 ₍₂₉₎ , 335 ₍₂₁₎ , 365 ₍₁₅₎	75
DX	437	307 ₍₃₃₎ , 361 ^a ₍₂₀₎ , 391 ₍₁₄₎	81
PL-d6	411	284 ₍₃₇₎ , 299 ₍₃₂₎ , 333 ^a ₍₁₉₎	77

Note: ^aMost abundant product ion.

Figure 3. HPLC-ESI-MS/MS chromatograms and related MS spectra of the studied corticosteroids in a blank urine sample spiked at the concentration of 5 ng ml⁻¹.



Attempts were made to acquire different fragmentation patterns and thus to distinguish aTHF from THF and aTHE from THE, but unfortunately these were without success. Therefore, the chromatographic column and gradient elution programme was carefully optimised until it permitted the complete separation of the isomers (Figure 3). For this purpose, we used a new Raptor Biphenyl Column that employs a phenyl-hexyl stationary phase, with an innovative biphenyl ligand, which is the first time it has been used in corticosteroid separation. The unique composition of biphenyl

groups in sterically favourable positions improves the interactions with steroid fused-ring moieties, resulting in baseline separation of very structurally similar corticosteroids, including two pairs of diastereoisomers. The separation of aTHF and THF was relatively easy to achieve, first of all because of the increased polarity due to presence of three hydroxyl groups. The OH-group from C₁₁ seems especially important as it interacts in a particular manner with the rest of the steroid moiety, which subsequently provokes distinct distribution of aTHF and THE between the mobile and stationary phases. Secondly, aTHF is a conformer where the A/B rings are joined through two equatorial-type bonds (*trans*-decalin type) whereas an axial-equatorial union gives the *cis*-decalin form. The separation of THE and its allo-isomer is an actual problem, not easily overcome and this is related to the C₁₁ keto function. That is why THE and aTHE frequently are determined in human clinical practice as a combination. More often, only THE is considered as the reduced metabolite of E while the level of aTHE is ignored (Główka et al. 2010 Główka F, Kosicka K, Karzniewicz-Łada M. 2010. HPLC method for determination of fluorescence derivatives of cortisol, cortisone and their tetrahydro- and allo-tetrahydro-metabolites in biological fluids. J Chromatogr B. 878:283–289). The reason for such simplification may be the much lower level of aTHE in comparison to THE in human urine (Vierhapper 2000 Vierhapper H. 2000. The stress of being a doctor: steroid excretion rates in internal medicine residents on and off duty. Am J Med. 109:492–494). However, with our chromatographic conditions, baseline separation of those isomers was fully accomplished because, analogously to aTHF/THF, the biphenyl column is capable to distinguish chromatographically A/B ring conformers.

Method validation

Specificity and selectivity were evaluated as described in the materials and methods section, applying the Commission Decision 2002/657/CE. Blank samples ($n = 10$) were analysed and did not show any interference (signals, peaks, ion traces) in the region of interest, where the target analytes were expected to be. The relative retention time of the analytes corresponded to that of the standards, with a tolerance of $\pm 2.5\%$. Relative intensities of the product ions, expressed as a percentage of the intensity of the most intense ion, corresponded to those of the reference analyte. The relative intensities of the detected ions were within the permitted tolerances: the ion ratio of the product ion with relative intensity greater than 50% did not exceed the tolerance of 20%. Nonetheless, if relative ion intensity was in ranges either 20–50% or 20–10% the deviance in intensity was not allowed to be higher than 25% and 30%, respectively.

Seven concentration levels with three replicates each were used to build the regression line using the least square method. The calibration curve equations were used to calculate unknown concentrations in real urine samples of bovines enrolled in the experimental study. Regression coefficients of the curves showed very good linearity for all compounds (Table 2). The LLOQ values (Table 2) indicated that this method could be used for confirmation purposes, as recommended maximal residual levels allowed in urine are set at 2 ng ml^{-1} for DX (CRL Guidance Paper 2007 CRL Guidance Paper. 2007. CRL Guidance paper (7 December 2007). CRLs view on state of the art analytical methods for national residue control plans. 8 pp. Available from: http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/EURL_Empfehlungen_Konzentrationsauswahl_Methodenvalidierungen.pdf?__blob=publicationFile) and 5 ng ml^{-1} for PL (PNR).

Table 2. Validation data summary: linearity, LLOQ and repeatability.

