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Study of Dexamethasone Urinary Excretion Profile in Cattle by LC−MS/MS: Comparison between Therapeutic and Growth-Promoting Administration

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Dexamethasone is a potent synthetic corticosteroid widely employed as a therapeutic agent in cattle. Besides this legal use, corticosteroids are also administered at low dosages as growth-promoters either alone or in combination with other steroids or with β-agonists. For this reason, appropriate control plans are established to survey corticosteroid misuse, using liver or urine as biological matrices. Since few data are available about the kinetics of dexamethasone excretion in meat cattle, an experimental study was designed to assess the drug residue levels in urines following either a therapeutic (60 μg of dexamethasone sodium phosphate/kg b.w., for three consecutive days) or a growth-promoting schedule (0.7 or 1.4 mg of dexamethasone sodium phosphate per capita/day for 60 days). The urinary elimination of dexamethasone, which was predominantly excreted in the unmodified form, was determined by high-performance liquid chromatography/tandem mass spectrometry at different time intervals, i.e. during the treatments and after appropriate withdrawal times. Our findings confirm the high and rapid rate of dexamethasone urinary excretion irrespective of the nature of the treatment, and provide useful reference values that can be conveniently employed for forensic purposes.

KEYWORDS: Dexamethasone; cattle; urine; excretion; growth promoters; liquid chromatography−tandem mass spectrometry

INTRODUCTION

Natural corticosteroids are hormones secreted by the adrenal cortex that are involved in a wide range of physiological processes, such as stress response, inflammation, immune function, hydro-electrolyte balance, reproduction and behavior. The discovery of their anti-inflammatory properties led to the chemical synthesis of more active compounds that are widely used in clinical practice. Among them, dexamethasone, a fluorinated hydro-cortisone derivative, is characterized by an increased glucocorticoid potency associated with a nearly complete loss of mineralcorticoid activity. In veterinary medicine, its use has a long record for the treatment of numerous metabolic and inflammatory diseases in both companion and farm animals. As regards cattle, therapeutic indications include primary ketosis, disorders of the musculoskeletal system, allergic reactions, skin diseases and shock. Furthermore, dexamethasone is often employed in conjunction with antimicrobial agents as supportive therapy to shorten the period of clinical signs, and it is also frequently administered in the last trimester of gestation to induce parturition (*1*).

On the other hand, it is well-known that synthetic corticosteroids, especially at low dosages and mostly through oral administration, are illicitly used as growth-promoters either alone or within protocols involving other active principles (i.e., steroid hormones and/or β-agonists). Although the effects of dexamethasone, the most illegally employed glucocorticoid, on cattle weight gain are controversial (*2*−*4*), there is evidence indicating that its slow release through intramuscular (IM) implants can improve the overall carcass quality traits, with an increase in subcutaneous fat deposition and longissimus dorsi muscle area. These effects are partly explained by the significant increase of serum insulin in treated animals, which is likely responsible for reduced protein catabolism and enhanced lipogenesis (*5*).

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In this respect, the relationship between dexamethasone and insulin level has been underlined by in vitro studies, demonstrating that the glucocorticoid drug is able to potentiate the proliferation of skeletal muscle cells, induced by both insulin and insulin growth factor-1 (*6*, *7*). The oral administration of dexamethasone at low dosages to veal calves can also positively influence both the feed conversion ratio and meat quality, leading to higher tenderness and paler color, two attributes particularly appreciated by the consumers (*8*). Synthetic glucocorticoids are also administered in combination with other active principles in order to take advantage of their synergistic effects with different illegal growth-promoting agents. In particular, dexamethasone can reverse the β_2-agonist-mediated down-regulation of β_2-adrenoreceptors, thereby enhancing the repartitioning effects of such β_2-adrenergic mimetics (*9*, *10*). In addition to these "favorable" pharmacological effects, the glucocorticoid-mediated electrolyte imbalance results in polydipsia and polyuria, which may actually decrease the concentration of other coadministered illegal substances in the urine.

The misuse of corticosteroids in meat cattle breeding appears to be a common practice in the EU; in particular, according to the results of the 2006 National Residue Monitoring Plans, Italy ranked first in EU, with 115 noncompliant results for dexamethasone in the bovine species (*11*). The strong pharmacological activity of synthetic corticosteroids makes the residues of these molecules potentially dangerous for meat consumers. As a consequence, the administration of such drugs for growth-promoting purposes has never been allowed in the EU, and their use in livestock is restricted to therapeutic indications that request an official record of the treatment by a licensed veterinarian and the application of appropriate withdrawal periods (up to several weeks), in order to comply with maximum residue limits (MRLs) established for bovine edible tissues (0.75 μg/kg in kidney and muscle, and 2 μg/kg in liver) and milk (0.3 μg/kg) (*12*). Such MRLs have not yet been set in the United States and in other non-EU countries like Japan, Canada, and Australia (*13*); a proposal for dexamethasone MRLs in cattle tissues has been recently made in the 70th Meeting Joint FAO/WHO Expert Committee on Food Additives (JECFA) based on an ADI as low as 0−0.015 μg/kg b.w. (*14*).

In living animals the official control for corticosteroid misuse is performed through the detection of the parent drug in the urine samples, which are first screened by immunoenzymatic methods with a cutoff level around 2 ng/mL. However, there is scant information about the kinetics of dexamethasone excretion in this biological fluid in the bovine species, especially in finishing bulls bred for meat production. Most published reports deal with therapeutic dosages, administered either to one animal or to a small number of individuals by the intravenous (*15*) or the oral route (*16*), to veal calves (*17*), or in combination with other drugs (*18*), respectively. Moreover, rather outdated and insensitive analytical methods were used in the past to detect the drug in urine samples. Lastly, no such information is available pertaining to dexamethasone administration to cattle with growth-promoting schedule, which, as far as we know, differs from therapeutic protocols in both dosing and route of administration.

As part of a larger project aimed at developing complementary biological assays for detecting the cattle exposure to corticosteroids, a study was designed to investigate the kinetics of dexamethasone urinary excretion in adult cattle using a LC−MS/MS validated method, according to the Commission Decision 2002/657/EC (*19*). To this aim, finishing bulls bred for meat production were experimentally treated with dexamethasone, either according to a pharmacological schedule typically employed in clinical practice or according to a growth-promoting protocol, in order to provide useful evidence that could possibly discriminate legal from illegal administrations.

