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Determination of prednisolone metabolites in beef cattle

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Prednisolone is a synthetic corticosteroid acting on both hydrosaline balance and metabolism that is liable to fraudulent administration to meat-producing animals for growth-promoting purposes. Its use outside strict therapeutic control and prescription is banned by the European legislation, but official controls are hampered by its negligible direct excretion into the urinary matrix. Recent studies reported on a potential endogenous origin of prednisolone in animals subjected to stressful conditions, accounting for its occasional detection in control urines. The objective of the present study was the identification and quantification of prednisolone urinary metabolites to be used as illicit treatment biomarkers in place of the parent drug. An LC-MS/MS screening was conducted on urine samples collected from a bullock intramuscularly administered with prednisolone acetate by using a therapeutic protocol (2 x 0.52 mg kg ^{l} at 48-hour interval). Four prednisolone metabolites were identified: 20β-dihydroprednisolone, 20α-dihydroprednisolone, 6β-hydroxyprednisolone and 20β-dihy-droprednisone; the first was detected at relatively high concentrations. An existing quantitative LC-MS/MS method was expanded and revalidated to include these metabolites. The new analytical method proved sensitive (LODs: 0.35-0.42 ng mL ^{l}*)* and specific and was applied to urine samples collected from eight beef cattle subjected to low-dosage oral administration of prednisolone acetate for a 35-day period, as in standard growth-promoting treatments. 20β-Dihydroprednisolone was detected in all urine samples collected during the treatment, at relatively high concentration (1.2-27 ng mL^{-1}), whereas the prednisolone concentration was virtually negligible (<0.7 ng mL^{-1}). 20β-Dihydroprednisolone was no longer present in almost all samples collected 6 days after the end of the treatment, but trace amounts of this metabolite were found in two urine samples from control animals. 20β-Dihydroprednisolone is proposed as an effective biomarker to test illegal growth-promoting treatments with prednisolone in meat cattle, alternatively to the parent drug.

Keywords: prednisolone; metabolites; urine; beef cattle; LC-MS/MS

Introduction

Natural corticosteroids are synthesised by the adrenal cortex and include two families of substances: mineralocorti-coids and glucocorticoids. Mineralocorticoids influence the electrolyte-water balance, while glucocorticoids act on carbohydrate and protein metabolism (Savu et al. 1996; Courtheyn et al. 2002). Glucocorticoids are known to be very potent anti-inflammatory drugs. Their pharmacological properties led to the synthesis of new molecules with similar structure and enhanced activity, which are extensively used in both human and veterinary clinical practice (Antignac et al. 2001; Deventer & Delbeke 2003). In recent years, a number of synthetic glucocorticoids have been illegally used at very low doses mainly by the oral route to improve the zootechnical performance in meat cattle (Courtheyn et al. 2002; Nebbia et al. 2010). The strong pharmacological activity of most synthetic glucocorticoids used in animal breeding makes the residues of these molecules potentially dangerous for meat and milk consumers. As a consequence, their administration for growth-promoting purposes is strictly banned in the European Union (Stolker & Brinkman 2005; De Brabander et al. 2007).

Prednisolone is among the most frequently detected synthetic glucocorticoids in bovine urine samples during Regione Piemonte (Italy) official controls (Leporati et al. 2012). Unlike other analogues, such as dexamethasone, prednisolone does not contain any halogen atom and its structure closely resembles that of other endogenous steroids, including cortisol. From this structural similarity arises the hypothesis that in meat cattle prednisolone could be generated by physiological metabolic processes, possibly under extremely stressful conditions, such as transport and slaughtering, 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 Union Reference Laboratory, the Italian Ministry of Health has recently enacted a new disposition: a bovine urine sample is considered non-compliant for prednisolone only when its concentration exceeds 5.0 ng mL ^{l} (Circular of Ministry of Health 2012). However, we recently published a study demonstrating that the urine of beef cattle treated with low doses of prednisolone acetate for extended periods of time, as occurs in growth-promoting illegal treatments, may contain prednisolone at 1 ng rnlT^{1} or even below. So far, the metabolic fate of the administered prednisolone acetate is relatively unknown and new approaches including metabolic studies are required to discriminate between exogenous and endogenous prednisolone origin, so as to clearly detect any illicit treatment (Blokland et al. 2007; Pinel et al. 2008; Destrez et al. 2009). The rapid conversion of prednisolone to prednisone is known to occur in cattle (EMEA 1999) as a result of the 11 β-hydro-xysteroid dehydrogenase-mediated oxidation of the -OH group at C11 (Divari et al. 2011). No further recent study is available concerning prednisolone metabolism in cattle, whereas many metabolic studies are reported in humans, starting more than 50 years ago. Vermeulen and Caspi (1959) isolated

20α-dihydroprednisolone, 20β-dihydropredni-solone and 20β-dihydroprednisone in the urines of leukaemic patients treated with prednisolone (see Figure 1).

The formation of the latter metabolites and 6β-hydroxypredniso-lone was also studied by Gray et al. (1956) and Garg and Jusko (1991).

Generally, the oxidative metabolism, catalysed by cytochrome P450 (-CH>-OH), is considered more important than the reductive one (-C = O> -CH-OH), although the latter represents a significant step of steroid hormones phase I biotransformation. The conversion of prednisolone in 20α- and 20β-dihydroprednisolone in man is catalysed by two hydroxysteroid dehydrogenases, namely, 20α-hydroxysteroid dehydrogenase and 3α,20β-hydroxysteroid dehydrogenase (Maser 1995). A scheme of the principal prednisolone metabolites is reported in Figure 1.