Analyte	Calibration curve (n = 3)	R ²	LLOQ	Spiked level (ng ml ⁻¹)	Repeatability	
					Intra-day (RSD; (n = 6))	Inter-day (RSD; (n = 6))
F	$y = 0.509 (\pm 0.011)x - 0.326 (\pm 0.247)$	0.9905	0.5	2.5	7	13
				10	5	17
E	$y = 0.179 (\pm 0.002)x - 0.080 (\pm 0.047)$	0.9971	0.5	2.5	8	12
				10	10	15
PL	$y = 0.082 (\pm 0.001)x - 0.060 (\pm 0.022)$	0.9972	0.5	2.5	6	17
				10	8	14
PN	$y = 0.286 (\pm 0.004)x - 0.258 (\pm 0.080)$	0.9968	0.5	2.5	10	17
				10	11	18
aTHF	$y = 0.074 (\pm 0.001)x - 0.041 (\pm 0.022)$	0.9957	0.5	2.5	7	20
				10	8	18
THF	$y = 0.090 (\pm 0.001)x - 0.065 (\pm 0.029)$	0.9944	0.5	2.5	6	12
				10	5	19
aTHE	$y = 0.484 (\pm 0.007)x - 0.319 (\pm 0.153)$	0.9960	0.5	2.5	5	12
				10	9	14
THE	$y = 0.405 (\pm 0.004)x - 0.206 (\pm 0.089)$	0.9980	0.5	2.5	9	16
				10	10	16
DX	$y = 0.632 (\pm 0.009)x - 0.598 (\pm 0.191)$	0.9963	0.5	2.5	7	12
				10	11	13

Precision, defined as the closeness of agreement between independent test results obtained under the stipulated conditions, is herein expressed as the repeatability. The intra-repeatability was satisfactory, with the RSD ranging from 6% to 11%, whereas analysis of inter-day repeatability resulted in slightly higher variability (up to 20%). Instrumental precision was between 8% and 17% for all analytes. These results show good repeatability, indicating a stable instrumental response as far as the performance of the HPLC-ESI-MS/MS system is concerned.

The overall recovery of the method, conducted on real urine samples with predetermined corticosteroid concentrations attained by adding known amounts of standards, ranged between 75% and 114% as presented in Table 3. The two main reduced steroids (THF and THE) had the lowest recovery rates, almost certainly because of its particular structure and conformation. Those steroids belong to the 5 α -cholestan series with the *cis*-positioning between A/B ring causing that steroidal structure is not linear as for F and E, e.g., but slightly bended. This reduction in overall molecule surface could, in some extent, compromise efficient retention on hydrophilic-lipophilic balanced polymers from SPE Oasis.

Table 3. Recovery data for natural and synthetic corticosteroids enrolled in study.

Analyte	Urine (ng ml ⁻¹)	Spiked (ng ml ⁻¹)	Urine + spike (ng ml ⁻¹)	Recovery (%)
F	1.4	2.5	2.4	114
		10	9.8	110
E	2.3	2.5	4.3	111
		10	12.4	116
PL	n.d.	2.5	2.8	112
		10	11	110
PN	n.d.	2.5	2.33	93
		10	8.9	89
aTHF	n.d.	2.5	2.4	75
		10	8.8	88
THF	7.2	2.5	9.85	105
		10	16.4	92
aTHE	n.d.	2.5	1.9	76
		10	8.9	89
THE	4.8	2.5	6.8	79
		10	15.0	102
DX	n.d.	2.5	2.1	84
		10	10.8	108

Note: Standard solutions (spike) were added to a urine sample with predetermined corticosteroid levels. The resulting concentration of the mixture (urine + spike) was determined. Concentrations presented are means of a triplicate analysis.

Assessment of matrix effects is mandatory in the validation of a quantitative assay using HPLC-ESI-MS/MS in biological samples, since endogenous impurities can affect the ionisation process and may reduce or increase the efficiency of formation of the desired analyte ions. At the same time, the SPE purification step can be indirectly validated. Our study documented the absence of a significant matrix effect, with aTHF being the analyte that had the most evident ion suppression. This is obviously affected by the presence of electrolytes and ionisable co-eluting species, as this steroid is first to be eluted. Nevertheless, as other validation data, such as sensitivity and linearity, were acceptable and indicated good performance of the developed method, this was not considered a drawback for accurate determination.

