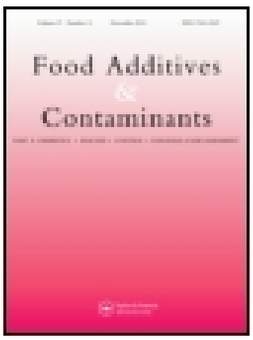

Food Additives & Contaminants: Part A

ISSN: 1944-0049 (Print) 1944-0057 (Online) Journal homepage: http://www.tandfonline.com/loi/tfac20

HPLC-ESI-MS/MS ASSESSMENT OF THE TETRAHYDRO-METABOLITES OF CORTISOL AND CORTISONE IN BOVINE URINE: PROMISING MARKERS OF DEXAMETHASONE AND PREDNISOLONE TREATMENT

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To cite this article: L. Chiesa, S. Panseri, R. Pavlovic, F.T. Cannizzo, B. Biolatti, S. Divari, R Villa & F. Arioli (2016): 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, DOI: 10.1080/19440049.2016.1202453

To link to this article: http://dx.doi.org/10.1080/19440049.2016.1202453

Accepted author version posted online: 16 Jun 2016.
Published online: 16 Jun 2016.

Publisher: Taylor & Francis

Journal: *Food Additives & Contaminants: Part A*

DOI: 10.1080/19440049.2016.1202453

HPLC-ESI-MS/MS ASSESSMENT OF THE TETRAHYDRO-METABOLITES OF CORTISOL AND CORTISONE IN BOVINE URINE: PROMISING MARKERS OF DEXAMETHASONE AND PREDNISOLONE TREATMENT

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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 11β-hydroxysteroid dehydrogenases (11βHSDs), special attention was paid to tetrahydrocortisol (THF), allo-tetrahydrocortisol (aTHF),

tetrahydrocortisone (THE) and allo-tetrahydrocortisone (aTHE), that are produced from F and E by catalytic activity of 5α and 5β 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 LOQQ 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 *p.o.* for 31 days); group DX (n = 5, 5 mg of estradiol (E2) *i.m.*, weekly, and 0.4 mg/day 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 production of F and E, leading to their complete depletion. Taken together, our results demonstrate the influence of DX and PL administration on 11βHSD 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.

1. 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). The most important glucocorticosteroid is cortisol (F), which exhibits hormonal activity; whereas, cortisone (E) is the inactive hormone that is produced from F by 11β-hydroxysteroid dehydrogenase (11β-HSD) (White et al, 2001) (**Fig. 1**.). There are two 11β-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 metabolized 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, catalyzed by 5α- or 5β-reductase, followed by the reduction of the 3-keto group, catalyzed by 3-oxoreductase (Draper & Stewart, 2005). Studies on human urine have shown that the F/E ratio can be used as a marker of inhibition of 11β-HSD type II (Tomlinson et al. 2007), while the (THF + aTHF)/THE ratio can be considered a marker of inhibition of 11β-HSD type I (Raffaelli et al. 2006; Courtney et al. 2008).

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, characterized by pronounced glucocorticoid efficacy associated with a nearly complete loss of mineralcorticoid 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; Pompa et al. 2011). 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 recognized is direct chemical analysis of the administrated 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; Divari et al. 2011; Vascellari et al. 2012; Pegolo et al. 2015, Pirro et al. 2015).

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). Quinkler et al. (2003) 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). 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; Vincenti et al. 2009; Capolongo et al. 2007; Ferranti et al. 2011; Vincenti et al. 2012; Ferranti et al. 2013; Nebbia et al. 2014). 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) that PL treatment influenced 11β-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 better understand the biological relevance of cattle's 11β-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; Pavlovic et al. 2012; Ferranti et al. 2013). 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.

2. MATERIALS AND METHODS

2.1 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, Quebec, Canada). All other chemicals were from Fluka Chemie GmbH (Buchs, Switzerland). Ultrapure water was obtained through a Milli-Q system (Millipore, Molsheim, France). Standard stock solutions were prepared in methanol (1 mg/mL) and stored at −40 °C. Working solutions were prepared daily by diluting the stock solutions with 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).

2.2 Sample preparation

The sample purification was analogous to that previously established for human urine by Cuzzola et al. (2009) with slight modifications regarding bovine urine (Pavlovic et al. 2012). 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 *Helix pomatia* at 45°C for 4 h. After cooling down to room temperature, 20 µL of 0.1 µg/mL 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.

