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Determination of flumethasone in calf urine and serum by liquid chromatography–tandem mass spectrometry

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Abstract

Corticosteroids can be illegally administered to cattle as growth promoting agents to improve meat production. We developed a liquid chromatography–atmospheric pressure ionization mass spectrometry–mass spectrometry (LC–MS–MS) method able to identify and quantify flumethasone, one of the most potent fluorinated synthetic corticosteroid, in serum and urine from treated calves. The analyte was purified from urine (conjugated and free, following enzymatic hydrolysis) and from serum by C_{18} solid-phase and liquid–liquid extractions, then analyzed by LC–MS–MS monitoring the product ions of an abundant precursor (SRM in negative ionization mode). Results on flumethasone residues in biological fluids in three calves treated at different levels are presented. This method allowed the detection of flumethasone in bovine urine and serum at the 30-pg/ml level.

Keywords: Flumethasone

1. Introduction

Corticosteroids can be illegally used in livestock production to improve the commercial quality of meat. To this aim they are often administered together with other unlicensed drugs and act on water retention in meat, and lipid, protein and carbohydrate metabolism [1,2].

Therefore, to trace as easily as possible the misuse of such molecules as growth-promoting agents, there is a strong demand to develop analytical procedures able to identify the parent drug either after its administration to animals or before via the analysis of feedstuffs.

One of the most frequently employed techniques to screen corticosteroids in large series of samples is enzyme-immunoassay (EIA) because of its easy and fast application. This technique has been proved to be very sensitive, nevertheless the cross-reactivity of the antibodies used with structural analogues of the molecules of interest precludes an unambiguous identification of the compound in the complex matrix

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of a biological sample [3,4,]. In consequence, more specific analytical techniques are mandatory for confirmation purposes. GC–MS-based methods, although very sensitive [5], seems to be somewhat impractical, as corticosteroids are only slightly volatile and could be denatured with heat. Derivatization is possible only for a few corticosteroids and requires a time-consuming additional step [6–8]. The best alternative to GC–MS is LC–MS [9,10], in particular reversed-phase (RP)-LC–MS [8,11], and to increase the specificity, LC–MS–MS with atmospheric pressure ionization (API) has been reported as the best choice [12,13].

Flumethasone, one of the most pharmacologically active drugs of this class, is registered in cattle for therapeutic use at a dosage of 1.25 mg/head per day. Such a low dosage can push unscrupulous farmers to choose such a drug for growth promotion to elude official controls [7,14–16]. As a consequence, the appropriate identification and determination of flumethasone is the most critical point in a monitoring strategy. We have developed a method for the identification of flumethasone in biological fluids based on a simple solid-phase and liquid–liquid extraction of the sample followed by semimicro-HPLC combined with on-line ion spray-tandem mass spectrometry in negative ionization mode.

Several experiments were also carried out in urine by enzyme immunoassay for the qualitative confirmation of the presence or absence of the analyte, and by HPLC–UV to make a comparison between the techniques.

2. Experimental

2.1. Chemicals and reagents

Flumethasone and dexamethasone standards were purchased from Sigma (Milan, Italy). The drug 'Fluvet' containing flumethasone (25 mg/100 ml) is produced by Gellini (Florence, Italy).

Methanol (HPLC grade), acetic acid and ammonium formate were obtained from Carlo Erba (Italy), *tert*.-butylmethylether (TBME, HPLC grade) and formic acid were from Fluka (Sigma–Aldrich, Milan, Italy).

All the other reagents were of analytical grade.

Ammonium formate 1 m*M* (pH 7.0) was prepared dissolving 63 mg of ammonium formate in 1000 ml of water. Ultrapure water was produced with a Pure Lab™ system (USF Elga, Ransbach-Baumbach, Germany).

Acetate buffer (pH 5.5) was prepared by mixing 11 ml of 0.2 *M* acetic acid, and 89 ml of 0.2 *M* sodium acetate.

The enzyme β-glucuronidase aryl sulfatase (type H-2) was purchased from Boehringer (Mannheim, Germany).

Sep-Pack C_{18} cartridges were obtained from Waters (Milan, Italy).

Stock standard solutions of flumethasone and dexamethasone (1 mg/ml) in methanol were prepared monthly and stored at −20°C in the dark. Working solutions were daily prepared in mobile phase by appropriate dilution.

The immunoassay kit applied was 'generic corticosteroid' by Elisa Technologies Division of Neogen Corporation (Lexington, KY, USA). Sensitivity I-50, as described in Ref. [4], in EIA buffer was 0.25 ng/ml for flumethasone. The test procedure was performed following the instructions provided with the kit, at a wavelength of 450 nm.

2.2. Instrumentation

The immunoassay apparatus was a Sanofi LP 400 (Diagnostic Pasteur, Marnes-La-Coquette, Paris, France). HPLC–UV analysis was carried out on Jasco two-pump system PU-980 (Jasco, Tokyo, Japan) at a flow-rate of 150 μl/min under isocratic elution: mobile phase, methanol–water (65:35). HPLC column (equipped with a guard column, Phenomenex C_{18}, 4 mm×2 mm I.D.) 250×2.1 mm Nucleosil C_{18}, 5 μm (Machery-Nagel, Dueren, Germany) was slurry packed in our lab. The spectrophotometric UV detector was a Jasco 875-UV (Jasco) set at a wavelength 240 nm. The injector was a Rheodyne 8125 with a sample loop of 5 μl.

LC–MS analysis was carried out by a Shimadzu two-pump system LC-10 AD (Shimadzu, Kyoto, Japan) under isocratic elution: mobile phase, methanol–ammonium formate (1 m*M*) (65:35); flow-rate, HPLC column, guard column and injector as above.

MS and MS–MS analyses were performed on a PE-Sciex API 365 (Perkin-Elmer Sciex Instruments, Foster City, CA, USA) equipped with a Turboion Spray interface in negative mode.

The API source voltage was set at −3.5 kV. The orifice potential (OR) was set at −35 V and the ring potential (RNG) to −280 V. Nitrogen was used as nebulizing gas, as curtain gas and as collisional gas The settings for the nebulizer, curtain and collision gas were 8, 8 and 3 (arbitrary) on the API 365. The collisional energy was adjusted by variation of the voltage difference between the high-pressure entrance quadrupole (Q0) and the collisional cell quadrupole (RO_2) and was found to give the highest sensitivity for the analyte at 28 eV. The vaporizer was set at 450°C.