In the present study, we reanalysed the urines of beef cattle experimentally treated with low dosages of prednisolone acetate according to a growth-promoting schedule, previously displaying levels of the parent drug either negligible or close to the detection limit, even when a sensitive LC-MS/MS confirmation method was used (Cannizzo et al. 2011; Vincenti et al. 2012). The main objective of our study was to verify the possible presence of prednisolone metabolites, which could justify the rapid disappearance of the parent drug and provide evidence of the low-dosage illicit-simulating treatment administered to the animals. To obtain clear evidence of the possible metabolites formed by prednisolone biotransformation in bovines, our work started with the analysis of the urines of a bullock treated with prednisolone acetate using a therapeutic protocol involving much higher drug dosages compared with the growth-promoting one.

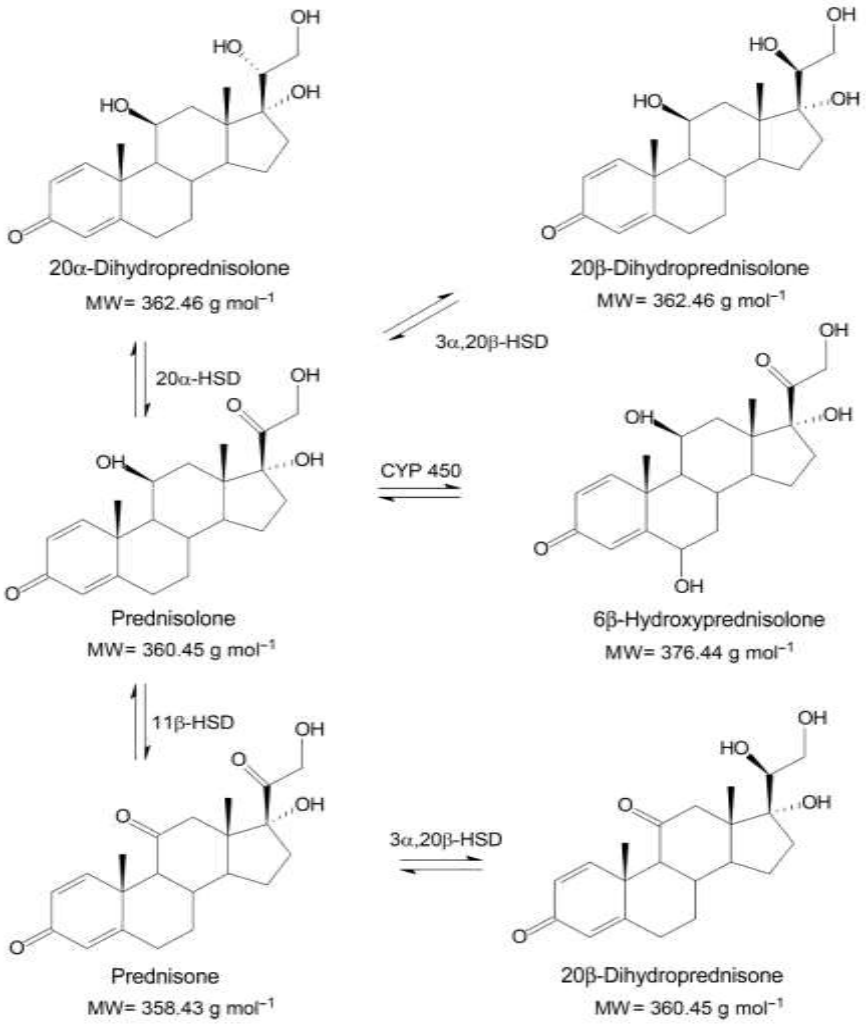

Figure 1. Hypothetical biotransformation pathways of prednisolone. CYP 450, cytochrome P450; HSD, hydroxysteroid dehydrogenase; MW, molecular weight.

Materials and methods

Urine sampling from a bullock pharmacologically treated with prednisolone acetate

A Charolais bullock was treated twice with prednisolone acetate (0.52 mg kg^{-1} intramuscularly at 2-day interval). Urine samples were collected before the treatment and 10 days after the first administration.

Urine sampling from beef cattle treated with low dosages of prednisolone acetate

Animal treatment and experimental design were described in a previous study (Cannizzo et al. 2011). We decided to re-analyse all the urine samples from group D animals (eight Friesian beef cattle, male, 10-17 months old, orally administered with prednisolone acetate 30 mg day^{-1} for 35 days) and the samples collected from two untreated animals (group K negative controls). Urine was collected at days 0, 10, 31 and 41 in early morning after spontaneous micturition by licensed veterinarians, taking care to prevent faecal contamination. The samples were divided into aliquots and stored at -80°C until the analysis was performed. The last sampling was carried out at the slaughterhouse. These samples were analysed once in 2010, and the results were published in Cannizzo et al. (2011). They were re-analysed in 2012, to detect the prednisolone metabolites. The stability of the prednisolone was evaluated by comparing the quantitative results: the repeated analysis of urine samples provided strictly comparable concentrations for prednisolone, as obtained in the previous study conducted on fresh aliquots.