MATERIALS AND METHODS

Chemicals, Reagents, and Standard Solutions. Methanol, β-glucuronidase (from *Escherichia coli*), dexamethasone, betamethasone, and triamcinolone acetonide-d_6 (Internal Standard, IS) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) was supplied by Carlo Erba (Milan, Italy). *t*-Butyl methyl ether, sodium carbonate, potassium dihydrogen phosphate and sodium phosphate monohydrate were from Riedel-de Haën (Seelze, Germany). Sodium hydrogen carbonate was supplied by Applichem (Darmstadt, Germany). Water was obtained by means of a Milli-Q system (Millipore Corporate Headquarters, Billerica, USA). A stock dexamethasone solution (1 mg/mL) was prepared monthly by dissolving the standard compounds in methanol and was stored at −20 °C in the dark. Calibration curves were obtained by diluting the stock standard solution in blank bovine urine, at six concentration levels (0.5, 1.0, 2.0, 5.0, 10.0, 20.0 ng/mL). Most validation experiments were performed using a negative reference urine obtained from control animals (5 mL), spiked with, respectively, 2.5, 5, 10, 15 μL of a 1 μg/mL working dexamethasone solution, yielding final concentrations of 0.5, 1.0, 2.0, 3.0 ng/mL. The analysis of each solution was replicated six times, and the whole set of experiments (four concentration levels replicated six times) was repeated three times. Working solutions of dexamethasone and triamcinolone acetonide-d_6, stored at −20 °C and analyzed regularly up to three months after the preparation, did not exhibit any degradation of the analytes.

Animals and Experimental Protocol. Twenty-four clinically healthy Friesian male cattle, with an initial average weight of 439.7 ± 53.4 kg, were used. The experimental plan was designed according to the guidelines of the Italian law for care and use of experimental animals (DLgs. 116/92), and the study was approved by the Ministry of Health and the local Committee for Animal Welfare. The animals were housed in outside pens with overhead shelter and fed a unifeed diet consisting of corn silage, cotton seal meal, corn meal, premix and concentrated meal (30% protein, 10% fiber, 6% ash, and 2.9% fats), with ad libitum access to fresh water. After an acclimatization period of four weeks, they were randomly divided into two groups, A and B, each consisting of 12 individuals. Group A (pharmacological treatment): Seven animals were injected IM once daily for three consecutive days with 60 μg/kg of dexamethasone as sodium phosphate salt (Dexadreson). The remaining five animals were used as controls. Group B (growth-promoting schedule): Animals received 0.7 (n = 4) or 1.4 mg (n = 4) dexamethasone per capita per day as sodium phosphate salt (Desashock) for sixty days. The lowest dosage was almost superimposable to that used in another trial aimed at investigating the growth-promoting effect of the glucocorticoid in meat cattle (*4*). The pharmaceutical preparation was diluted with tap water to a volume of 10 mL and orally administered by means of a plastic syringe without needle. The remaining four animals were used as controls.

Sample Collection. Group A: Urine samples were collected on days 1, 2, 3, 5, and 7, after the first dexamethasone administration (T_0). Group B: Urine collection started 8 days after the beginning of the treatment (T_0), and continued with approximately a once-per-week sampling frequency, until two weeks after the end of the treatment. Samples were collected at early morning after spontaneous micturition, divided in aliquots and stored at −20 °C until analysis.

Sample Preparation. Urine samples and spiked urine samples (5 mL) were inserted into glass tubes (30 mL), and 10 μL of the internal standard solution (1 μg/mL) was added. The pH was adjusted to approximately 6.5 by adding a phosphate buffer. Then, samples were treated with 50 μL of β-glucuronidase (from *E. coli*) and incubated for 1 h at 42 °C. For 32 urine samples from treated animals, the preparation was repeated on two separate aliquots, respectively including or omitting the β-glucuronidase addition and incubation, so as to calculate the amount of the free vs conjugated dexamethasone. The pH was adjusted to 9.2−9.8 by adding 3 mL of carbonate buffer. Subsequently, 5 mL

of *t*-butyl methyl ether was added twice. The centrifuge tube was shaken vigorously for 5 min by means of a vortex multimixer (Tecnovetro, Monza, Italy) and then centrifuged at 3500 rpm for 5 min (model Megafuge 1.0 Heraeus from ASHI, Milan, Italy). The supernatant organic fraction was collected and evaporated to dryness under a gentle stream of nitrogen at 50 °C, using a Techne Sample Concentrator (Barloworld Scientific, Stone, U.K.). The residue was last dissolved into 50 μL of mobile phase (28% acetonitrile + 72% H_2O) for the LC−MS/MS analysis.

LC−MS/MS Conditions. All analyses were performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA), including a vacuum degasser, a binary pump, an autosampler and a column thermostat. The liquid chromatograph was equipped with a Synergi 4u Fusion (150 × 2.0 μm, Phenomenex, Torrence, CA). The chromatographic run was carried out with a binary mobile phase of water and acetonitrile, using a linear gradient (acetonitrile from 28% to 35% in 27 min). The flow rate was 200 μL/min. The chromatograph was interfaced to an Applied Biosystems API 4000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada), operating in the positive ion mode by an atmospheric pressure chemical ionization (APCI) source. The injection volume was 10 μL. The nitrogen nebulizing gas was operated at 50 psi, and the turbo gas temperature was 300 °C. Ion acquisition was operated at unit mass resolution in the selected reaction monitoring (SRM) mode, using the transitions from the protonated molecular ion of dexamethasone to the three most abundant fragments, as follows: *m*/*z* 393.3 → 373.3; *m*/*z* 393.3 → 355.3; *m*/*z* 393.3 → 337.3. For triamcinolone acetonide d_6 the following transitions were selected: *m*/*z* 441.4 → 403.4; *m*/*z* 441.4 → 357.3; *m*/*z* 441.4 → 339.3; *m*/*z* 441.4 → 421.4. Mass parameters such as declustering potential (DP), entrance potential (EP), collision gas (N_2) pressure and collision energy (CE) were optimized for each MS/MS transition to achieve the maximum fragment ion abundance. Collision energies varied from 13 to 19 eV. The instrument was interfaced to Applied Biosystems Analyst version 1.4 software.