Acquisition parameters were optimized in ion spray mode by direct continuous pump infusion of standard working solution (10 ng/μl in ammonium formate 1 m*M*) at a flow-rate of 10 μl/min in the mass spectrometer.

Data acquisition were performed preliminarily on the standard compound of flumethasone in full scan, in negative mode (mass range 60–440 Da) using the first quadrupole to choose an abundant precursor (*m*/*z* 379). MS/MS product ion scans were then recorded from *m*/*z* 90–410 Da. Finally all the analyses, both on standard and on samples, were carried out by LC–MS–MS in SRM mode monitoring the product ions *m*/*z* 310 and 305 from *m*/*z* 379 to obtain a high specificity and sensitivity. For the I.S. dexamethasone the precursor ion *m*/*z* 361 and product ions *m*/*z* 307 and 292 were chosen for the SRM experiments.

2.3. Dosing of calves and sampling protocol

Two different dosages of Fluvet ('low' and 'high') were administered by intramuscular injection to a group of three calves to simulate both therapeutic and illegal use of this drug.

Two calves, identified, respectively, with Code 114 and Code 131, weighing about 400 kg body weight (bw) were treated with Fluvet at a low dosage (5 μg/kg bw per single administration corresponding to a therapeutic dose) [17]. The urine was collected by catheter before the administration at time 0 (blank), and after 2, 4, 8 and 24 h and a week after the administration. A third calf (Code 150) weighing about 50 kg bw was treated at high dosage (50 μg/kg bw per single administration, corresponding to 5 times the therapeutical dose of Fluvet), under veterinary control. Urine was collected before the administration at time 0 (blank), and after 5.7, 6.3,7.2, 10.7, 23, 24, 25.2 and 45 h. Sample serum of calf Code 150 were also collected at the following times: 0, 0.5, 1, 2, 4, 8, 24 and 48 h. All samples were stored at −20°C until analysis. Analyses were performed on the urine of all treated animals searching for total flumethasone (after enzymatic hydrolysis) and on the calf Code 150 also for free flumethasone both in serum and urine.

2.4. Sample preparation

Five ml of urine were adjusted to pH 5.2 adding acetate buffer and a few droplets of glacial acetic acid, and then hydrolyzed by 50 μl of β-glucuronidase–aryl sulfatase from Elix Pomatia (overnight, *t*= 37°C). The digested sample was loaded on a Sep-Pack C_{18} SPE column (previously conditioned with 3 ml of methanol and washed with 6 ml of aqueous acetic acid, pH 3.0). After a washing step with 6 ml of aqueous acetic acid (pH 3.0) the sample was eluted by 5 ml of MeOH. The extract was then evaporated at 45°C by nitrogen stream to dryness. The residue, dissolved with 1.5 ml of acetate buffer at pH 5.2, was extracted by 3 ml of TBME twice. The organic layers were collected and evaporated to dryness under nitrogen stream at 40°C. The residue was dissolved in 100 μl of a MeOH–water (1:1) mixture containing 100 ng of the I.S. dexamethasone and analysed by LC–MS–MS.

Urine samples of calf Code 150 were also analysed, without hydrolysis (free fraction), directly after SPE purification, elution with MeOH, evaporation and extraction by TBME. The residue was dissolved in 100 μl of a MeOH–water (1:1) mixture containing 100 ng of the I.S. dexamethasone and analysed by LC–MS–MS and by LC–UV. Serum samples (2 ml each) of this last calf were also treated as with urine for free fraction.

Urine samples of calf Codes 114 and 131 were also tested directly by enzyme-immunoassay after collection for qualitative purposes only.

2.5. Recovery

The recovery of the methods used was tested on a pool of calf blank urine spiked with the flumethasone standard compound.

Recovery was tested by immunoassay in spiked blank urine with concentration of flumethasone between 2.5 and 10 ng/ml. The antibody was reactive towards total flumethasone (free and conjugated).

Recovery was tested also on spiked blank urine of calf both by HPLC–UV, and by LC–MS–MS in SRM-negative mode. The samples were hydrolyzed as described above. Both spiked samples and standard (with the same final concentration in mobile phase) were run in triplicate.

For LC–UV, spiked urines were prepared at concentrations from 400 ng/ml up to 4 μg/ml of flumethasone corresponding to a final concentration in mobile phase, respectively, of 20 and 200 ng/μl. For the recovery by LC–SRM–MS–MS spiked urine were prepared at concentrations from 400 pg/ml up to 1 ng/ml of flumethasone corresponding to a final concentration in MeOH, respectively, of 40 and 100 pg/μl. For serum the recovery was tested by LC–MS–MS on a calf blank serum spiked with the same amount reported for urine.

2.6. Calibration and quantitation procedure

Calibration curves were prepared by SRM analyses of standard mixtures of flumethasone and I.S. The standards were injected in triplicate into the LC–MS–MS system. The amount of flumethasone in the mixture was chosen according to the estimated concentration of flumethasone in urine and serum samples. Graphs were calculated by least-squares linear fitting of the peak area ratio of the analyte to I.S. (using the most abundant ions) versus flumethasone concentration.

Two quality control samples (spiked urine and serum), prepared as recovery samples (concentration from 400 pg/ml to 1 ng/ml), were analyzed in