Chemicals, reagents and standard solutions

Diethylether and acetonitrile were supplied by Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide and hydrochloric acid were purchased from Carlo Erba Reagenti (Milan, Italy). Betaglucuronidase/aryl-sulfatase was from Roche Diagnostics (Mannheim, Germany). 20α-Dihydroprednisolone, 20β-dihydroprednisolone, 6β-hydroxyprednisolone and 20β-dihydroprednisone were supplied by Steraloids (Newport, RI, USA). Triamcinolone acetonide-d6 (internal standard (IS)) was from RIVM (Bilthoven, The Netherlands). Stock standard solutions of 20α-dihydroprednisolone, 20β-dihydro-prednisolone, 6β-hydroxyprednisolone and 20β-dihydropred-nisone were prepared in acetonitrile at a concentration of 1 mg $I/^{1}$ and stored at -20°C in the dark.

Sample preparation and instrumental analysis

Sample preparation and instrumental analysis were conducted as reported in Vincenti et al. (2012). Briefly, urine samples were subjected to a liquid/liquid extraction at pH 8.5-9.5 with diethylether after β-glucuronidase/aryl-sulfatase deconjugation. After centrifugation, the supernatant organic phase was transferred into a 10-mL glass tube and evaporated to dryness under nitrogen by heating at 40°C. The residue was re-dissolved in 50 μL of H_2O/ CH_3CN (70/30) solution and transferred into analytical vials for analysis. Instrumental analysis was performed by LC-MS/MS using the chromatographic conditions previously described (Cannizzo et al. 2011). The LC was interfaced to an Applied Biosystems API 4000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in atmospheric pressure chemical ionisation positive ion mode.

Mass spectrometric conditions for qualitative and quantitative analysis

The qualitative search for prednisolone metabolites was conducted on the urine samples collected from the bullock treated with prednisolone acetate using a therapeutic schedule. The tandem mass spectrometer was operated in the product ion scan mode, that is, the protonated molecular ions of hypothetical prednisolone metabolites were selected with the first quadrupole (Q1), then fragmented in the intermediate cell upon collision with nitrogen molecules (Q2) and finally the product ions were analysed by the third quadrupole (Q3) under continuous scanning conditions.

Quantitative analyses were performed in the SRM mode by using three SRM transitions for each analyte, and the experimental conditions are reported in Table 1.

Method validation

The analytical method was originally developed and fully validated for prednisolone and prednisone according to Commission Decision 2002/657/EC. The validation procedure is described in a previous study (Cannizzo et al. 2011), reporting CCα values of 0.67 and 0.66 ng rnL^{-1}, respectively. A simplified validation was executed on the present analytical method, which was modified to include 20β-dihydroprednisolone, dihydroprednisolone, 6β-hydroxyprednisolone and 20β-dihy-droprednisone. Specificity, linearity, LOD, LOQ, precision (in terms of intra-laboratory repeatability), accuracy and trueness were estimated for these metabolites.

Table 1. Mass spectrometric acquisition parameters for SRM operating mode.

Analyte	Retention time (min)	Precursor ion *m/z*	Declustering potential (V)	Product ions	Collision energy (V)
6β-Hydroxyprednisolone	4.25	377.3	65	**377.3→341.2 Q**	15
				377.3→323.2	21
				377.3→359.3	13
20α-Dihydroprednisolone	10.32	363.3	43	**363.3→267.4 Q**	18
				363.3→291.3	20
				363.3→309.3	17
20β-Dihydroprednisolone	11.26	363.3	37	**363.3→345.2 Q**	15
				363.3→267.3	20
				363.3→291.3	22
20β-Dihydroprednisone	12.23	361.3	55	**361.3→153.2 Q**	16
				361.3→297.3	25
				361.3→313.2	11
Prednisolone	16.95	361.3	55	**361.3→279.2 Q**	24
				361.3→265.2	18
				361.3→223.2	29
Prednisone	17.70	359.3	70	**359.3→313.2 Q**	19
				359.3→295.2	20
				359.3→267.2	21
Triamcinolone acetonide D6	26.97	441.4	60	**441.4→421.3 Q**	13

Note: Q, Quantifier transition.

.Identification criteria and specificity

Identification criteria for the analytes were established according to international guidelines (European Commission 2002). Three SRM transitions were considered for each analyte. Variations in relative SRM transition intensities were judged compliant with respect to the corresponding standard within a ±20% error. Ten different blank urine samples were analysed. The occurrence of possible interferences from endogenous substances was tested by monitoring the SRM profiles characteristic for each investigated compound at the retention time interval expected for their elution. A signal-to-noise ratio of <3 was considered satisfactory to verify the method specificity.

Linearity

The linear calibration model was checked by analysing (three replicates) blank urine samples spiked with standard solutions at six final concentrations (0, 1, 2 3, 5 and 10 ng mL ^{l}*)*. Linearity fit was roughly verified by using squared correlation coefficients *(R^2).*

LOD and LOQ

The LOD was calculated by extrapolation from the three lowest non-zero levels, as the concentration of the analyte that gives a signal (peak height) equal to the average background of the blank (Sblank) plus three times its standard deviation (LOD = Sblank + 3Sblank). LOQ values were verified experimentally as the first non-zero level of the calibration curves for each analyte

Precision, accuracy and trueness

Precision (in terms of intra-laboratory repeatability), accuracy and trueness were estimated as a percentage of the theoretic value, by quantifying the analytes using the Internal Standard (IS) correction factor. Five replicates at three concentration levels (1, 3 and 10 ng mL ^{h}*)* were averaged for trueness evaluation.