RESULTS AND DISCUSSION

Analytical Performance. A variety of materials and techniques are commonly used to extract corticosteroids from biological samples, including solid-phase (SPE) (*20*), liquid−liquid (LLE) (*21*), accelerated solvent (ASE) (*22*), matrix solid-phase dispersion (MSPD) (*23*). We decided to use liquid−liquid extraction, because of its simplicity, speed, and repeatability (i.e., no dependence on the producer, batch and packing of SPE columns), provided that the process is fully standardized. For the instrumental determination of dexamethasone, GC−MS was mainly used in the past (*24*−*26*) either after trimethylsilyl-derivatization or after oxidation with chromates, followed by electron-capture chemical ionization. More recently, corticosteroids are generally analyzed by LC−MS, which allows direct, rapid and sensitive detection (*27*−*29*). Due to the continuous improvement of instrumentation technology, LC−MS performances tend to improve rapidly, assuring increasingly sensitive and selective determinations. The most recent literature (*30*−*33*) reports detection limits for dexamethasone in various biological samples in the fraction of ppb range (ng/mL or μg/kg), depending on the specific matrix studied. In particular, CCα and CCβ values of 0.5 and 0.8 ng/mL, respectively, were estimated in bovine urine (*31*). In the present study, a quantitative LC−MS/MS determination method was developed and validated according to the Commission Decision 2002/657/EC (*19*). Typical chromatographic profiles obtained during the present study are reported in **Figures 1** and **2**. The parameters measured in the validation process included selectivity, decision limit (CCα), detection capability (CCβ), trueness, precision, recovery, ruggedness, limits of detection (LOD) and quantitation (LOQ), as is described in the following sections.

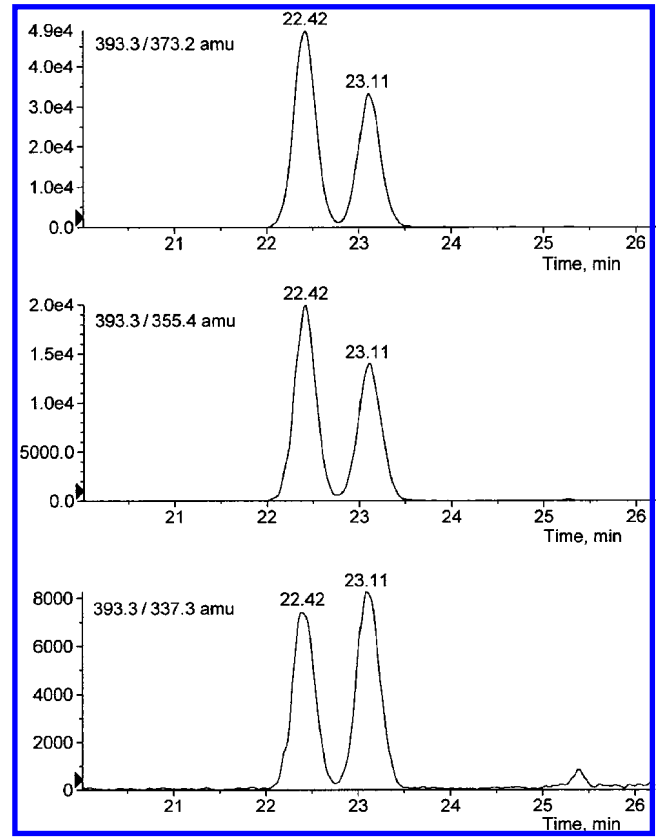

Figure 1. Selected reaction monitoring chromatographic profiles of a blank bovine urine sample spiked with 0.5 ng/mL dexamethasone (retention time 23.11 min) and betamethasone (retention time 22.42 min). The SRM transitions depicted are characteristic for both compounds.

Selectivity. **Figure 1** reports the three characteristic SRM chromatograms obtained from a blank urine spiked with dexamethasone (retention time 23.11 min) and betamethasone (retention time 22.42 min) at a 0.5 ng/mL concentration. The two isomeric corticosteroids were added to the same urine sample in order to demonstrate that the chromatographic conditions adopted were adequate to fully separate the peaks, thus preventing the risk of misrecognition and possible interference.

Twenty different blank bovine urine samples were extracted and analyzed with the method described in Materials and Methods. The occurrence of possible interference from endogenous substances was tested by plotting the selected-ion chromatograms characteristic for dexamethasone (3 plots) and triamcinolone acetonide-d_6 (4 plots), at the retention time interval (±2.5%) expected for their elution. Ion chromatograms exhibited no extraneous peaks (i.e., peaks with a S/N > 2), indicating that the method is selective for both the analyte and the internal standard.

Linearity, Calibration. A six-point calibration curve was established, which proved linear throughout the entire concentration range studied, from 0.5 ng/mL to 20 ng/mL (squared correlation coefficient $r^2 = 0.9966$; average standard deviation from the curve = 0.14 ng/mL). The curve was repeated and verified at the beginning of each working session involving the analysis of urinary excretion samples.

Different calibration curves for dexamethasone were built during three separate working sessions, involving validation experiments on spiked urine samples. Peak areas from six replicates were averaged, at four concentration levels (0.5, 1.0,

2.0, 3.0 ng/mL). A restricted calibration range was selected so as to provide accurate determinations around the cutoff concentration level (2.0 ng/mL).