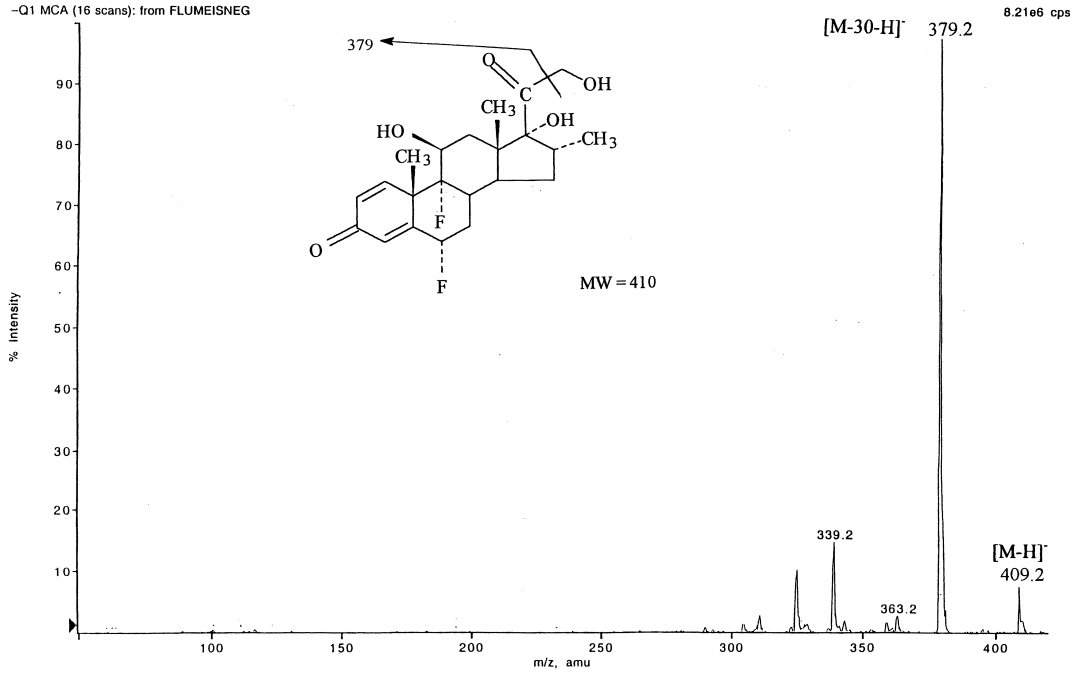

Fig. 1. Full scan API–MS spectrum, negative ions, obtained by infusion (10 μl/min) of flumethasone standard (10 ng/μl in ammonium formate 1 m*M*). MS conditions: I.S., −3500 V; OR, −35 V; RNG, −280 V.

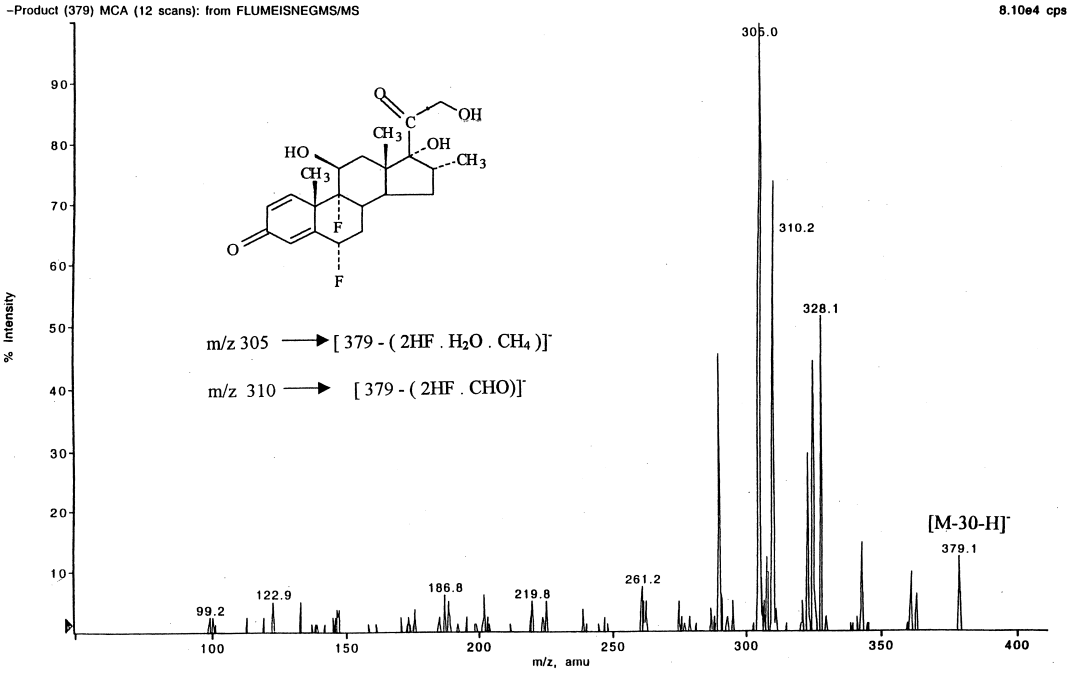

Fig. 2. Full scan MS–MS spectrum of flumethasone standard. Precursor ion m/z 379.1. RO_2, 38 V; IQ3, 53 V; RO_3, 41 V. The standard was infused as indicated in Fig. 1.

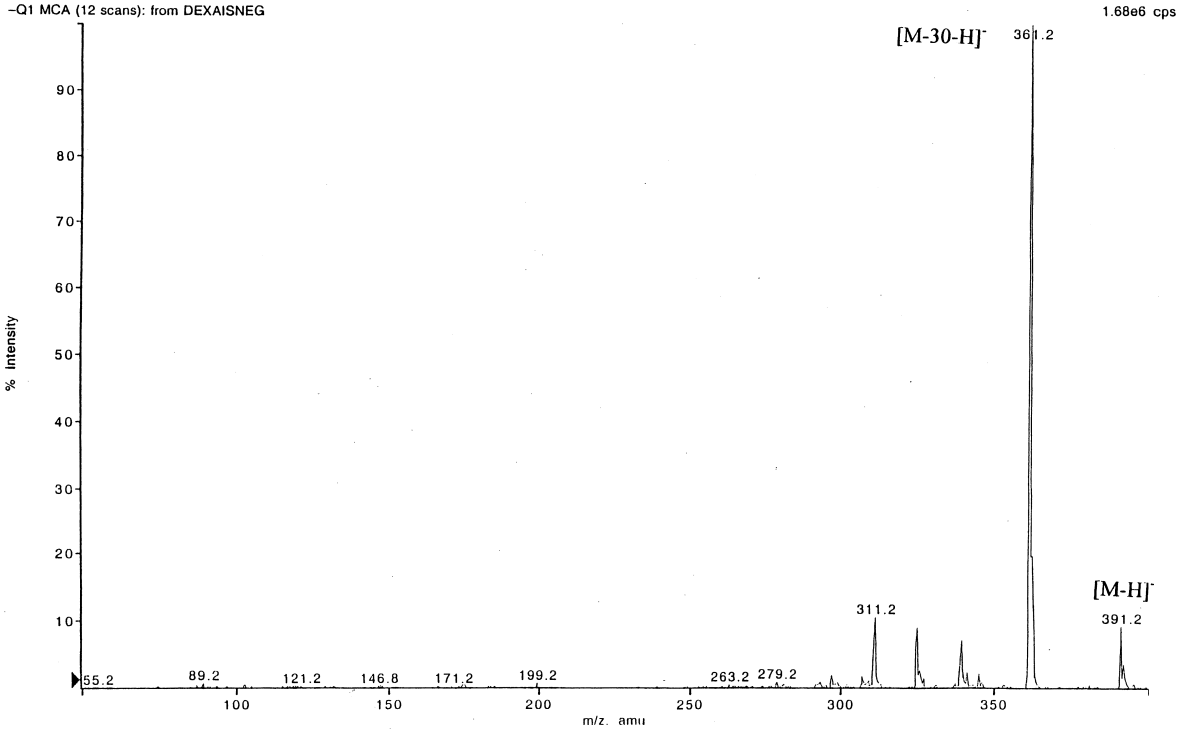

Fig. 3. Full scan API–MS spectrum, negative ions, obtained by infusion (10 μl/min) of dexamethasone standard (10 ng/μl in ammonium formate 1 m*M*). MS conditions as in Fig. 1.