Results and discussion

Qualitative analysis of urine samples

The qualitative exploration of potential prednisolone metabolites was carried out on the urine samples collected from a bullock treated twice with dosages of prednisolone acetate higher than those used for growth-promoting purposes, following standardised conditions for pharmacological treatments. Under these conditions, it was expected that the hypothetical metabolites could be found in urine at higher concentrations than after a long-term low-dosage treatment.

Using a standard chromatographic run, the tandem mass spectrometer was programmed so as the first quad-rupole (Q1) sequentially selected the protonated molecular ions of the potential metabolites depicted in Figure 1, while the second quadrupole (Q3) was operated in the continuous scanning mode to collect the corresponding product ion spectra. Following this procedure, the most interesting results were observed from the product ion spectra obtained after the isolation of *m/z* 363.5 on Q1. Figure 2(A) reports the comparison of the *m/z* 363.5 chromatographic profiles obtained from the urines collected before the treatment and 10 days after the first administration, respectively. A high chromatographic peak is evident at the retention time of 11.26 minutes only in the profile from the urine collected after the treatment. The corresponding product ion spectrum, depicted in Figure 2(B), exhibits the consecutive losses of four water molecules from the protonated molecular ion, which are compatible with the structure of both 20α-dihydroprednisolone and 20β-dihydroprednisolone, that is, two metabolites already found in the studies conducted on humans (Vermeulen & Caspi 1959).

Confirmation that the chromatographic peak detected at a retention time of 11.26 minutes could be attributed to 20β-dihydroprednisolone was obtained by the analysis of a 20β-dihydroprednisolone analytical standard solution at a concentration of 10 μg mL^{-1}. Identical retention time (Figure 2C) and product ion spectrum (Figure 2D), within the random experimental error, were observed from the pure standard with respect to the real urine sample. To exclude possible co-elution and interference from the 20α-dihydroprednisolone isomer, the latter was also analysed as a pure analytical standard solution at 10 μg mL^{-1} concentration. The results are reported in Figure 2(E) and (F), showing similar product ion spectrum, but significantly different retention time with respect to the 20β-isomer, as expected. It is also evident that the chromatographic separation between the two isomers is so large that easy recognition of the two potential metabolites is possible, but 20α-dihydroprednisolone could not be detected in these preliminary experiments. Likewise, neither 6β-hydroxyprednisolone nor 20β-dihydropredni-sone could be picked out from product ion scanning experiments, possibly because of the limited sensitivity of the technique. Thus, the analysis on the same urine samples was repeated using the SRM mode of operation, to increase the sensitivity, after appropriate method validation.

Validation of the quantitative analytical method

An overview of the results obtained for analytical method validation is given in Table 2.

Specificity

The SRM chromatographic profiles obtained from 10 blank matrix samples did not show the presence of any significant signal (S/N < 3) at the relative retention time typical of the studied compounds, indicating that the method is specific, and no interfering substance is present in the biological matrice.

Linearity

Calibration curves were linear in the range 1-10 ng mL^{-1}. The calibration fit showed squared correlation coefficients (R^2) of 0.9973, 0.9980, 0.9973 and 0.9949 for 20β-dihy-droprednisolone, 20α-dihydroprednisolone, 6β-hydroxy-prednisolone and 20β-dihydroprednisone, respectively.

LOD and LOQ

LOD values, obtained by extrapolation from the first three levels of the calibration curves, ranged between 0.35 and 0.42 ng mL \ These values are very similar to one another and demonstrated that the sensitivity of the method for structurally similar substances is quite homogeneous. Therefore, LOQ values were also consistently verified at 1 ng mL^{-1} for all analytes, making it possible to experimentally test precision, accuracy and trueness at the same LOQ value.

Precision, accuracy and trueness

Precision (as within-laboratory repeatability) was expressed by the variation coefficient from five replicated samples: it ranged between 2% and 8%. Accuracy was satisfactory for all analytes, as it showed a maximum error of ±13%, and proved not to depend on their concentration. Trueness ranged from 96% to 108% of the expected value, confirming the appropriateness of the present method for quantitative determinations.

Analysis of urine samples from high- and low-dosage-treated animals

The urine samples used for qualitative analysis were re-analysed by using the SRM mode of operation. The increased sensitivity allowed by the SRM technique is expressed in Figure 3, showing the chromatographic profiles of the three SRM transitions specifically selected to highlight the possible presence of 20α-dihydropredniso-lone. Alongside the out-of-scale peak found at about 11.20 minutes, owing to the 20β-isomer, a new peak appeared in all profiles at the retention time of 10.32 minutes, matching that of the 20β-dihydroprednisolone pure standard. Likewise, the chromatographic profiles of the six SRM transitions that selectively monitored the presence of 6β-hydroxyprednisolone and 20β-dihydro-prednisone (three SRM transitions each) showed coincident peaks for the real urine sample and pure standard solutions of the same substances (Figure 4). This demonstrated that 20β-dihydroprednisolone, 20α-dihydro-prednisolone, 6β-hydroxyprednisolone and 20β-dihydro-prednisone are all bovine metabolites of prednisolone, but the first compound is produced to a much greater extent than the other metabolites.