Precision, Decision Limit, Detection Capability. All three chromatograms depicted in **Figure 1** show excellent signal-to-noise ratio, allowing accurate quantification at a concentration largely below the cutoff value generally used in immunoenzymatic screening procedures. From the results obtained for building the four-point calibration curves, the standard deviation (STD) together with the interassay repeatability was calculated for each concentration level. The experimental coefficients of variation (CV%) were 21 at 0.5 ng/mL, 19 at 1.0 ng/mL, 5 at 2.0 ng/mL and 4 at 3.0 ng/mL. These precision values proved entirely satisfactory with respect to the conditions posed by the Commission Decision 2002/657/EC. The same did not apply at a 0.1 ng/mL concentration level, where a CV% of 36 was calculated. Thus, 0.5 ng/mL was safely set as the lowest concentration at which accurate quantitation is guaranteed, even if less-accurate determinations are still possible at lower concentration levels. The decision limit (CCα) at $\alpha = 1\%$ was calculated from the STD value at the lowest calibration level:

$$CC\alpha = 0.5 + 2.33 \times STD_{0.5} = 0.75 \text{ ng/mL}$$

Similarly, the detection capability (CCβ) at $\beta = 5\%$ was calculated from the STD value obtained by pooling the replicated results from the first (0.5 ng/mL) and second (1.0 ng/mL) calibration levels:

$$CC\beta = CC\alpha + 1.64 \times STD_{0.5+1.0} = 1.00 \text{ ng/mL}$$

As mentioned by the Commission Decision 2002/657/EC and subsequent explication notes (SANCO/2004/2726 rev.1), the calculation of CCα incorporated the method uncertainty value. As is evident from **Figure 1**, the limit of detection for dexamethasone, using the present analytical method, lies largely below the CCα value, since the latter is calculated from the lowest point of the calibration curve. Using the common LOD calculation methods (the analyte concentration whose response yielded a signal-to-noise ratio (S/N) equal to 3 on the least abundant SRM transition), a value below 0.05 ng/mL was determined, which was confirmed by injecting a blank urine sample spiked at the same concentration. Similarly, an LOQ value of 0.10 ng/mL was estimated. Evidence for the method sensitivity is provided in **Figure 2**, representing the SRM chromatographic plots obtained from a real urine sample collected from a group B bovine, on day 67 since the beginning of the growth-promoting treatment: the approximate dexamethasone concentration in the urine is equal to 0.07 ng/mL.

Trueness. The data obtained from the three working sections described above were tested also for trueness. The resulting experimental values (0.50 ± 0.02 ng/mL; 1.04 ± 0.07 ng/mL; 1.97 ± 0.04 ng/mL; 3.01 ± 0.02 ng/mL) were consistent with the theoretical values of 0.5, 1.0, 2.0 and 3.0 ng/mL and largely within the accepted error limits (from −30% to +10% for a concentration of 1.0 ng/mL and above; from −50% to +20% for the calibration point at 0.5 ng/mL).

Extraction Recovery. The extraction recovery was calculated from the percentage ratio between the quantitation results obtained respectively (i) when the blank urine is spiked and then submitted to extraction and analysis and (ii) when the same amount of spiking analytes was added after the extraction step. In both cases, the internal standard was added before the extraction step. Replicated determinations were performed at three spiking levels (0.5, 1.0, and 5.0 ng/mL). An average extraction recovery of 108 ± 9% was found, with values ranging from 99 to 119%.

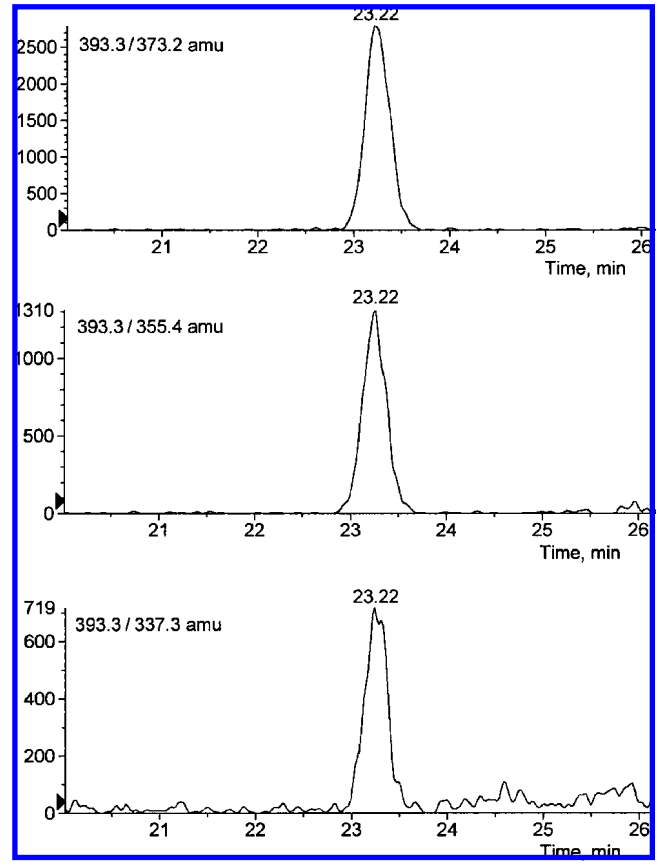

Figure 2. Selected reaction monitoring chromatographic profiles of a urine sample collected from a group B bovine, on day 67 from the beginning of the growth-promoting treatment. The same SRM transitions of **Figure 1** were used.

Ruggedness. A Youden test was set up using eight urine aliquots spiked at 1.0 ng/mL. A ±10% variation of optimal values was imposed to the following parameters: (i) pH during the extraction step; (ii) deconjugation temperature; (iii) composition of the solvent added to the residue to reconstitute the final analytical solution. The quantitative results did not show any significant change with respect to the standard conditions, proving that limited variation of the experimental parameters under study had no influence on the method performance.