triplicate to ensure that the method produced satisfactory results in terms of precision and accuracy. The precision was determined by calculating the relative standard deviation for the repeated measurements, and the accuracy of the method was determined by assessing the agreement between the measured and nominal concentrations of quality control samples.

The amounts of corticosteroids present in real samples were estimated from calibration graphs.

2.7. Limit of detection and quantification

The limit of detection (LOD) was calculated with spiked urine on the basis of a signal to noise ratio (S/N) 3:1.

The limit of quantification (LOQ) was estimated as the sample concentration of flumethasone resulting in a signal to noise of 10.

3. Results and discussion

3.1. LC–MS and LC–MS–MS analysis

The screening test made by immunoassay (calves Code 114 and 131) showed the presence of flumethasone in all collected urine fractions except the blank. The highest concentration were found in first fraction (time 2 h) and in the second fraction (time 4 h).

First acquisitions with mass spectrometer were made on quadrupole 1 (Q1) in full scan mode on flumethasone standard in continuous infusion. Fig. 1 shows the relative mass spectrum in negative mode. Fig. 2 shows the MS–MS collision-induced decomposition (CID) full scan spectrum of the m/z 379 fragment ions of the flumethasone standard under the same experimental conditions.

The abundant ion m/z 379 $[M–30–H]^-$, due to the loss of formaldehyde CH_2O (30 Da) from the

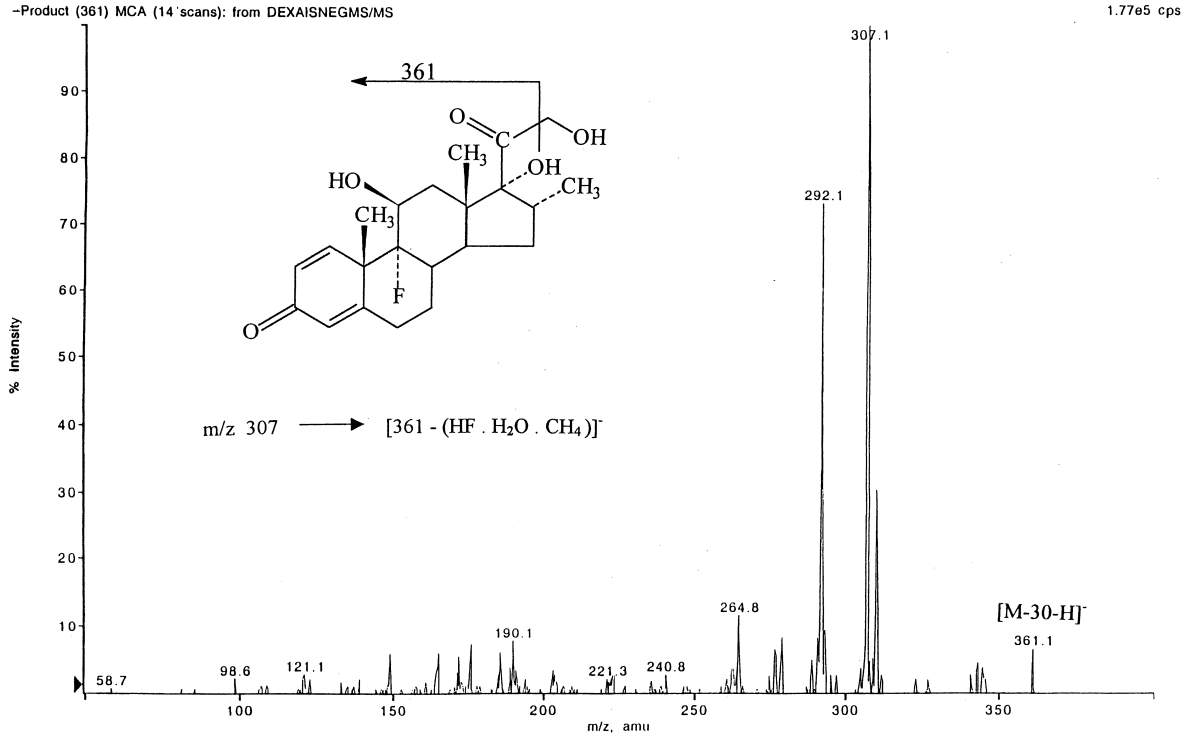

Fig. 4. Full scan MS–MS spectrum of dexamethasone standard. Precursor ion m/z 361.1. MS–MS condition as in Fig. 2. The standard was infused as indicated in Fig. 3.

hydroxymethyl group C_{21}, was chosen as a precursor for this experiment. We tried to use a lower orifice potential, to try to increase the signal of the deprotonated molecular ion $[M–H]^-$, but the most abundant ion was always $[M–30]^-$. Two product ions, m/z 310 $[379–2HF–CHO]^-$, and m/z 305 $[379–H_2O–CH_4–2HF]^-$ [11], were monitored in the SRM analysis.

Figs. 3 and 4 show, respectively, the MS spectrum and the MS/MS spectrum of the I.S., dexamethasone, in continuous infusion. The ion m/z 361 $[M–30–H]^-$ was chosen as a precursor and the ions m/z307 $[361–HF–H_2O–CH_4]^-$ and m/z 292 were monitored in the SRM analysis. The ions used for quantitation were, respectively, m/z 305 for flumethasone and m/z 307 for I.S.