2.3 LC-MS/MS instrumentation and analytical conditions

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

Chromatographic separation was achieved using a Raptor (Restek) biphenyl column (150 x 2.1 mm, 2.7 µm) with a Raptor (Restek) biphenyl 2.7 (5 x 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 µL/min. The gradient program began at 75% A changing to 40% A in 20th min. Subsequently, the mobile phase A rapidly decreased to 10% in next two minutes, 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 28th to 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 µg/mL at a flow rate of the syringe of 10 µL/min and a pump flow rate of 100 µL/min in the ion source of the mass spectrometer. The optimised parameters were the following: capillary voltage 4000V; 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.

2.4 Assay validation

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

2.4.1 Specificity

During preliminary experiments, urine from bovines that were variable in age, gender and breed were randomly analyzed. 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 2002/657/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 ± 2.5%. Three/four transitions were monitored for each analyte with a signal-to-noise ratio greater than three. All ion ratios of compounds from real samples were within the recommended tolerances when compared with the standards. A signal-to-noise (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.

2.4.2 Calibration curve preparation, linearity, and sensitivity

A standard stock solution (1 mg/mL) of each corticosteroid was prepared in methanol. Standard spiking solutions at concentrations of 1 μg/mL 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 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 $Y = m * X + b$. 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 5 times the response compared to blank response; and (2) the analyte peak should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% (FDA: 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.

2.4.3 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 relative standard deviations (RSD), 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.

2.4.4 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 × series 1/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).

2.4.5 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: Recovery (%) = 100 × [$Conc_{(urine + spike)}$ − $Conc_{(urine)}$] / $Conc_{(spike)}$.

2.5 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 *p.o.* for 31 days; group DX (n = 5) was administered 5 mg of estradiol (E2) *i.m.*, weekly for six weeks, and 0.4 mg/day 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 (one 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 three days after treatment suspension. The samples were divided into aliquots and stored at -80 °C until the analysis was performed.

2.6 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) were 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.

3. RESULTS AND DISCUSION

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) with some aspects that were studied in more detail.

3.1 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). Arioli et al.

(2012) 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 (*Escherichia 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 *Helix pomatia* juice as the enzyme source. The concentration did not vary significantly (less 5% for all compounds), while heterogeneous properties of the *Helix 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) and because in our previous study it was not found (Pavlovic et al. 2012). Hydrolysis experiments of urine samples were carried out varying the incubation time (4h, 8h and 12h) and the temperature (37^{o}C, 45^{o}C and 55^{o}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 (**Fig. 2)**. No substantial enhancement in yield of any corticosteroids was observed when any of these parameters was increased.

3.2 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), with necessary adjustments for bovine urine (Pavlovic et al. 2012). 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.

3.3 Optimization 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 **Fig. 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 careful 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). Special consideration was focused on fragmentation of the tetrahydro-metabolites, as there is limited number of publication that deal with the behaviour of their formic acid adducts in an ESI(-) source (Cuzzola et al. 2009). 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 m/z = 335, respectively, for THF and THE, which is in agreement with the recently published paper from Zhai et al. (2015). The selection of the most abundant product ion was the best option in order to optimize the sensitivity and specificity of the tetrahydro-metabolite characterisations. Interestingly, it was not 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 m/z = 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 (16Da) giving $[M–CH_2O–CH_4–H]^-$ which is similar to the finding reported for PL (Ferranti et al. 2011; Savu et al. 1996). 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 analogues with those that Mitamura et al. (2014) 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 m/z = 365) were identified in second transition, and their appearance was enabled by applying a low collision energy. Turpeinen et al. (2006), 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.

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 (**Fig. 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 C_{11} 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 though 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 C_{11} 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). The reason for such simplification may be the much lower level of aTHE in comparison to THE in human urine (Vierhapper, 2000). However, with our chromatographic conditions, baseline separation of those isomers was fully accomplished because, analogously to aTHF/THF, the biphenyl column is capable of chromatographically distinguish A/B ring conformers.

3.4 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 ± 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) and 5 ng mL^{-1} for PL (PNR).

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 relative standard deviation (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 it particular structure and conformation. Those steroids belong to the 5α-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 extend, compromise efficient retention on hydrophilic–lipophilic balanced polymers from SPE Oasis.

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 ionization 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.

3.5 The 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; Ferranti et al. 2011; Pompa et al. 2011). 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). 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). 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.