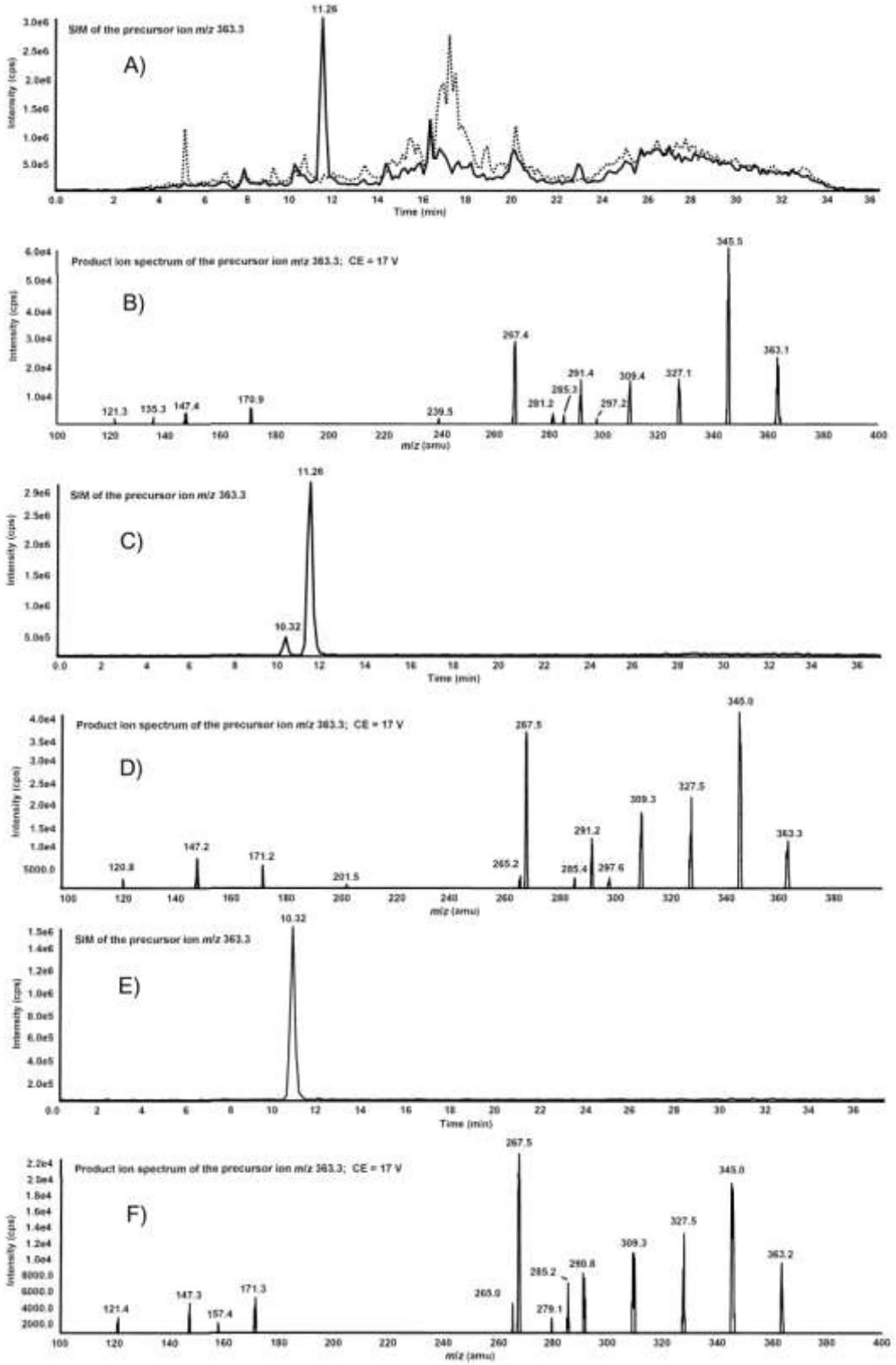

Figure 2. (A) Superimposed mass chromatograms of the *m/z* 363.3 precursor ion, obtained from the urine samples of a pharmacologically treated bullock, collected before the treatment (dotted line) and 10 days after the first treatment (continuous line). (B) Product ion spectrum of the selected *m/z* 363.3 precursor, collected at 11.26 minutes, from the latter analysis. (C) Mass chromatogram of the *m/z* 363.3 precursor ion, obtained from a 10 μg mL^{-1} solution of 20β-dihydroprednisolone. (D) Product ion spectrum of the selected *m/z* 363.3 precursor, collected at 11.26 minutes, from the 10 μg mL^{l} 20β-dihydroprednisolone standard solution. (E) Mass chromatogram of the *m/z* 363.3 precursor ion, obtained from a 10 μg mL~ solution of 20α-dihydroprednisolone. (F) Product ion spectrum of the selected *m/z* 363.3 precursor, collected at 10.32 minutes, from the 10 μg mL^{l} 20α-dihydroprednisolone standard solution. CE, collision energy.

Table 2. Validation results.