Impact of dexamethasone and prednisolone on endogenous corticosteroids production: real sample analysis

An important objective of this study was to apply the developed method to real urine samples from veal calves enrolled in an experimental treatment consisting of long-term PL and DX administration. The first group that we considered was the control group that did not receive any therapy (Table 4). It is worth noting that PL was detected in urine of control animals: median values were 0.72 and 0.74 ng ml⁻¹ at the second and third time points, respectively. This supports the theory that PL can be produced as an endogenous substance, at least for healthy Friesian veal calves. The concentrations found are extremely low and, in some of our samples, barely detectable. Up to now, it has been demonstrated that PL could be generated in food-producing cattle by physiological metabolic processes, possibly under extremely stressful conditions, such as transport and slaughter, or by faecal contamination, as is supported by a few recent studies (Arioli et al. 2010 Arioli F, Fidani M, Casati A, Fracchiolla ML, Pompa G. 2010. Investigation on possible transformations of cortisol, cortisone and cortisol glucuronide in bovine faecal matter using liquid chromatography–mass spectrometry. *Steroids*. 75:350–354.; Ferranti et al. 2011 Ferranti C, delli Quadri F, Palleschi L, Marchiafava C, Pezzolati M, Bozzeta E, Carmelli M, Draisi R. 2011. Studies on the presence of natural and synthetic corticosteroids in bovine urine. *Steroids*. 76:616–625.; Pompa et al. 2011 Pompa G, Arioli F, Cassati A, Fidani M, Bertocchi L, Dusi G. 2011. Investigation of the origin of prednisolone in cow urine. *Steroids*. 76:104–110.). On the basis of these results and the opinion of the European Reference Laboratory, the Italian Ministry of Health has recently enacted a new disposition: a bovine urine sample is considered non-compliant for PL

only when its concentration exceeds 5.0 ppb (Circular of Ministry of Health 2012 Circular of Ministry of Health, Department of Public and Veterinary Health about the opinion of the Consiglio Superiore di Sanità. Sezione IV. 2012 May 22.). Recently, evidence has been provided that veal calves, besides endogenous PL production, revealed remarkable F and E urine concentrations as a response to extremely stressful conditions (Ferranti et al. 2013 Ferranti C, Famele M, Palleschi L, Bozzetta E, Pezzolato M, Draisci R. 2013. Excretion profile of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-promoting administration of dexamethasone. *Steroids*. 78:803–812.). The presumption was made that PL might be formed by the dehydrogenation of F due to the enterohepatic circulation process. Although the determination of the PL biosynthetic route was beyond the aim of this research, and bearing in mind that our experiment was designed to avoid any type of stress, it can be noticed that in the control group PL appeared along with higher F and E concentrations: on day 15, those levels were 4.07, 9.89 and 0.72 ng ml⁻¹ for E, F and PL respectively. PN residue was found only in one urine sample without any correlation to the highest levels of PL, F or E and it is probably due to some individual metabolic state of the animal at this time point.

Table 4. Concentrations of corticosteroids in control bovine urine collected during the experiment.

Corticosteroids	Day 0	Day 15	Day 29	Day 40	Statistical differences
THF	13.00 7.91–23.04 (8/8)	17.75 8.42–49.22 (8/8)	17.26 7.54–24.03 (8/8)	12.36 9.78–17.43 (8/8)	n.s.
THE	6.65 2.74–9.70 (8/8)	6.99 2.77–14.95 (8/8)	7.20 1.80–10.89 (8/8)	15.42 9.15–22.38 (8/8)	n.s.
THF/THE	2.36 1.65–3.82 (8/8)	3.04 2.50–3.89 (8/8)	2.76 2.21–4.18 (8/8)	0.94^{a,b,c} 0.71–1.13 (8/8)	<i>p</i> = 0.003
F	3.70 1.42–5.80 (8/8)	4.07 2.82–5.27 (8/8)	1.7 1.77–3.80 (8/5)	3.13 2.71–6.55 (8/8)	n.s.
E	6.99 2.29–9.15 (8)	9.89 6.29–13.34 (8)	3.28^d 1.56–9.13 (8)	2.18^a 1.60–3.01 (8)	<i>p</i> = 0.011
F/E	0.65 0.53–0.69 (8/8)	0.41 0.39–0.44 (8/8)	0.53 0.45–3.76 (8/5)	1.78^{a,b,c} 1.47–2.25 (8/8)	<i>p</i> = 0.003
PL	0 0–0.76 (8/2)	0.72 0.63–0.79 (8/7)	0.74 0–1.32 (8/5)	0 (8/0)	n.s.
PN	0 (8/0)	0 (8/8)	5.6 (8/1)	0 (8/0)	n.p.
DX	0 (8/0)	0 (8/0)	0 (8/0)	0 (8/0)	n.p.