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 7.54–49.22 and 1.80–22.38 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; Angeletti et al. 2006; Pleadin et al. 2011). In our research, aTHF and aTHE were below the detection limit, unlike results obtained for human urine (Cuzzola et al. 2014). These findings imply that for the natural corticosteroids in bovine, 5β-reduction predominates over the 5α-reductase activity. The

virtual absence of 5α-reduction of F and E is represented visually in the MRM chromatograms illustrated in **Fig. 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). 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.

PL significantly interfered with the production of F and E, leading to complete depletion of both. Nebbia et al. (2014) 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) 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 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 vs. 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; Ferranti et al. 2013), 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 three 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; Ferranti et al. 2013; Nebbia et al. 2014).

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 to 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). The values obtained are comparable with that obtained in beef cattle (Cannizzo et al. 2011) especially when it comes to the amount of drug dosed per kg of animal body weight: 0.4 mg/day for veal calves vs. 0.7 mg/day for beef. The main differences were observed in the samples taken after suspension of the treatment: in previous work no detectable residues were found six days after drug withdrawal, whereas in this study, approximatively the same quantity detected on day 25 was also measurable three days after the end of treatment (day 40). This might be due to the slightly different experimental design, such as the cattle breeds (Friesian vs. Charolaise) and animal age (veal calves vs. beef). However, the sampling schedule after the end of the treatment (sixth vs. 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; Vincenti et al. 2009; Ferranti et a 2011; Ferranti et al. 2013), 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). The same studies 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 resent 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).

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 three 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 5β-reductase, followed by the reduction of the 3-keto group catalyzed by 3α-oxoreductase (Draper & Stewart 2005). 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 11β-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 11β-HSDs in adrenal tissue (Cannizzo et al. 2013).

4. 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 5β-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.

Acknowledgments

This work was partially funded by the Ministero delle Politiche Agricole Alimentari e Forestali SAFORISK project.