Kinetics of Dexamethasone Excretion Following the Therapeutic Treatment. The kinetics of dexamethasone excretion was determined by averaging the urine concentration values obtained from the seven treated animals (group A) at each time interval (**Figure 3**). The urinary samples showing dexamethasone concentration exceeding the calibration range were occasionally diluted in order to obtain accurate quantitation, even if the interindividual variability largely compensated for the hypothetical calibration inaccuracy. The progress of urinary dexamethasone excretion, during and after the therapeutic schedule, revealed a very rapid decrease of the drug concentration, beginning already throughout the treatment. Indeed, as early as 24 h after the second administration, the level of dexamethasone was reduced by a factor of almost 5, i.e. from a mean value of 164 ng/mL to 35 ng/mL. Afterward, the dexamethasone elimination rate proceeded relatively slower, even though values approaching the detection limit for routine immunoenzymatic screening tests (2 ng/mL) were reached after 3 days from the end of the treatment. All the urine samples proved to be virtually free from dexamethasone (concentrations <0.015 ng/mL) as soon as 5 days after the last injection. Taken into account the limitations of the cited previously published studies concerning

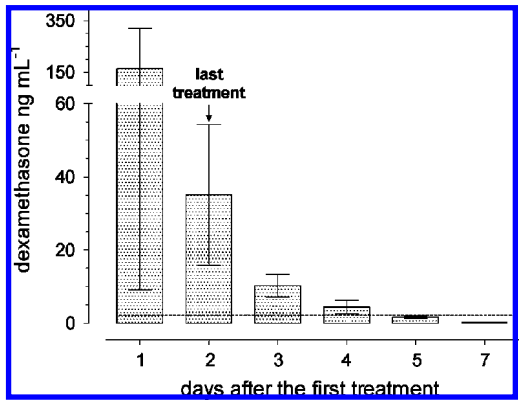

Figure 3. Daily excretion profile of dexamethasone in urines from cattle treated with the therapeutic protocol (60 μg/kg b.w. for three consecutive days). Values are reported as mean ± standard deviation. The broken line indicates the detection limit of routine immunoenzymatic screening tests (2 ng/mL).

the excretion kinetics of the glucocorticoid in cattle, our results confirm the rapid urinary excretion rate of dexamethasone. Adult cattle of either sex receiving four IM injections of a dexamethasone sodium phosphate (150 μg/kg b.w.) and 19-nortestosterone (1 mg/kg b.w.) combination, at one week intervals, displayed a peak for urinary dexamethasone concentration immediately after the second injection (171 ng/mL), followed by a drop to less than 10 ng/mL after 3 days. Subsequently, considerably lower values were recorded after the remaining two treatments (*18*). As mentioned before, the glucocorticoid-mediated polyuria and polydipsia, leading to an enhanced urine dilution, could explain the decrease in the urinary dexamethasone concentration already in the course of that treatment, as was also observed in our study. Since the urinary excretion profile obtained during the study may represent a semiquantitative assessment of the amount of drug actually cleared from the body, however, it is not possible to exclude that such a phenomenon may arise, to some extent, from dexamethasone accumulation in liver and other target tissues (*34*). Our data differ from the data reported previously (*18*), in that we found the urinary dexamethasone level to drop to below 2 ng/mL as early as 3 days after the last (i.e., the third) treatment, whereas Calvarese et al. indicated that a similar urinary concentration was reached only 11 days after the last (of four) administration. This discrepancy could be explained by the higher dosage used, which was nearly three times higher than in our case, the different treatment schedule, and the less accurate analytical method (RIA instead of LC−MS/MS). In addition, the concomitant administration of 19-nortestosterone may have influenced the elimination rate of dexamethasone as well. In another study, in which three calves were treated IM with a single dose of a dexamethasone sodium phosphate (20 μg/kg b.w.) and dexamethasone phenylpropionate (40 μg/kg b.w.) combination, the drug concentration in the urine, 72 h after the treatment, was on average 26 ng/mL (*17*), a value more than 10-fold higher than that recorded in our experiment after a comparable time delay. Besides the use of a long-acting compound like the phenylpropionate ester, this discrepancy might be also due to the young age and the health conditions of treated animals, which were diagnosed with a respiratory disease: actually, both factors are reported to significantly depress the elimination rate of drugs and xenobiotics (*35*).

Kinetics of Dexamethasone Excretion Following the Growth-Promoting Schedule. The bovine urinary excretion of dexamethasone, during and after two different growth-promoting treatments, is illustrated in **Figure 4**. The drug concentrations obtained from the animals treated with 1.4 mg of dexamethasone/day showed mean values ranging between 8 and 11 ng/mL up to 25 days after the beginning of the treatment (**Figure 4**, top). Thereafter, the glucocorticoid urinary concentration tended to decrease progressively during the remaining experimental weeks, with average levels declining from 5 ng/mL to about 2.5−3 ng/mL on days 53 and 60, close to the cutoff levels used in immunoenzymatic screening tests. The rapid excretion of dexamethasone was confirmed by the finding of drug concentrations below 0.5 ng/mL in all samples collected one week after treatment withdrawal. The dexamethasone urinary concentrations in bulls treated with 0.7 mg/day consistently proved roughly halved with respect to those determined in animals receiving the higher (double) dexamethasone dosage (**Figure 4**, bottom). Urinary mean concentrations around 5 ng/mL on days 8 and 11 were measured, with a progressive decline observed thereafter. Consistently with the lower drug dosage, urinary mean values around or below 2 ng/mL were reached as early as 25 days after the beginning of the treatment, and constantly maintained throughout the rest of the experiment. Therefore, unlike what was observed in the animals treated with 1.4 mg/day, it is worth noting that the group administered with the lower dosage yielded urinary samples that would test negative to the common screening for corticosteroids, for at least one-half of the treatment period. Accordingly, dexamethasone concentrations largely below 2 ng/mL (actually, lower than 0.2 ng/mL) were found in samples collected one week after treatment withdrawal. The results obtained in the present part of our experiments cannot be directly compared to the ones observed in previous studies, mostly because of the striking differences in both dosage and duration of the administration. This notwithstanding, a qualitatively similar urinary excretion pattern was noted in a cow supplied with 50 mg of dexametha-