Notes: Data are reported as the median (ng ml⁻¹) with corresponding 25th–75th percentile. The number of samples/number of positive findings is given in parentheses.

The data obtained from the control group provides preliminary information regarding the physiological levels of A-ring-reduced F and E in non-treated, healthy veal calves, which has not been reported in the literature up to now. The measured values exhibited a high intergroup variation having THF and THF values ranging from 7.54 to 49.22 and from 1.80 to 22.38 ng ml⁻¹, respectively. In general, no official threshold has been established for natural hormone concentrations in bovine urine, mainly due to the fact that concentrations of naturally occurring hormones are highly variable and depend on the type of animal product, breed, gender, age, disease, medication and physiological condition (Antignac et al. 2002 Antignac JP, Le Bizec B, Monteau F, André F. 2002. Study of natural and artificial corticosteroid phase II metabolites in bovine urine using HPLC-MS/MS. *Steroids*. 67:873–882.; Angeletti et al. 2006 Angeletti R, Contiero L, Gallina G, Montesissa C. 2006. The urinary ratio of testosterone to epitestosterone: a good marker of illegal treatment also in cattle? *Vet Res Comm*. 30:127–131; Pleadin et al. 2011 Pleadin J, Terzić S, Peršić N, Vulić A. 2011. Evaluation of steroid hormones anabolic use in cattle in Croatia. *Biotechnol Anim Husb*. 7:147–158). In our research, aTHF and aTHE were below the detection limit, unlike results obtained for human urine (Cuzzola et al. 2014 Cuzzola A, Mazzini F, Petri A. 2014. A comprehensive study for the validation of a LC-MS/MS method for the determination of free and total forms of urinary cortisol and its metabolites. *J Pharm Biomed Anal*. 94:203–209). These findings imply that for the natural corticosteroids in bovine, 5 β -reduction predominates over the 5 α -reductase activity (Figure 1). The virtual absence of 5 α -reduction of F and E is represented visually in the MRM chromatograms illustrated in Figure 3. This indicates that there are substantial differences between human and bovine production of F and E A ring metabolites.

Data obtained from veal calves treated with PL revealed the low concentrations of this glucocorticoid in most, but not all, urine samples collected in the course of treatment (Table 5), which confirms previous findings (Cannizzo et al. 2011 Cannizzo FT, Capra P, Divari S, Ciccotelli V, Biolatti B, Vincenti M. 2011). Effects of low-dose dexamethasone and prednisolone long-term administration in beef calf: chemical and morphological investigation. *Anal Chim Acta*. 700:95–104). Despite the significantly 20-times higher dose administered with respect to DX, it is metabolised much faster and apparently, the depletion pattern does not depend on the administered dose. Three days after treatment suspension, PL was still detected in half of the samples. In the middle of treatment a very low concentration of PN was found (day 25), as it is formed as a metabolite from the interconversion of PL.

Table 5. Urinary concentrations of corticosteroids collected during prednisolone treatment.

Corticosteroids	Day 0	Day 15	Day 29	Day 40	Statistical differences
THF	14.26 8.06–18.62 (6/6)	6.18 4.74–8.75 (6/6)	2.90^a 1.11–4.87 (6/6)	2.40^b 1.96–6.94 (6/6)	<i>p</i> = 0.004
THE	11.93 5.73–20.31 (6/6)	3.86 1.87–4.80 (6/6)	2.40 2.01–3.30 (6/6)	4.40 1.9–6.9 (6/6)	n.s.
THF/THE	1.09 0.79–2.66 (6/6)	2.10 1.48–4.80 (6/6)	0.74 0.53–1.93 (6/6)	1.10 0.54–1.26 (6/6)	n.s.
F	4.41 2.69–8.27 (6/6)	1.07 0.91–1.28 (6/6)	0^a (6/0)	0^b (6/0)	<i>p</i> = 0.001
E	4.27 1.83–8.25 (6/6)	1.49 1.01–2.04 (6/6)	0^a (6/0)	0^b (6/0)	<i>p</i> = 0.001
F/E	1.66 0.44–2.33 (6/6)	0.76 0.66–0.97 (6/6)	–	–	n.p.
PL	0 (6/0)	0.67 0.46–1.19 (6/5)	2.48^a 1.05–6.18 (6/6)	0.39^c 0–1.07 (6/3)	<i>p</i> = 0.001
PN	0 (6/0)	0 (6/0)	0.80 0–2.34 (6/3)	0 (6/0)	n.s.
DX	0 (6/0)	0 (6/0)	0 (6/0)	0 (6/0)	n.p.