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Figure captions

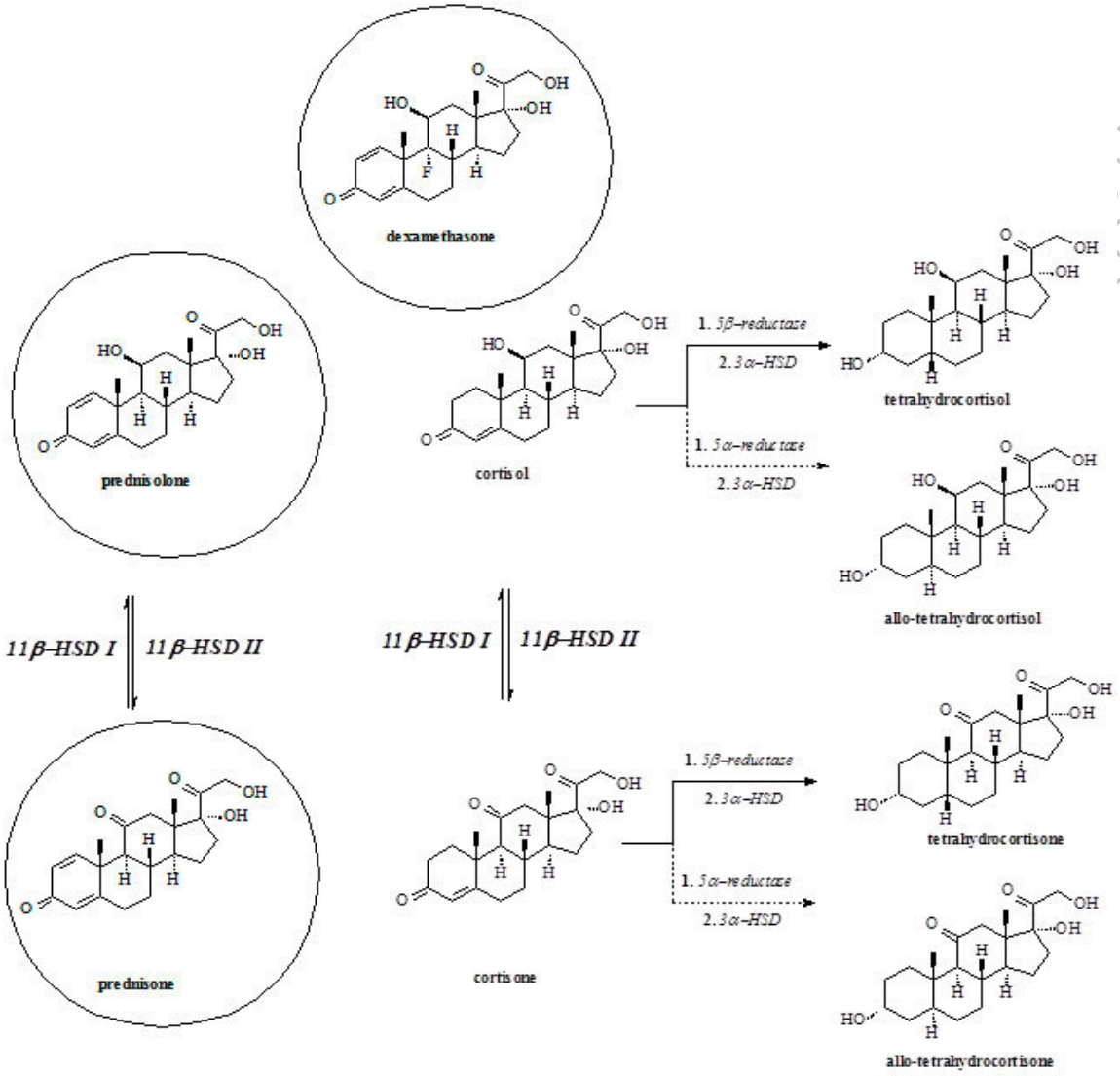

Fig. 1. Interconversion of cortisol/cortisone by 11β-hydroxysteroid dehydrogenases (11β-HSDs) and formation of their main A ring reduced metabolites: tetrahydrocortisol and tetrahydrocortisone. Dashed arrows denote the probable metabolic route that would lead to corresponding diastereoisomers allo-tetrahydrocortisol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

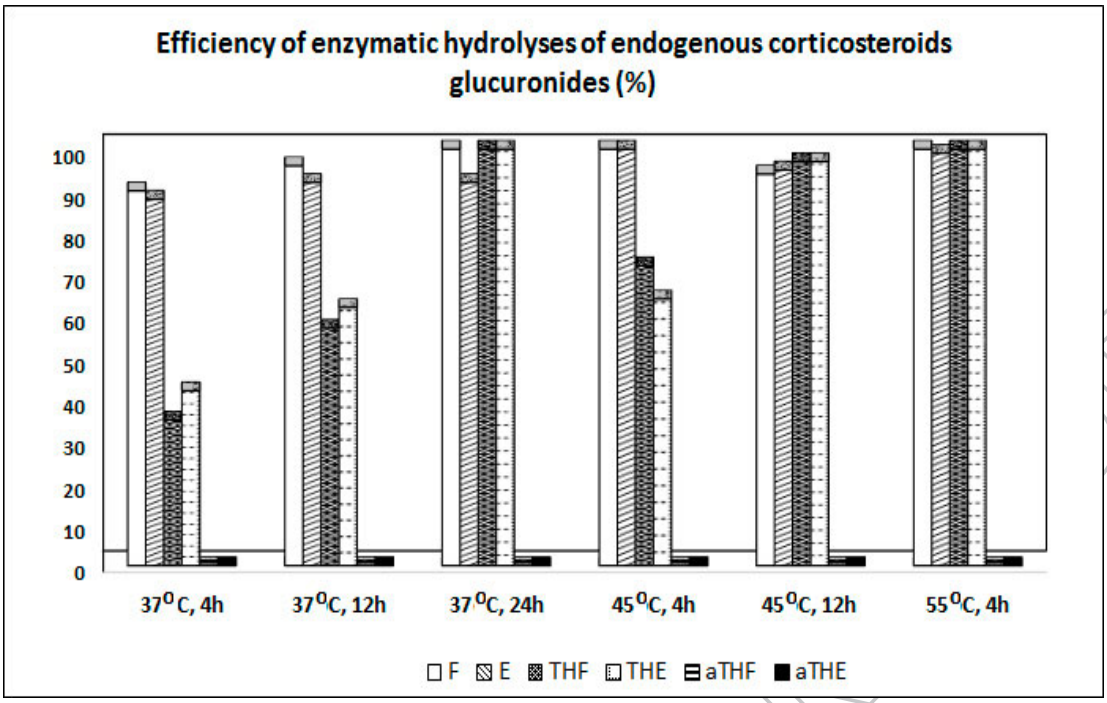

Fig. 2. Influence of temperature and incubation time on the extent of enzymatic hydrolysis of conjugated corticosteroids in 1 mL of calves' urine at pH 6.2 with 40 µL β-glucuronidase from *Escherichia coli* K 12 (EC 3.2.1.31). The highest yield of hydrolysis for each compound is fixed as 100% and all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