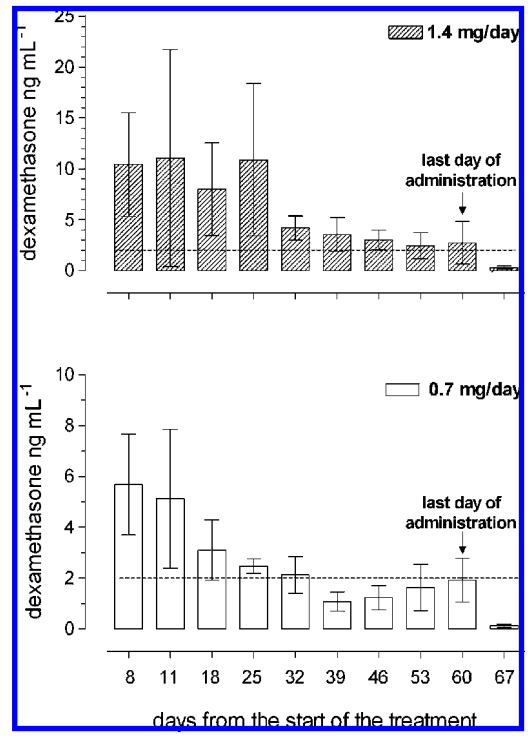

Figure 4. Time-dependent excretion profile of dexamethasone in urines from cattle administered with the growth-promoting schedule (1.4, top, or 0.7, bottom, mg per capita per day for 60 days). Values are reported as mean ± standard deviation. The broken lines indicate the detection limit of routine immunoenzymatic screening tests (2 ng/mL).

sone/day for 7 days by the oral route: the urinary drug concentration maintained an approximately constant value, ranging from 669 to 980 ng/mL, for the first 4 days of the administration, but then rapidly declined to about one-half, in the subsequent 3 days of treatment, and reached values one hundred times lower as soon as 7 days after treatment withdrawal (*16*).

Free vs Conjugated Dexamethasone Occurrence. Despite its extensive use, little is known about dexamethasone biotransformation in cattle. In vitro and in vivo studies performed in camels revealed two phase I metabolites, the major one arising from reduction of the 3-carbonyl group in ring A, mediated by hydroxysteroid dehydrogenases, the minor one resulting from the cytochrome P450-dependent hydroxylation of the same ring. Glucuronidation involved both phase I metabolites and the parent compound (*36*). It is thought that any backbone structural modification introduced in synthetic corticosteroids, with respect to natural ones, makes them more refractory to phase I and possibly phase II biotransformations. Indeed, the free fraction proved largely predominant (72% to 96%) over the conjugated one, in urine samples collected from cows that were IM treated with a single dose of approximately 10 μg/kg b.w. of different dexamethasone esters (*37*). The growth-promoting schedule carried out in the present experiments implies a repeated animal exposure to the drug, which could hypothetically enhance the activity of phase I and/or phase II enzymes, catalyzing dexamethasone biotransformation and thereby diminishing the relative amount of the parent compound excreted in the urines. In contrast with this hypothesis, however, we determined percentages of free urinary dexamethasone averaging from 74% to 89% in all urine samples, in good agreement with results from the cited study (*37*). We found similar percentages of free drug for both finishing bulls subjected to the administration of large doses of dexamethasone (60 μg/kg b.w.) for 3 consecutive days (**Figure 5A**) and animals treated with small daily amounts of the glucocorticoid (0.7 or 1.4 mg per capita) over an extended time period (60 days) (**Figure 5B**). It can be therefore concluded that, under the experimental conditions adopted in the present study, dexamethasone is mainly excreted in cattle urine as unmodified parent drug, suggesting that neither the therapeutic nor the growth-promoting schedules are apparently able to induce the enzymes involved in the oxidative or conjugative biotransformations of the drug.

Comparison between the Urinary Excretion Profiles of Pharmacological vs Growth-Promoting Treatments. In order to draw useful conclusions from this study, it should be reminded that, according to the EU legislation in force, every therapeutic intervention in food producing species should be prescribed by a licensed veterinarian and annotated in an official register, and that any omission to comply with this procedure de facto represents an illicit treatment. Since urine is the official matrix for controlling dexamethasone misuse in living bovines, it would be useful, for forensic purposes, to ascertain whether the urinary excretion profile could assist in identifying the phase and the intention of the treatment. Although a relatively high individual variability characterizes the specimens collected at the beginning of drug administration (**Figures 3** and **4**), the overall data collected from all experimental groups of the present study (**Figure 6**) show that, in agreement with the results of earlier reports (*16*−*18*), urinary levels above 20 ng/mL strongly suggest that sampled animals are experiencing a dexamethasone therapy. On the other hand, the detection of drug concentrations below 20 ng/mL points to either the post-treatment phase of a therapeutic intervention or to the whole period of a growth-promoting treatment. Nevertheless, it should be noted that all but two samples from bulls receiving daily doses of 0.7 mg/per capita displayed urinary dexamethasone values below 5 ng/mL, and even close to or less than 2 ng/mL, for the whole sampling period. Interestingly, this dosage is believed to closely approximate the one commonly used in the illegal practice (*4*). In contrast, the time interval, during which the urines of bulls administered with the therapeutic protocol exhibit such a range of glucocorticoid concentrations, is extremely limited, being restricted to the two days after the drug withdrawal.

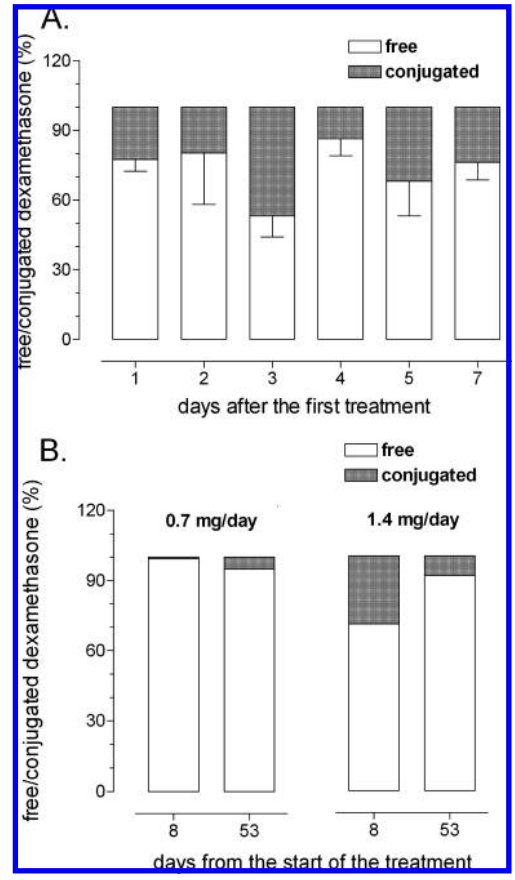

Figure 5. Percentages of free vs conjugated dexamethasone in urine specimens collected at different time points from cattle administered (**A**) a therapeutic treatment (60 μg/kg b.w. for three consecutive days, mean $\pm$ standard deviation, $n = 4$) or (**B**) a growth-promoting schedule (1.4 or 0.7 mg per capita per day for 60 days, mean values, $n = 2$).