Analyte	Repeatability (CV%)			Measured concentrations		
	1 ng mL^{-1}	3 ng mL^{-1}	10 ng mL^{-1}	1 ng mL^{-1}	3 ng mL^{-1}	10 ng mL^{-1}
20β-Dihydroprednisolone	5%	7%	5%	1.05	3.04	9.93
				1.09	3.21	9.42
				0.97	3.31	10.0
				1.02	3.29	9.07
				0.99	2.81	10.2
			Trueness (%)	102%	104%	97%
	LOD (ng mL^{-1}) = 0.35					
	LOQ (ng mL^{-1}) = 1.00					
20α-Dihydroprednisolone	8%	5%	2%	0.93	3.09	9.69
				1.06	3.38	10.1
				0.88	3.17	9.92
				0.99	3.45	9.73
				1.04	3.12	9.50
			Trueness (%)	98%	108%	98%
	LOD (ng mL^{-1}) = 0.36					
	LOQ (ng mL^{-1}) = 1.00					
6β-Hydroxyprednisolone	4%	6%	5%	1.00	2.93	9.90
				1.05	3.30	10.0
				1.01	2.86	9.90
				1.06	3.18	9.09
				1.12	3.11	9.13
			Trueness (%)	105%	103%	96%
	LOD (ng mL^{-1}) = 0.41					
	LOQ (ng mL^{-1}) = 1.00					
20β-Dihydroprednisone	6%	8%	2%	1.14	2.91	10.5
				1.07	3.43	10.0
				0.97	3.26	10.0
				1.01	2.93	9.90
				1.05	2.97	10.1
			Trueness (%)	105%	103%	101%
	LOD (ng m^{L-1}) = 0.42					
	LOQ (ng mL^{-1}) = 1.00					

The subsequent step of our work was to explore the possible presence of the same metabolites in the trial urine samples of beef cattle treated with a low dosage (30 mg day^{1}) of prednisolone acetate for an extended period of time. In our previous work (Cannizzo et al. 2011), we found that the extremely low concentration of prednisolone detected in the urine samples contributed only to a negligible extent to the overall drug excretion. Even the expected partial interconversion of prednisolone to prednisone could not compensate for the missing parent drug, but no other metabolites could be put forward at that time.

The extended analytical protocol for metabolites' monitoring developed in the present study was applied to the urine samples collected from a group of eight treated beef cattle (identified as group D, in the preceding study) plus two untreated animals, used as negative controls. Among the target metabolites, none of the minor ones (i.e., 20α-dihydroprednisolone, 6β-hydroxyprednisolone and 20β-dihydroprednisone) could be detected. In contrast, the major prednisolone metabolite, namely, dihydroprednisolone, was also found in these urine samples, at concentrations largely exceeding the residual parent drug content.

The sensitivity of the analytical method towards the target metabolites can be appreciated from the left side of Figure 5, which presents the SRM chromatographic profiles characteristic for 20β-dihydroprednisolone and obtained from a blank urine sample spiked with 20α-and 20β-dihydroprednisolone at the final concentration of 1 ng mL '. Much larger concentrations of 20β-dihydro-prednisolone are observed in the urine samples of the treated animal, as is evident from the SRM chromatographic profiles obtained from the urine sample collected at the 10th day of treatment from the animal identified as 28 D (Figure 5, central column). However, trace amounts of 20β-dihydroprednisolone were also occasionally detected in the urine samples collected from control animals, as is shown on the right side of Figure 5.

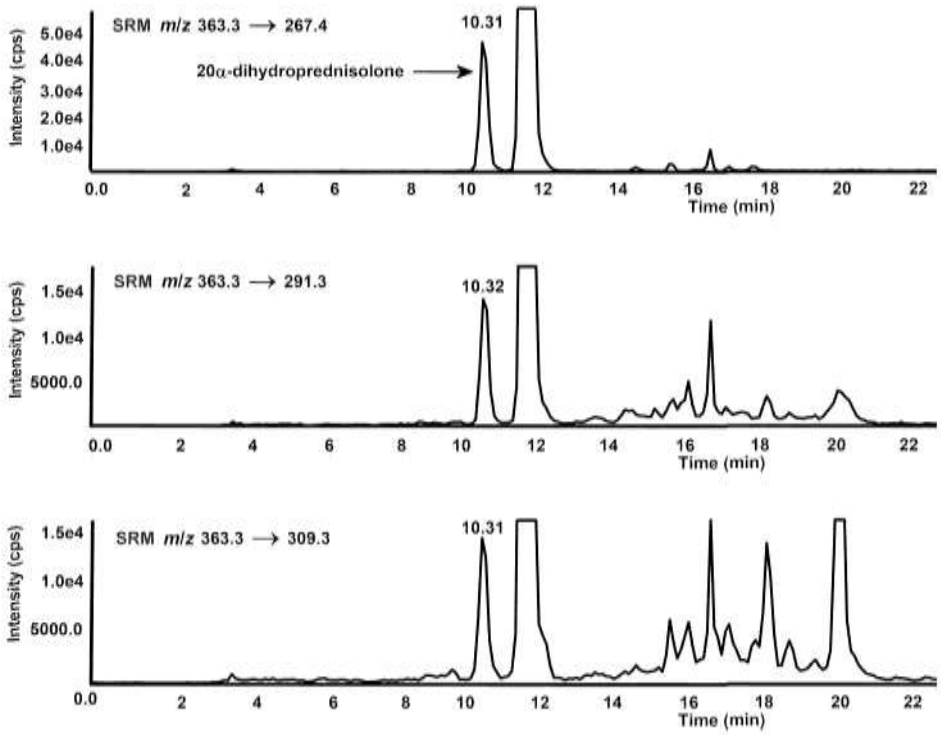

Figure 3. Chromatographic profiles of the three SRM transitions distinctive for 20α-dihydroprednisolone from the urine sample collected 10 days after the first pharmacological administration of prednisolone acetate to a bullock.