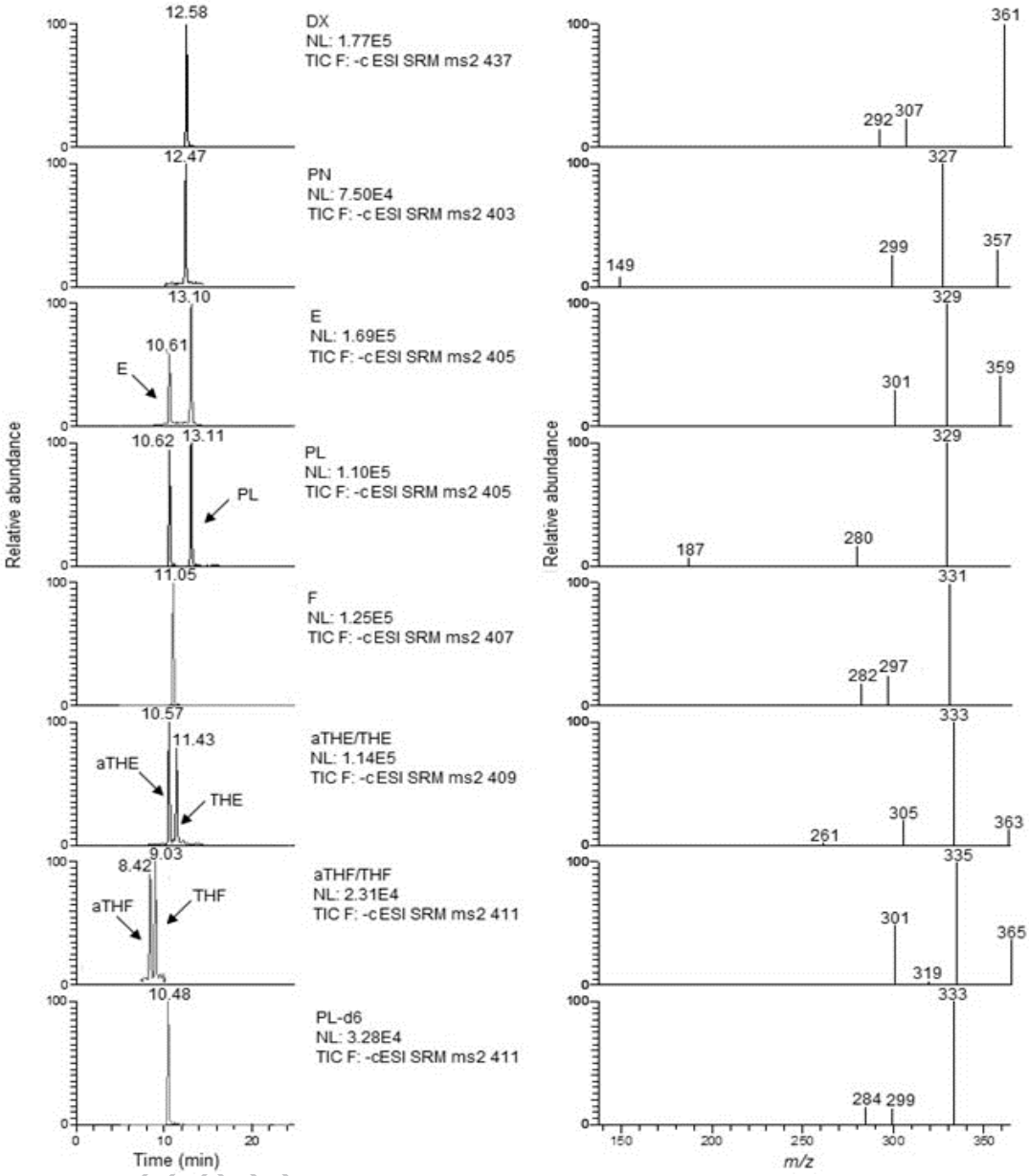

Fig. 3. HPLC–ESI-MS/MS chromatograms and related MS spectra of the studied corticosteroids in a blank urine sample spiked at the concentration of 5 ng mL^{-1}.