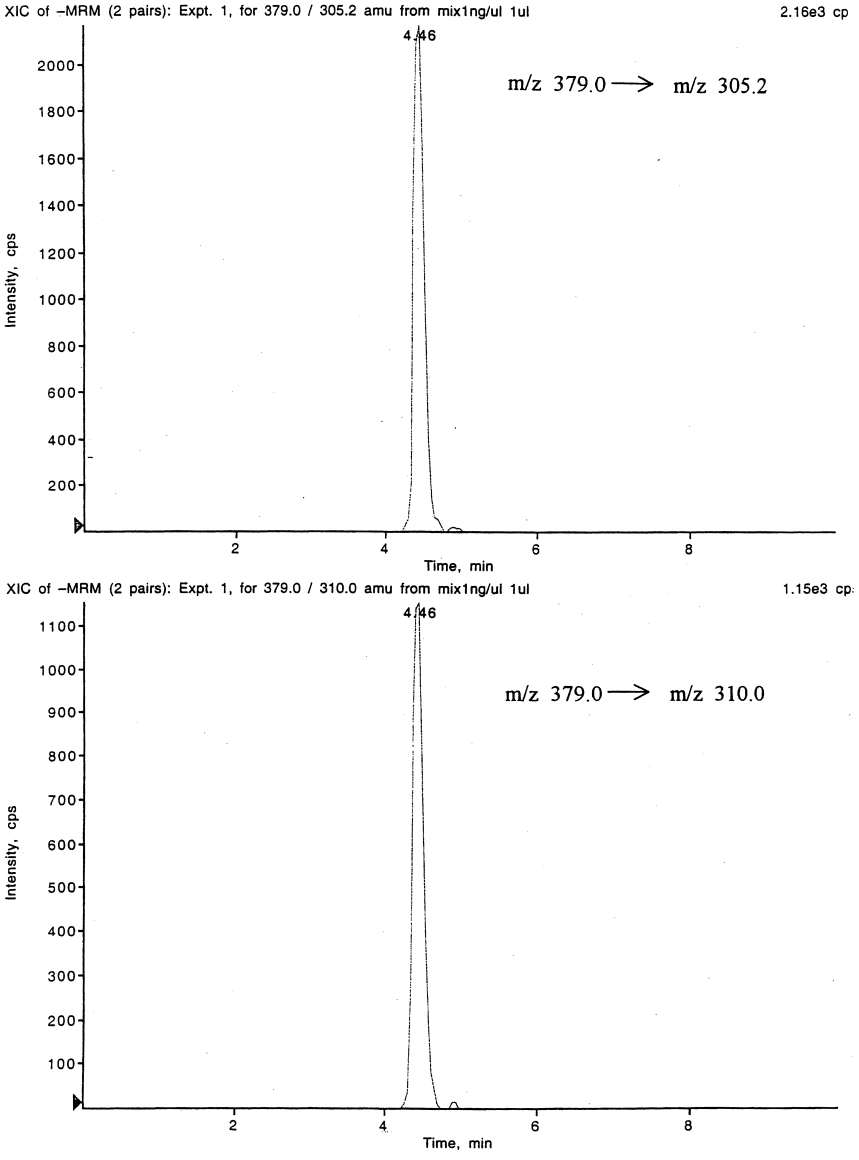

Fig. 5. SRM chromatogram in negative ionization of flumethasone standard (0.2 ng/μl). HPLC conditions: column, C_{18} Nucleosil 250 mm×2.1 mm I.D; mobile phase, MeOH–ammonium formate 1 m*M* (65:35). Flow, 150 μl/min. MS–MS conditions as in Fig. 2.

Figs. 5 and 6 show, respectively, the product ion chromatogram of a standard of flumethasone (1 ng/μl) and of a urine sample (Code 150 free fraction at time 25.2 h) injected onto the HPLC column monitoring the two product ions m/z 305 and m/z 310 of the precursor ion m/z 379 in SRM analysis. For qualitative purposes EU criteria [18] were used (retention times, number of diagnostic ions and ion ratios). Our analyses showed, after the application of the criteria for confirmation, that the samples fulfilled these requirements within day. Fig. 7 presents the results obtained, under the same experimental conditions, from the blank hydrolyzed bovine urine, showing no interfering peaks present at the flumethasone retention time. Working in negative SRM we obtained very high selectivity and sensitivity

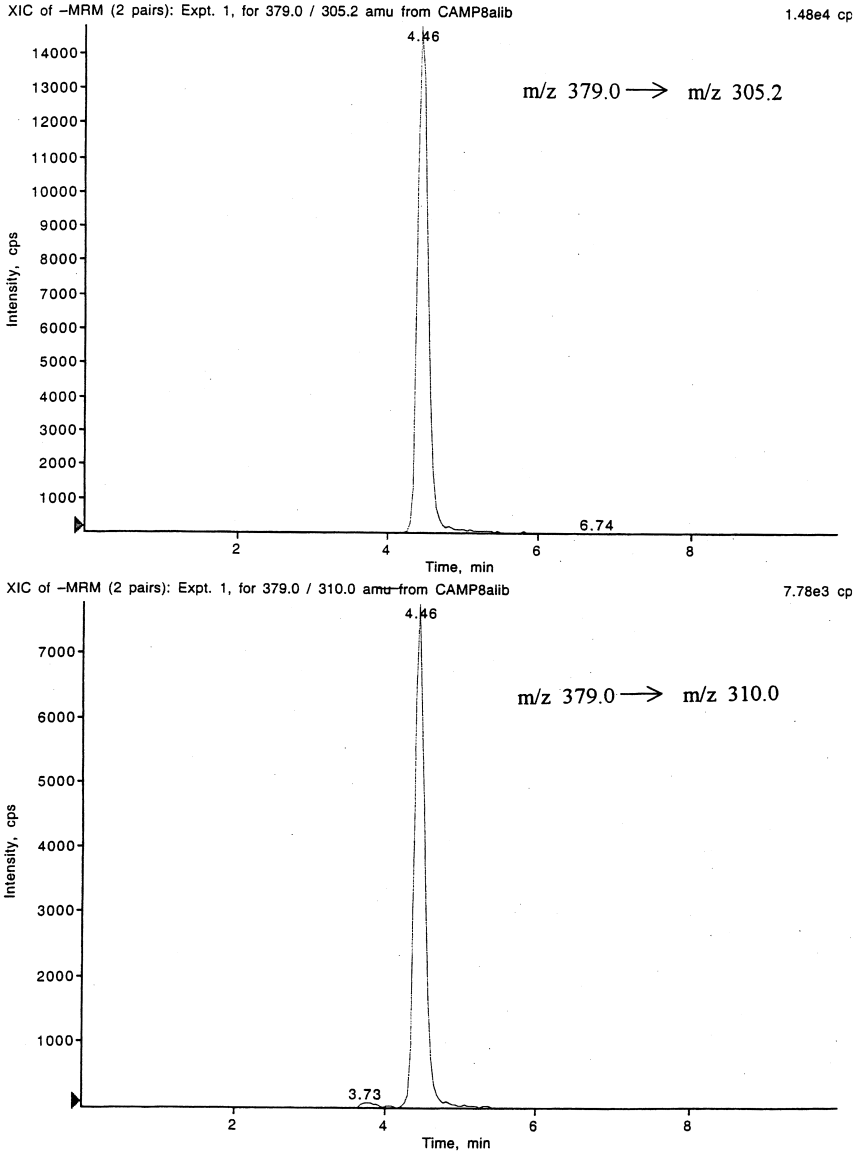

Fig. 6. SRM chromatogram in negative ionization of a urine sample (free fraction at time 25.2 h) of calf Code 150. Experimental conditions as in Fig. 5.