Notes: Data are reported as the median (ng ml⁻¹) with corresponding 25th–75th percentiles. The number of samples/number of positive findings are given in parentheses.

n.s., Not significant; n.p., not performed.

Friedman repeated measures analysis of variance on ranks was used to test the significance between the groups. When the analysis was significant, to isolate the group that differs from the others, pairwise multiple comparison procedures (Tukey's test) were applied. The following subscripts refer to statistically significant pairwise difference: ^aday 0 versus day 29; ^bday 0 versus day 40; ^cday 29 versus day 40.

PL significantly interfered with the production of F and E, leading to complete depletion of both. Nebbia et al. (2014 Nebbia C, Capra P, Leporati M, Girolami F, Barbarino G, Gatto S, Vincenti M. 2014). Profile of the urinary excretion of prednisolone and its metabolites in finishing bulls and cows treated with a therapeutic schedule. *BMC Vet Res.* 10:237) also observed a decrease in F and E levels in finishing bulls and cows after therapeutic treatment with PL acetate. This is to some extent in contrast with our preliminary study (Pavlovic et al. 2013 Pavlovic R, Cannizzo FT, Panseri S, Biolatti B, Trutic N, Biondi PA, Chiesa L. 2013). Tetrahydro-metabolites of cortisol and cortisone in bovine urine evaluated by HPLC-ESI-mass spectrometry. *J Steroid Biochem Mol Biol.* 135:30–35) where no differences were found in the F and E excretion time course during PL treatment in male cattle. The plausible explanation for this discrepancy is that those experimental subjects differed in age and consequently in metabolism (veal calves are not yet ruminant). Although in both experiments the same dosage (15 mg day^{-1} for 30 consecutive days) of PL was administered, in veal calves it resulted in approximately double the dose relative to the animals' weight, which averaged 60 kg for veal calves versus 120 kg for beef cattle. Furthermore, considerable impact could be attributed to a different sampling schedule, since the last time point in our previous experiment was at slaughter, which intensified the extremely stressful situation. As was mentioned above, heavy stress conditions apparently favour endogenous PL production that is usually accompanied by an increase in urinary concentrations of F and E (Bertocchi et al. 2013 Bertocchi L, Dusi G, Ghidelli V, Hathaway T, Nassuato C, Casati A, Fidani M, Pompa G, Arioli F. 2013). Investigation of the origin of prednisolone in urine and adrenal glands of cows. Excretion profile of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-promoting administration of dexamethasone. *Steroids.* 78:803–812), while exogenous administration of PL is likely to reduce the production of these main endogenous glucocorticoids. Our results confirm this hypothesis and are enriched with monitoring of the levels of F and E A-ring-reduced metabolites.

As far as THF is concerned, its concentration significantly and gradually decreased during PL treatment, and remained low 3 days after the drug was discontinued. The THE levels showed a tendency to decline, but without statistical significance. Consequently, the THF/THE ratio remained unaffected. This indicated that it was better to consider the absolute values of THF and THE instead of their molar ratio. For this purpose, reference or threshold levels need to be introduced. So far, the metabolic fate of exogenously administered PL is relatively unknown, although some very important studies have been conducted recently (Vincenti et al. 2012 Vincenti M, Leporati M, Capra P, Gatto S, Attucci A, Barbarino G, Nebbia C. 2012). A field survey on the presence of prednisolone and prednisone in urine samples from untreated cows. Excretion profile of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-promoting administration of dexamethasone. *Steroids.* 78:803–812; Nebbia et al. 2014 Nebbia C, Capra P, Leporati M, Girolami F, Barbarino G, Gatto S, Vincenti M. 2014. Profile of the urinary excretion of prednisolone and its metabolites in finishing bulls and cows treated with a therapeutic schedule. *BMC Vet Res.* 10:237). The evident decrease in A-ring-reduced F and E metabolites during the course of PL treatment may represent a new approach that would discriminate between exogenous and endogenous PL origin.