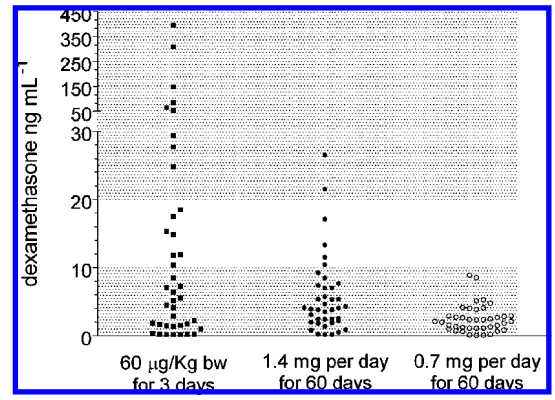

Figure 6. Dexamethasone concentrations (raw values) in urines from cattle administered with the therapeutic (60 μg/kg bw for 3 consecutive days) or the growth-promoting schedule (1.4 or 0.7 mg per capita per day for 60 days).

In conclusion, results from the present study confirm that sodium phosphate dexamethasone ester administered to finishing bulls is rapidly excreted in the urines, irrespective of the dosage and the route of administration employed. The excretion profile of the therapeutic protocol is characterized by the occurrence of relatively high drug concentrations (>20 ng/mL) during the course of the treatment, followed by levels between 5 and 2 ng/mL for a short time period, namely a couple of days after drug withdrawal, after which the drug rapidly disappears from the urine. The finding of urinary drug concentrations averaging 2−10 ng/mL with a regular declining trend, throughout most of the administration period (60 days), appears to characterize the growth-promoting schedule, making it therefore difficult to distinguish unambiguously such values from those occurring in the post-therapeutic phase, due also to the interindividual variability. Based on our results, in the attempt to discriminate the legal use of dexamethasone from its misuse, it is suggested that urine sampling for surveillance policy purposes should be performed on several animals to account for the interindividual variability; in addition, sampling should be ideally repeated after two or three days to better characterize the drug excretion profile, although this procedure may be of limited feasibility under field conditions.

It has also to be stressed that shrewd administration of dexamethasone for performance enhancing purposes may result in urinary drug concentrations lower than the ones detectable by the common immunoenzymatic screening methods, even during the treatment period. Therefore, efficient and highly sensitive multitarget screening protocols based on LC−MS/MS have to be implemented and increasingly applied to urinary field samples, in order to detect the most sophisticated illegal growth-promoting treatments in meat cattle breeding, which often involve low dosages and smart drug cocktail administration, yielding negative results from routine screening tests. The latter objective represents one of the major tasks of our forthcoming research.

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LITERATURE CITED

(1) Ferguson, D. C.; Hoenig, M. Glucocorticoids, mineralcorticoids and steroid synthesis inhibitors. In *Veterinary Pharmacology and Therapeutics*, 7th ed.; Adams, H. R. Ed.; Iowa State University Press: Ames, IA, 1995; pp 622−637.

(2) Johnson, B. H.; Silcox, R. W. The utilization of dexamethasone for feedlot bulls: a preliminary investigation. Anim. Sci. Rep. North Carolina State Univ, Raleigh, 1986.

(3) Renaville, R.; Massart, S.; Devolder, A.; Sneyers, M.; Burney, A.; Portetelle, D. Effects of dexamethasone treatment on growth, sexual axis and hormonal status in finishing bulls. *J. Anim. Sci.* **1993**, *71* (Suppl. 1), 232.

(4) Gottardo, F.; Brscic, M.; Pozza, G.; Ossensi, C.; Contiero, B.; Marin, A.; Cozzi, G. Administration of dexamethasone *per os* in finishing bulls. I. Effects and productive traits, meat quality and cattle behaviour as indicator of welfare. *Animal* **2008**, *2*, 1073–1079.

(5) Corah, T. J.; Tatum, J. D.; Morgan, J. B.; Mortimer, R. G.; Smith, G. C. Effects of dexamethasone implant on deposition of intramuscular fat in genetically identical cattle. *J. Anim. Sci.* **1995**, *73*, 3310–3316.

(6) Dodson, M. V.; Allen, R. E.; Hossner, K. L. Ovine somatomedin, multiplication-stimulating activity, and insulin promote skeletal muscle satellite cell proliferation in vitro. *Endocrinology* **1985**, *117*, 2357–2363.

(7) Giorgino, F.; Smith, R. J. Dexamethasone enhances insulin-like growth factor-I effects on skeletal muscle cell proliferation. Role of specific intracellular signaling pathways. *J. Clin. Invest.* **1995**, *96*, 1473–1483.

(8) Tarantola, M.; Schiavone, A.; Preziuso, G.; Russo, C.; Biolatti, B.; Bergero, D. Effects of low doses of dexamethasone on productive traits and meat quality of veal calves. *Anim. Sci.* **2004**, *79*, 93–98.

(9) Abraham, G.; Gottschalk, J.; Ungemach, F. R. Possible role of dexamethasone in sensitizing the beta-2 adrenergic receptor system in vivo in calves during concomitant treatment with clenbuterol. *Pharmacology* **2004**, *72*, 196–204.

(10) Odore, R.; Badino, P.; Pagliasso, S.; Nebbia, C.; Cuniberti, B.; Barbero, R.; Re, G. Changes in lymphocyte glucocorticoid and beta-adrenergic receptors in veal calves treated with clenbuterol and steroid hormones for growth-promoting purposes. *J. Vet. Pharmacol. Ther.* **2006**, *29*, 91–97.

(11) http://ec.europa.eu/food/food/chemicalsafety/residues/workdoc_2006_en.pdf.

(12) Commission of the European Communities, Regulation 1999/508/EC Off. J. Eur. Communities: Legis. 1999, L60, 16−52.