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

Analyte	Precursor ion (m/z)	Product ions (m/z)	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

[a]Most abundant product ion

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

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

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	ND	2.5	2.8	112
		10	11	110
PN	ND	2.5	2.33	93
		10	8.9	89
aTHF	ND	2.5	2.4	75
		10	8.8	88
THF	7.2	2.5	9.85	105
		10	16.4	92
aTHE	ND	2.5	1.9	76
		10	8.9	89
THE	4.8	2.5	6.8	79
		10	15.0	102
DX	ND	2.5	2.1	84
		10	10.8	108

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

Table 4. Concentrations of corticosteroids in control bovine urine collected during the experiment. Data are reported as the **median** (ng mL^{-1}) with corresponding 25^{th}–75^{th} percentile. Number of samples/number of positive findings are given in parenthesis.

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)	NS
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)	NS
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)	$P = 0.003$
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)	NS
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)	$P = 0.011$
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)	$P = 0.003$
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)	NS
PN	**0** (8/0)	**0** (8/8)	**5.6** (8/1)	**0** (8/0)	NP
DX	**0** (8/0)	**0** (8/0)	**0** (8/0)	**0** (8/0)	NP

NS – not significant, NP – 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. Following subscripts refer to statistically significant pairwise difference: [a] Day 0 vs Day 40; [b] Day 15 vs Day 40; [c] Day 29 vs Day 40; [d] Day 0 vs Day 29

Table 5. Urinary concentrations of corticosteroids collected during prednisolone treatment. Data are reported as the median (ng mL^{-1}) with corresponding 25^{th}–75^{th} percentiles. Number of samples/number of positive findings are given in parenthesis.

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)	NS
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)	NS
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)	*P* = 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)	*P* = 0.001
F/E	**1.66** 0.44-2.33 (6/6)	**0.76** 0.66-0.97 (6/6)	/	/	NP
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)	*P* = 0.001
PN	**0** (6/0)	**0** (6/0)	**0.80** 0-2.34 (6/3)	**0** (6/0)	NS
DX	**0** (6/0)	**0** (6/0)	**0** (6/0)	**0** (6/0)	NP

NS – not significant; NP – 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. Following subscripts refer to statistically significant pairwise difference: [a] Day 0 vs Day 29; [b] Day 0 vs Day 40; [c] Day 29 vs Day 40

Table 6. Urinary concentrations of corticosteroids collected during dexamethasone treatment. Data are reported as the median (ng mL^{-1}) with corresponding 25^{th}–75^{th} percentiles. Number of samples/number of positive findings are given in parenthesis.

Corticosteroids	*Day 0*	*Day 15*	*Day 29*	*Day 40*	*Statistical differences*
THF	**19.21** 10.86-25.1 (5/5)	**18.83** 5.41-29.34 (5/5)	**0** [a] (5/0)	**0** [b, d] (5/0)	*P* = 0.004
THE	**10.25** 8.41-16.61 (5/5)	**9.09** 1.24-14.25 (5/5)	**0** [a, c] (5/0)	**5.08** 4.00-5.51 (5/5)	*P* = 0.011
THF/THE	**1.76** 0.99-2.24 (5/5)	**2.31** 1.93-8.06 (5/5)	/	/	**NP**
F	**4.40** 3.50-9.90 (5/5)	**1.00** 0.81-9.22 (5/5)	**0** [a, c] (5/0)	**0** [b, d] (5/0)	*P* < 0.001
E	**4.30** 1.30-8.21 (5/5)	**1.30** 0.73-1.94 (5/5)	**0** [a, c] (5/0)	**0** [b, d] (5/0)	*P* < 0.003
F/E	**0.43** 0.99-2.24 (5/5)	**0.93** 0.37-1.45 (5/5)	/	/	**NP**
PL	**0** (5/0)	**0** (5/0)	**0.66** 0-1.24 (5/3)	**0** (5/0)	*P* = 0.029
PN	**0** (5/0)	**0** (5/0)	**0** (5/0)	**0** (5/0)	NP
DX	**0** (5/0)	**0** (5/0)	**1.65** [a, c] 1.47-1.71 (5/5)	**1.46** [b, d] 1.32-1.60 (5/5)	*P* = 0.003

NS – not significant; NP – not performed

Friedman Repeated Measures Analysis of Variance on Ranks was used to test the significance between groups.

When the analysis was significant, to isolate the group that differs from the others, pairwise multiple comparison procedures (Tukey's Test) were applied. Following subscripts refer to statistically significant pairwise difference: [a] Day 0 vs Day 29; [b] Day 0 vs Day 40; [c] Day 15 vs Day 29; [c] Day 15 vs Day 40

Graphical Abstract