Quantitative data from the urine samples in animals treated with DX are reported in Table 6. This drug was not identified at the first check-up (day 15), but a substantial amount was found in all samples collected at day 25. The results obtained in our experiments could be, only in limited extent, compared with the ones observed in previous studies, mostly because of the striking differences in both dosage and duration of DX administration. The elimination kinetics were in line with previously published data regarding the grow-promotion protocol (Vincenti et al. 2009 Vincenti M, Girolami F, Capra P, Pazzi M, Carletti M, Gardini G, Nebbia C. 2009. Dexamethasone urinary excretion in cattle. *J Agric Food Chem.* 57:1299–1306). The values obtained are

comparable with that obtained in beef cattle (Cannizzo et al. 2011 Cannizzo FT, Capra P, Divari S, Ciccotelli V, Biolatti B, Vincenti M. 2011). Effects of low-dose dexamethasone and prednisolone long-term administration in beef calf: chemical and morphological investigation. *Anal Chim Acta*. 700:95–104) especially when it comes to the amount of drug dosed per kg of animal body weight: 0.4 mg day⁻¹ for veal calves versus 0.7 mg day⁻¹ for beef. The main differences were observed in the samples taken after suspension of the treatment: in previous work no detectable residues were found 6 days after drug withdrawal, whereas in this study, approximately the same quantity detected on day 25 was also measurable 3 days after the end of treatment (day 40). This might be due to the slightly different experimental design, such as the cattle breeds (Friesian versus Charolaise) and animal age (veal calves versus beef). However, the sampling schedule after the end of the treatment (sixth versus third day) has to be taken as the more plausible reason for those findings. Apart from the fact that it is well known that DX reduces F levels (Ljung et al. 1996 Ljung T, Andersson B, Bengtsson B, Bjorntorp P, Marin P. 1996. Inhibition of cortisol secretion by dexamethasone in relation to body fat distribution: a dose-response study. *Obes Res*. 4:277–282; Vincenti et al. 2009 Vincenti M, Girolami F, Capra P, Pazzi M, Carletti M, Gardini G, Nebbia C. 2009. Dexamethasone urinary excretion in cattle. *J Agric Food Chem*. 57:1299–1306.; Ferranti et al. 2011 Ferranti C, delli Quadri F, Palleschi L, Marchiafava C, Pezzolati M, Bozzeta E, Carmelli M, Draisci R. 2011). Studies on the presence of natural and synthetic corticosteroids in bovine urine. (*Steroids*. 76:616–625; Ferranti et al. 2013 Ferranti C, Famele M, Palleschi L, Bozzetta E, Pezzolato M, Draisci R. 2013). Excretion profile of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-promoting administration of dexamethasone. *Steroids*. 78:803–812. the withstanding result from this group is that the low dosage of DX given daily p.o. was capable of completely depleting E and F. This method of drug administration could accomplish circulating steady state concentrations of DX capable of influencing the production of endogenous F much more than the higher intermittent intramuscular dose applied by Capolongo et al. (2007 Capolongo F, Tapparo M, Merlanti R, Ravarotto L, Tealdo E, Gallina G, Montesissa C, Dacasto M. 2007. Illicit treatments in cattle and urinary 6beta-hydroxycortisol/cortisol ratio. *Anal Chim Acta*. 586:228–232). The same study also indicated the urinary free 6β-F/F as new promising screening test for detection of long-term DX illicit administration in cattle, but very recent data suggest that it could not be a reliable biomarker of illicit DX treatment as presence of 6β-F was not always registered (Famele et al. 2015 Famele M, Ferranti C, Palleschi L, Abenavoli C, Fidente R, Pezzolato M, Botta M, Bozzetta E, Draisci R. 2015). Quantification of natural and synthetic glucocorticoids in calf urine following different growth-promoting prednisolone treatments. *Steroids*. 104:196–202.

Table 6. Urinary concentrations of corticosteroids collected during dexamethasone treatment.