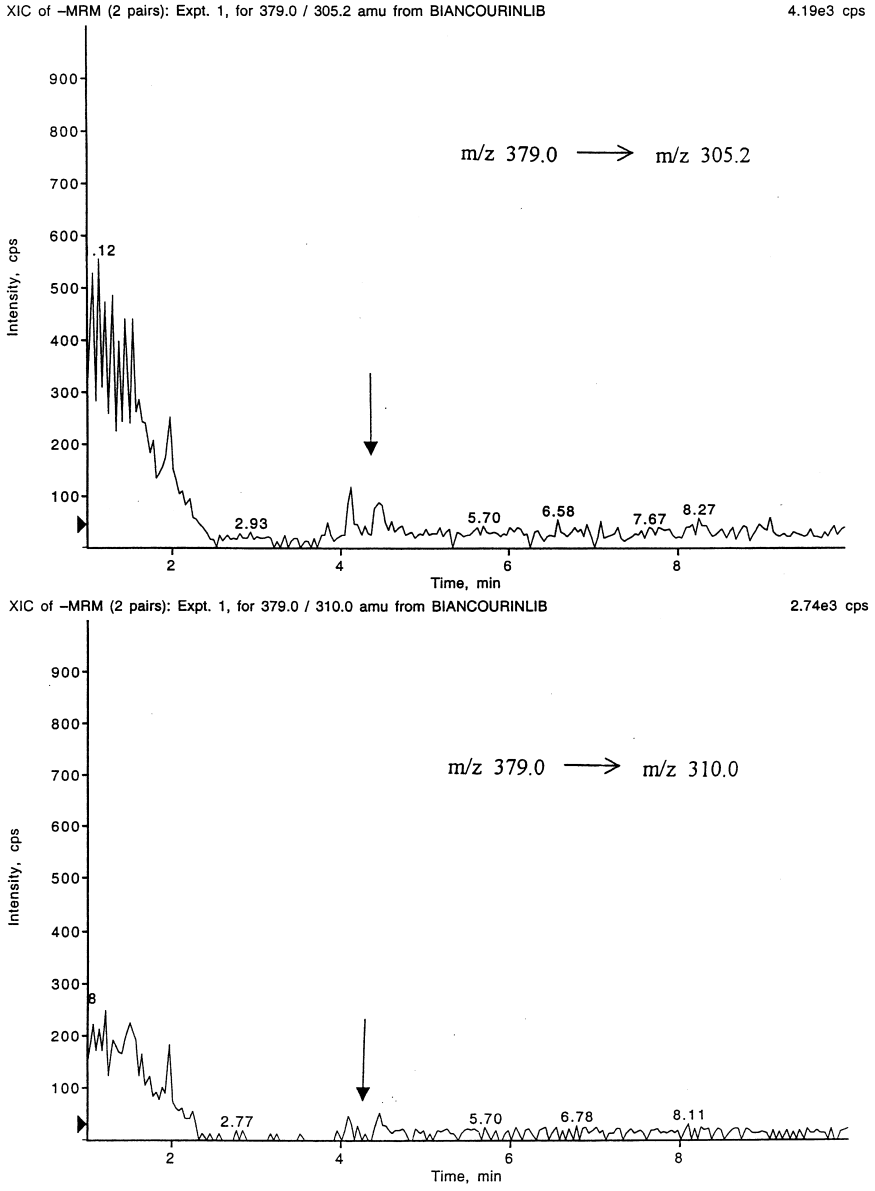

Fig. 7. SRM chromatogram in negative ionization of urine blank (free fraction) of calf Code 150. Experimental conditions as in Fig. 5.

and the analyses were very fast (about 5 min). Acquisitions in positive mode were also tried, but the sensitivity was not so good. Also selected ion monitoring (SIM) of the m/z 379.2 ion resulted in a good sensitivity for the detection of flumethasone. However, an endogenous compound in flumethasone-free urine interfered with its detection in the biological sample; therefore this method could not be used for the analysis of flumethasone in the urine.

Fig. 8 shows a SRM profile in negative ionization of a positive serum sample (Code 150, serum collected at time 1 h). Also in this case blank bovine serum (not reported) did not give any interfering peaks at the retention time of flumethasone.