It should be noted that control animals were breeded under strictly controlled conditions and exogenous contribution can be excluded. However, this result has to be confirmed on a larger set of samples and animals by using a dedicated analytical method. If confirmed, the estimation of a threshold value for 20β-dihydroprednisolone concentration in the urine of untreated animals will provide sure discrimination between treated and untreated animals.

The quantitative results obtained from the urine of treated and untreated beef and analysed in duplicate are reported in Table 3. Taking into account that the low-dosage treatment with prednisolone acetate lasted 35 consecutive days, the most striking result deduced from Table 3 is that the urine of treated beef cattle showed virtually no presence of prednisolone even during the treatment, whereas its main metabolite, namely, 20β-dihydropredni-solone, was detected at relatively high concentrations (up to 27 ng mL ^{l}) in all treated animals. Another notable observation is that the urine samples of almost all treated beef cattle became negative to both 20β-dihydropredniso-lone and its precursor just 6 days after the end of the treatment. For comparison, low but measurable urinary levels (0.5-1.5 ng mL ^{l}) of either prednisolone or prednisone were found 31 days after the second administration in bullocks and cows subjected to the same pharmacological protocol described in the bullock from this study (Nebbia et al. unpublished results). The only positive sample at day 41 was observed for animal 31 D (1.9 ng mL ^{l}), also presenting a metabolite peak delayed (maximum at day 31) with respect to other animals (with a concentration of almost 21 ng mL ^{l}).

Conclusions

The identification of 20β-dihydroprednisolone as a major prednisolone metabolite in cattle, together with the assessment that its concentration in the urine of treated animals is relatively high, on the one hand provides a way to describe the depletion and fate of prednisolone and may explain the limited urinary excretion of the parent compound in such species. On the other hand, it suggests a possible approach to detect illegal pharmacological treatments. Both perspectives need further investigation to make the fundamental metabolic pathways of prednisolone in bovines clear, and further metabolites are likely to be identified in the future to possibly be used as biomarkers for discriminating exogenous administration of the drug from alleged endogenous production under stress conditions. However, we believe that a first crucial step was accomplished towards the recognition of illegal growth-promoting prednisolone treatments of beef cattle in animal breeding that could modify the monitoring strategy of official controls, most likely shifting from the parent drug to its metabolites. Our data show that dihydroprednisolone is detected and measured in all treated animals, unlike prednisolone, which is detected only in a few cases and, notably, in concentrations largely below the threshold of 5 ppb, established by the Italian Ministry of Health. Therefore, all the studied samples are compliant with the current legislation, although they come from surely treated animals. It stems that new markers able to clearly detect illicit treatments are needed.