Corticosteroids	Day 0	Day 15	Day 29	Day 40	Statistical differences
THF	14.26 8.06–18.62 (6/6)	6.18 4.74–8.75 (6/6)	2.90^a 1.11–4.87 (6/6)	2.40^b 1.96–6.94 (6/6)	$p = 0.004$
THE	11.93 5.73–20.31 (6/6)	3.86 1.87–4.80 (6/6)	2.40 2.01–3.30 (6/6)	4.40 1.9–6.9 (6/6)	n.s.
THF/THE	1.09 0.79–2.66 (6/6)	2.10 1.48–4.80 (6/6)	0.74 0.53–1.93 (6/6)	1.10 0.54–1.26 (6/6)	n.s.
F	4.41	1.07	0^a	0^b	$p = 0.001$

Corticosteroids	Day 0	Day 15	Day 29	Day 40	Statistical differences
	2.69–8.27 (6/6)	0.91–1.28 (6/0) (6/6)		(6/0)	
E	4.27	1.49	0^a	0^b	<i>p</i> = 0.001
	1.83–8.25 (6/6)	1.01–2.04 (6/0) (6/6)		(6/0)	
F/E	1.66	0.76	–	–	n.p.
	0.44–2.33 (6/6)	0.66–0.97 (6/6)			
PL	0	0.67	2.48^a	0.39^c	<i>p</i> = 0.001
	(6/0)	0.46–1.19 (6/5)	1.05–6.18 (6/6)	0–1.07 (6/3)	
PN	0	0	0.80	0	n.s.
	(6/0)	(6/0)	0–2.34 (6/3)	(6/0)	
DX	0	0	0	0	n.p.
	(6/0)	(6/0)	(6/0)	(6/0)	

Notes: Data are reported as the median (ng ml⁻¹) with corresponding 25th–75th percentiles. The number of samples/number of positive findings are given in parentheses.

n.s., Not significant; n.p., not performed.

Friedman repeated measures analysis of variance on ranks was used to test the significance between the groups. When the analysis was significant, to isolate the group that differs from the others, pairwise multiple comparison procedures (Tukey's test) were applied. The following subscripts refer to statistically significant pairwise difference: ^aday 0 versus day 29; ^bday 0 versus day 40; ^cday 29 versus day 40.

No detectable residues were found as far as THF is concerned, at the same sampling points as for F and E whereas THE expressed an interesting behaviour: after complete depletion at day 25, it appeared again in urine 3 days after the withdrawal of DX. This indicates that low dose administration of DX reversibly influences the metabolic pathway, which involves the two-step enzymatic transformation of E to THE by 5 β -reductase, followed by the reduction of the 3-keto group catalysed by 3 α -oxoreductase (Draper & Stewart 2005 Draper N, Stewart PM. 2005. 11beta-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. J Endocrinol. 186:251–271). The short withdrawal period was enough to restore enzymatic activity responsible for E to THE transformation, while it was not the case for the corresponding F to THF conversion. In addition, appearance of THE after DX removal is attributable to prompt reestablishment of 11 β -HSDII activity, as well. This finding qualifies THF and THE as promising biomarkers that could be useful to the evaluation of DX abuse in cattle. Also, it remains to be established whether the complete disappearance of four natural corticosteroids in the course of DX treatment is associated with up-regulation of both 11 β -HSDs in adrenal tissue (Cannizzo et al. 2013 Cannizzo FT, Crispo M, Divari S, Starvaggi L, Cucuzza M, Biolatti B. 2013. Corticosteroid

hormone receptors and prereceptors on hypothalamus-pituitary-adrenal axis of veal calves. LXVII Convegno Nazionale S.ISVet, Società Italiana delle Scienze Veterinarie. Book of abstracts 77.).

Conclusions

The development of analytical procedures based on the HPLC-ESI-MS/MS technique was indispensable to carry out investigations regarding the effects of DX and PL on the metabolism of the main endogenous glucocorticosteroids. The method validation requirements were completely fulfilled: the method was precise, accurate, sensitive and reliable for the above mentioned scope. The presented data provide an indication of the utility of the method for the assessment of 11-hydroxysteroid dehydrogenases and 5 β -reductase activity associated with DX or PL treatment. Further studies are needed to understand the mechanisms of DX and PL action on F and E. In particular, it would be interesting to study more in-depth the origin of the specific behaviour of the corresponding tetrahydro-metabolites during DX and PL administration. Furthermore, it remains to be clarified whether results observed herein might represent a promising tool for the detection of illicit treatment of bovine with DX or PL.