The average recovery calculated in urine was

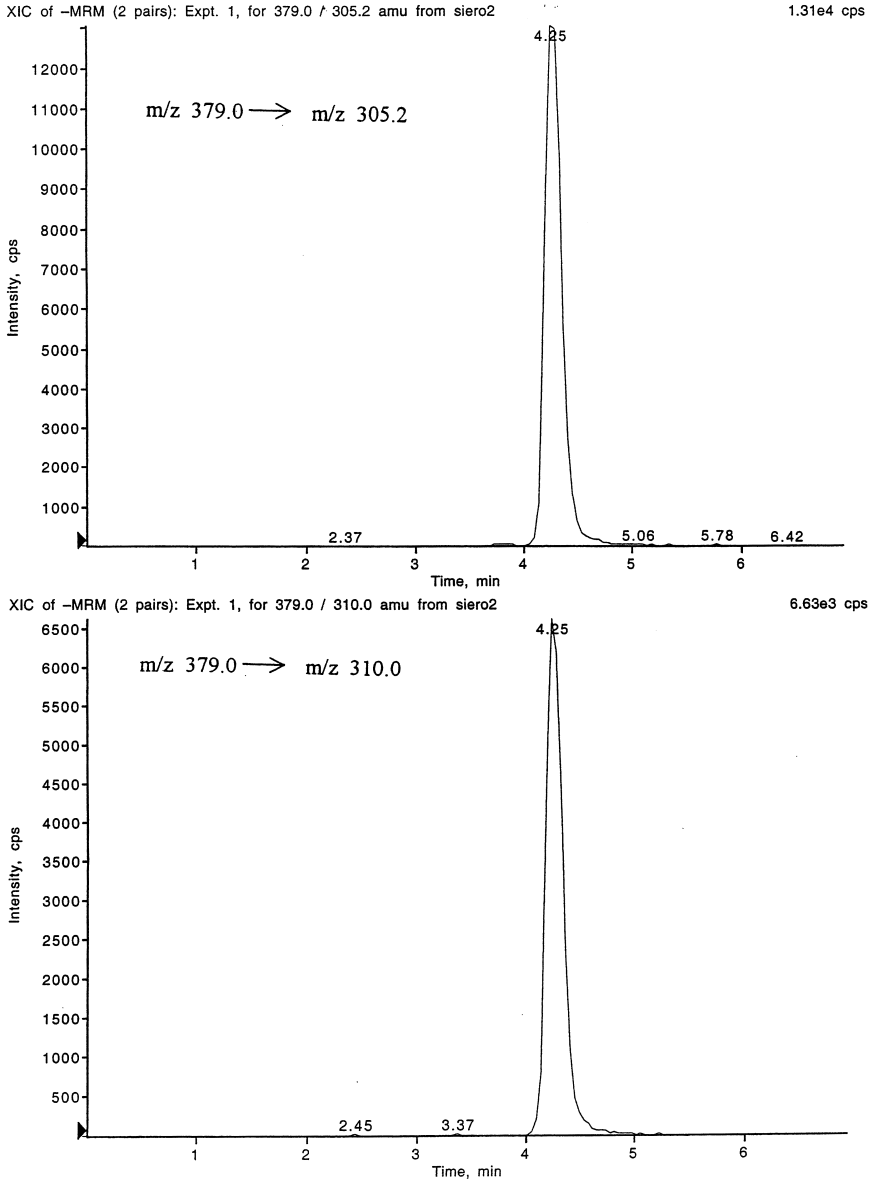

Fig. 8. SRM chromatogram in negative ionization of a serum sample (fraction at time 1 h) of calf Code 150. Experimental conditions as in Fig. 5.

87%±5% (n=3) and in serum was 85%±5%; no significant differences in the extraction efficiency were observed at the lowest concentration.

The precision was 10% for each sample.

The minimum amount detectable (LOD) by LC–MS–MS in SRM was 30 pg/ml and the limit of quantification (LOQ) was 100 pg/ml both in serum and urine.

Fig. 9 shows the product ion chromatogram of the internal standard dexamethasone (1 ng/μl) monitoring the two product ions m/z 307 and m/z 292 of the precursor ion m/z361 in SRM analysis.

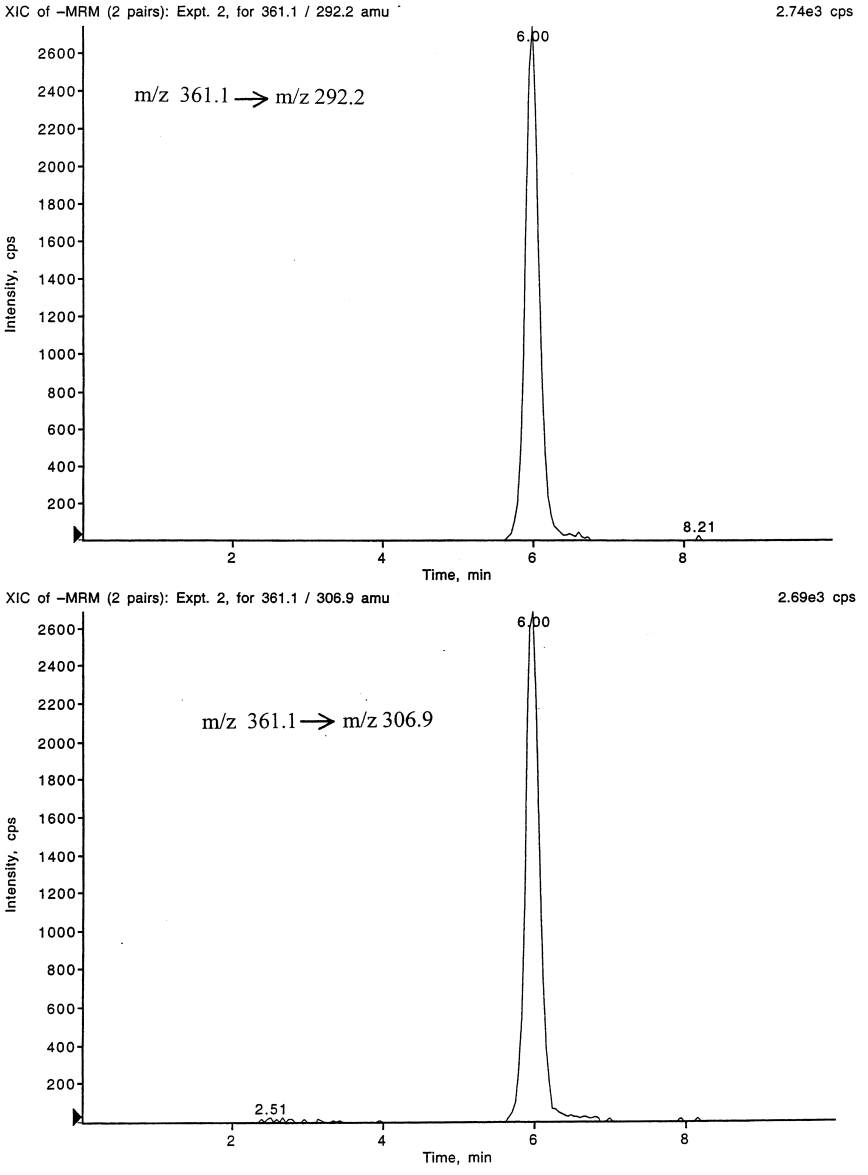

Fig. 9. SRM chromatogram in negative ionization of dexamethasone standard (0.2 ng/μl). Experimental conditions as in Fig. 5.