(13) http://www.daff.gov.au/agriculture-food/nrs/industry-info/mrl/cattle-sheep-pigs.

(14) http://www.fao.org/ag/agn/agns/files/JECFA70summaryreportEfinal.pdf.

(15) Gaignage, P.; Lognay, G.; Bosson, D.; Vertongen, D.; Dreze, P.; Marlier, M.; Severin, M. Dexamethasone bovine pharmacokinetics. *Eur. J. Drug. Metab. Pharmacokinet.* **1991**, *16*, 219–221.

(16) Courtheyn, D.; Vercammen, J.; De Brabander, H.; Vandenreyt, I.; Batjoens, P.; Vanoosthuyze, K.; Van Peteghem, C. Determination of dexamethasone in urine and faeces of treated cattle with negative chemical ionization-mass spectrometry. *Analyst* **1994**, *119*, 2557–2564.

(17) Van Den Hauwe, O.; Schneider, M.; Sahin, A.; Van Peteghem, C. H.; Naegeli, H. Immunochemical screening and liquid chromatographic-tandem mass spectrometric confirmation of drug residues in edible tissues of calves injected with a therapeutic dose of the synthetic glucocorticoids dexamethasone and flumethasone. *J. Agric. Food Chem.* **2003**, *51*, 326–330.

(18) Calvarese, S.; Rubini, P.; Urbani, G.; Ferri, N.; Ramazza, V.; Zucchi, M. Experimental administration of 19-nortestosterone and dexamethasone in cattle: elimination of the two drugs in different biological matrices. *Analyst* **1994**, *119*, 2611–2615.

(19) Commission of the European Communities, Council Directive 2002/657/EC Off. J. Eur. Communities: Legis. 2002, L221, 8−36.

(20) Delahaut, P.; Jacquemin, P.; Colemonts, Y.; Dubois, M.; De Graeve, J.; Deluyker, H. Quantitative determination of several synthetic corticosteroids by gas chromatography-mass spectrometry after purification by immunoaffinity chromatography. *J. Chromatogr., B: Biomed. Sci. Appl.* **1997**, *696*, 203–215.

(21) Tang, P. W.; Law, W. C.; Wan, T. S. Analysis of corticosteroids in equine urine by liquid chromatography-mass spectrometry. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *754*, 229–244.

(22) Draisci, R.; Marchiafava, C.; Palleschi, L.; Cammarata, P.; Cavalli, S. Accelerated solvent extraction and liquid chromatography-tandem mass spectrometry quantitation of corticosteroid residues in bovine liver. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *753*, 217–223.

(23) Kristenson, E. M.; Ramos, L.; Brinkman, U. A. T. Recent advances in matrix solid-phase dispersion. *Trends Anal. Chem.* **2006**, *25*, 96–111.

(24) Hamalainen, E.; Fotsis, T.; Adlercreutz, H. A gas chromatographic method for the determination of neutral steroid profiles in urine, including studies on the effect of oxytetracycline administration on these profiles in men. *Clin. Chim. Acta* **1991**, *199*, 205–220.

(25) Savu, S. R.; Silvestro, L.; Haag, A.; Sörgel, F. A confirmatory HPLC-MS/MS method for ten synthetic corticosteroids in bovine urines. *J. Mass Spectrom.* **1996**, *31*, 351–363.

(26) Shibasaki, H.; Furuta, T.; Kasuya, Y. Quantification of corticosteroids in human plasma by liquid chromatography-thermospray

mass spectrometry using stable isotope dilution. *J. Chromatogr., B: Biomed. Sci. Appl.* **1997**, *692*, 7–14.

(27) Niessen, W. M. Analysis of antibiotics by liquid chromatography-mass spectrometry. *J. Chromatogr A.* **1998**, *812*, 53–75.

(28) Antignac, J. P.; Le Bizec, B.; Monteau, F.; Poulain, F.; Andre, F. Multi-residue extraction-purification procedure for corticosteroids in biological samples for efficient control of their misuse in livestock production. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *757*, 11–19.

(29) Ramos, M.; Muñoz, P.; Aranda, A.; Rodriguez, I.; Diaz, R.; Blanca, J. Determination of chloramphenicol residues in shrimps by liquid chromatography-mass spectrometry. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2003**, *791*, 31–38.

(30) Gentili, A. LC-MS methods for analyzing anti-inflammatory drugs in animal-food products. *Trends Anal. Chem.* **2007**, *26*, 595–608.

(31) Andersen, J. H.; Hansen, L. G.; Pedersen, M. Optimization of solid phase extraction clean up and validation of quantitative determination of corticosteroids in urine by liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta* **2008**, *617*, 216–224.

(32) Luo, U. C.; Soma, L. R.; Guan, F.; Rudy, J. A.; Tsang, D. S. Resolution, quantification and confirmation of betamethasone and dexamethasone in equine plasma by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 825–832.

(33) Goyal, N.; El Achhabi, A.; Goldberg, E.; Hochhaus, G. Simultaneous determination of Dexamethasone, Dexamethasone 21-Acetate, and Paclitaxel in a simulated biological matrix by RP-HPLC: assay development and validation. *J. Liq. Chromatogr. Relat. Technol.* **2008**, *31*, 1478–1491.

(34) Van den Hauwe, O.; Dumoulin, F.; Elliott, C.; Van Peteghem, C. Detection of synthetic glucocorticoid residues in cattle tissue and hair samples after a single dose administration using LC-MS/MS. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci* **2005**, *817*, 215–223.

(35) Baggot, D. J. *The Physiological Basis of Veterinary Clinical Pharmacology*; Blackwell Science: Oxford, United Kingdom, 2001.

(36) Al Katheeri, N. A.; Wasfi, I. A.; Lambert, M.; Giuliano Albo, A.; Nebbia, C. In vivo and in vitro metabolism of dexamethasone in the camel. *Vet. J.* **2006**, *172*, 532–543.

(37) Antignac, J. P.; Le Bizec, B.; Monteau, F.; André, F. Study of natural and artificial corticosteroid phase II metabolites in bovine urine using HPLC-MS/MS. *Steroids* **2002**, *67*, 873–882.

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