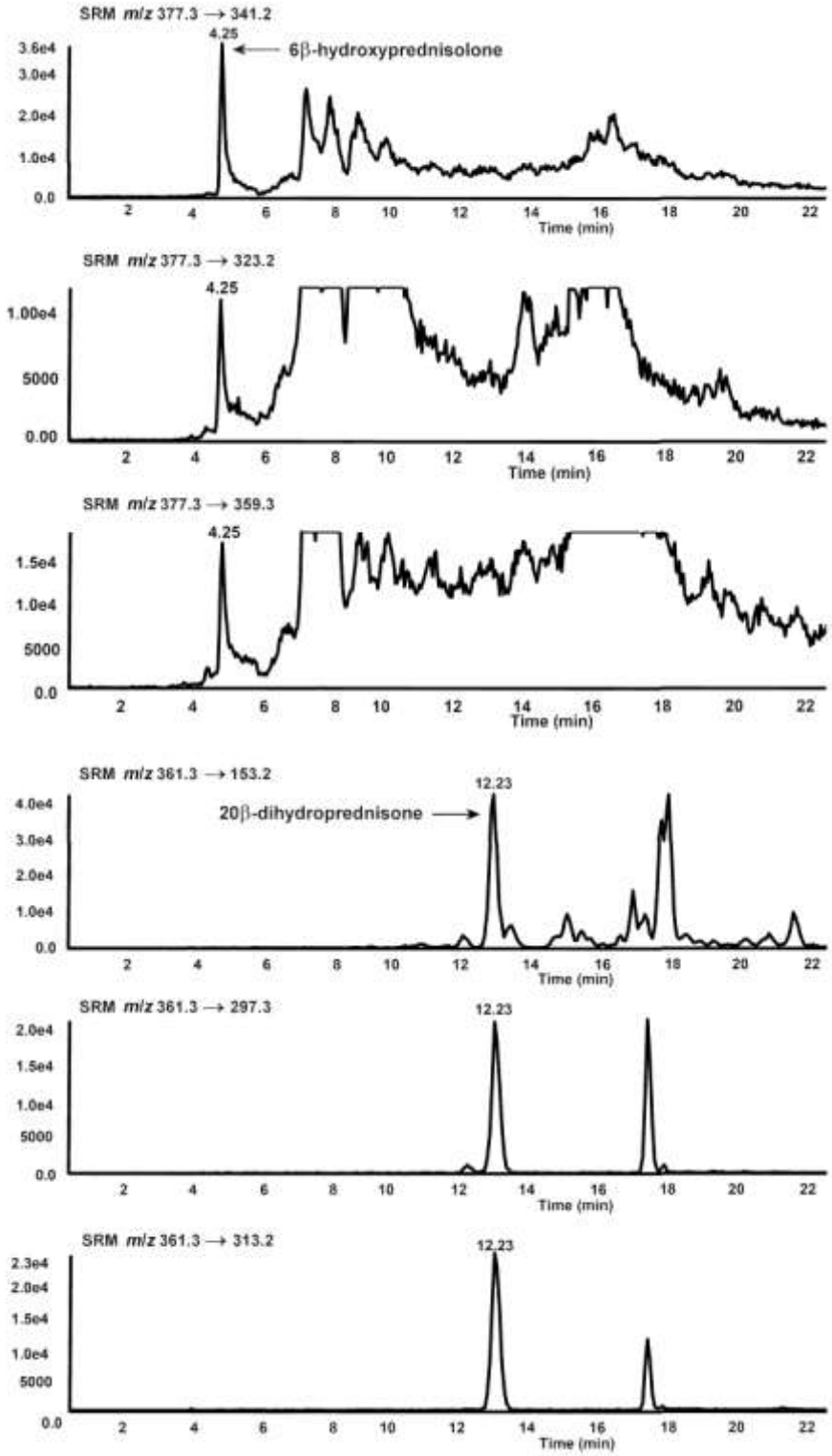

Figure 4. Chromatographic profiles of three SRM transitions distinctive for 6β-hydroxyprednisolone and 20β-dihydroprednisone, respectively, from the urine sample collected 10 days after the first pharmacological administration of prednisolone acetate to a bullock.

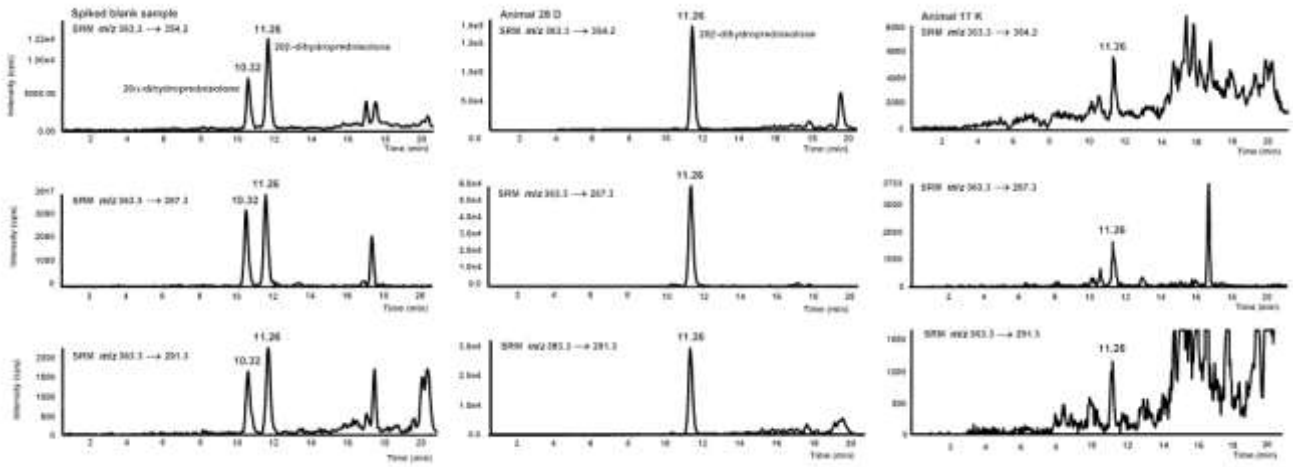

Figure 5. Chromatographic profiles of the three SRM transitions distinctive for 20β-dihydroprednisolone. (Left) Blank urine spiked with 20α- and 20β-dihydroprendisolone at the final concentration of 1 ng mL^{-1}. (Centre) Urine sample from animal 28 D, collected at the 10th day of low-dosage treatment. (Right) Real urine sample collected from untreated animal 17 K.

Table 3. Prednisolone and 20β-dihydroprednisolone levels (ng mL) in urine samples from beef cattle treated with low dosages of prednisolone acetate to mimic a growth-promoting protocol.

	Prednisolone				20β-Dihydroprednisolone			
Animal	Day 0	Day 10	Day 31	Day 41	Day 0	Day 10	Day 31	Day 41
25 D	n.d.	0.51	<0.10	n.d.	n.d.	2.7	5.1	n.d.
26 D	n.d.	<0.10	n.d.	n.d.	n.d.	3.4	4.6	n.d.
27 D	n.d.	<0.10	<0.10	n.d.	n.d.	1.8	2.6	n.d.
28 D	n.d.	n.d.	0.68	n.d.	n.d.	27.2	8.4	n.d.
29 D	n.d.	n.d.	0.67	n.d.	n.d.	9.9	14.6	n.d.
30 D	n.d.	n.d.	n.d.	n.d.	n.d.	1.2	4.5	n.d.
31 D	n.d.	n.d.	n.d.	n.d.	n.d.	6.0	20.6	1.9
32 D	n.d.	n.d.	n.d.	n.d.	n.d.	3.3	3.9	n.d.
17 K	n.d.	n.d.	n.d.	n.d.	n.d.	Traces	n.d.	n.d.
18 K	n.d.	n.d.	n.d.	n.d.	n.d.	Traces	n.d.	n.d.

Note: n.d., not detected.

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