3.2. Quantitation results

3.2.1. Quantitation results for calf Code 150

3.2.1.1. Urine by LC–MS–MS. Six calibration levels were used injecting directly three times 5 μl of standard solutions of flumethasone (with dexamethasone 1 ng/μl) at the following concentrations 0.5, 1.0, 2.0, 6.0, 10 and 20 ng/μl in MeOH–water (1:1) as shown in Table 1. The relative calibration graph is given by the equation $y=(0.66\pm0.70)+(1.45\pm0.03)x$ with $R^2=0.9974$.

Table 2 shows the concentrations found in urine on the fractions collected from calf Code 150. Two

Table 1
Calibration table of flumethasone standard in MeOH–water (1:1) for urine sample analyses

Absolute conc. (ng/μl)	Conc. referred to urine (ng/ml)	Injected amount (ng)	Area ratio (analyte versus I.S.) average (n=3)
0.5	10	2.5	0.67
1.0	20	5.0	2.40
2.0	40	10.0	4.17
6.0	120	30.0	9.64
10.0	200	50.0	14.47
20.0	400	100.0	29.80

aliquots of each sample were prepared and injected three times each. The data shown in the table were obtained on the average of six injections (n=6).

After 45 h, flumethasone is still present in urine in both free and conjugated forms. The difference between the free and hydrolyzed fractions is about 20–35%. Considering this small difference, and that preparation of the free fraction is faster and cleaner than that of the total flumethasone, we think that for a screening procedure it can be sufficient to analyze only the free fraction.

3.2.1.2. Urine by LC–UV. Considering the results of Table 1 we decided to examine several fractions of urine of calf Code 150 by LC–UV to make a comparison between quantitative results. Due to the low sensitivity, just a few fractions of calf Code 150 were analysed. We analyzed only free fractions, because the total fractions showed interfering peaks at the same retention time as flumethasone. Table 3 shows the results. The concentrations found by LC–UV, are, except for the first fraction, a bit lower than

Table 2
Flumethasone concentration in calf urine (Code 150) after administration of flumethasone (five times the therapeutic dose) (n=6)

Time (h)	Free fraction (urine conc.) mean±SD (ng/ml)	C.V. (%)	Total fraction after hydrolysis (urine conc.) mean±SD (ng/ml)	C.V. (%)
0	–	–	–	–
5.7	285±5	2	373±15	4
6.4	30.3±2.4	8	43.45±1.7	4
7.2	61.3±0.7	1	77.4±1.2	2
10.7	219±4	2	266±7	3
23	137± 4	3	163±5	3
24	25.1±1.2	5	37.9±1.9	5
25.2	21.2±1.2	6	27.1±1.7	6
45	27.3±2.8	10	33.4±0.6	2

Table 3
Flumethasone concentration by HPLC–UV in calf urine (Code 150) (n=6)

Time (h)	Free fraction (urine conc.) mean±S D (ng/ml)	C.V. (%)
0	–	–
5.7	329±18	5
6.3	29.7±2.3	8
7.2	41.6±3.1	8
10.7	150±7	5
23	90.4±1.4	2

Table 4
Calibration table of flumethasone standard for serum sample analyses

Absolute conc. (pg/μl)	Conc. referred to serum (ng/ml)	Injected amount (ng)	Area ratio (analyte versus I.S.) average (n=3)
5	0.25	0.03	0.013
125	6.25	0.63	0.29
250	12.5	1.25	0.52

Table 5
Flumethasone concentration in calf serum (Code150) after administration of flumethasone (five times the therapeutical dose) (n=6)

Time (h)	Serum conc. (mean±SD, ng/ml)	C.V. (%)
0	–	–
0.5	9.74±0.16	2
1	8.73±0.12	1
2	7.68±0.11	1
4	5.21±011	2
8	4.88±0.09	2
24	1.58±0.05	3
48	n.d.	–

Table 6
Flumethasone concentration in calf urine Code 114 and Code 131 after an administration of a therapeutic dose ($n=6$)

Time (h)	Sample Code 114 Total fraction after hydrolysis (urine conc.) mean±SD (ng/ml)	C.V. (%)	Sample Code 131 Total fraction after hydrolysis (urine conc.) mean±SD (ng/ml)	C.V. (%)
0	–		–	
2	7.55±0.50	7	8.10±0.45	6
4	6.90±0.14	2	4.60±0.33	7
8	3.48±0.10	3	3.40±0.19	6
24	0.09±0.01	10	0.10±0.01	10
A week after administration	n.d.[a]		n.d.[a]	

[a] n.d., not detected (<0.01).

by LC–MS–MS (Table 1). The minimum detectable amount (LOD) by LC–UV was 15 ng/ml ($S/N=3$). The average recovery was 97%±5% ($n=3$).

3.2.1.3. Serum by LC–MS–MS. Three calibration levels were used injecting for three times 5 μl of standard solutions of flumethasone (with dexamethasone 1 ng/μl) at the following concentrations 5, 125 and 250 pg/μl in MeOH–water (1:1) as shown in Table 4. The relative calibration graph is given by the equation $y=(0.012\pm0.015)+(0.0021\pm5.52\times10^{-5})x$ with $R^2=0.9957$. The results obtained on serum samples are shown in Table 5. The amount of flumethasone found in serum is about 10–40 times less than in urine. The concentration decreases from the first to the sixth fraction, and 48 h after it is below the limit of detection. This consideration makes urine the most suitable biological fluid to search for the illegal use of flumethasone.

3.2.2. Quantitation results for calves Code 114 and 131

In Table 6 the comparison between hydrolyzed urine samples, obtained for two calves after therapeutic treatment, is reported. 5 μl of each sample (prepared in duplicate) were injected for three times (total $n=6$).

The comparison between the samples collected at different time in both the calves is in good agreement and shows that the highest excretion of flumethasone is 2 h after administration. Then the analyte concentration in urine decreases quite rapidly and after 24 h the presence of flumethasone is going to disappear according to the data obtained by immunoassay.

4. Conclusions

The method described for the determination and confirmation of flumethasone in animal biological fluids is easy, highly specific and very sensitive (<0.05 ng/ml of flumethasone can be easily detected). Quantitative data obtained by this method showed an acceptable precision and accuracy.

The sensitivity reached is better than GC–MS and immunoassay methods and in LC–MS–MS there are no problems due to the derivatization and to possible interferences typical of immunoassay. As the administration of corticosteroids in cattle is not allowed by health institutions and official authorities for fattening purposes the use of LC–MS–MS based on monitoring two characteristic ions coming from the same precursor ion gives the strong evidence of the drug treatment. For forensic purposes, urine seems to be the biological fluid most suitable for flumethasone detection.

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