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Detection of synthetic glucocorticoid residues in cattle tissue and hair samples after a single dose administration using LC–MS/MS

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Abstract

A sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for the detection of several synthetic glucocorticoids in kidney, muscle and hair samples of cattle after a single intramuscular injection is described. After a dichloromethane wash of the hair samples, analytes were released from the hair matrix by enzymatic digestion. Muscle samples were also digested enzymatically using proteinase, while kidney samples were deconjugated by Helix pomatia juice. These preliminary steps were followed by a methanol extraction and a solid phase extraction (SPE) clean up step for all matrices. Chromatographic separation was achieved on a Hypersil Hypercarb column and MS/MS data were obtained in the multiple reaction monitoring mode using negative electrospray ionization. The developed protocols were evaluated by assessing residue concentrations in muscle, kidney and hair samples of thirteen calves, treated with a particular intramuscular injection of glucocorticoid. The lowest residue levels were found in muscle samples (approximately 5% of the residue levels in kidney), while high residue levels were obtained in hair samples. Hair is an interesting matrix since the sampling is non-invasive and the drugs may stay incorporated for a longer period of time.

Keywords: Glucocorticoids; LC–MS/MS; Food safety; Growth-promoters

1. Introduction

The application of synthetic glucocorticoids as growth promoting additives is banned within the European Union [1]. For therapeutic indications only, the use of dexamethasone, betamethasone, prednisolone and methylprednisolone is allowed and therefore maximum residue limits (MRLs) have been established in milk and tissues intended for human consumption [2]. Betamethasone and dexamethasone have identical MRLs i.e. 2 μg/kg in liver, 0.75 μg/kg in muscle and kidney and 0.3 μg/kg in milk samples. A maximum residue limit of 10 μg/kg prednisolone is allowed in liver and kidney, 4 μg/kg in muscle and fat and 6 μg/kg in milk. Methylprednisolone has MRLs of 10 μg/kg for all matrices, but may not be used in animals that produce milk for human consumption. In the European monitoring programmes urine is the most commonly used analytical matrix for the detection of glucocorticoids and thus methods based on LC–MS are described frequently [3–5]. Previously, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for synthetic glucocorticoid detection in liver samples was described [6]. In this study other matrices such as kidney, muscle and hair were explored. The analysis of hair samples for the detection of glucocorticoids has been described, although mainly for human hair samples in doping control [7–11]. The advantage of hair analysis over other matrices is that the collection of the samples is non-invasive, and that once the drug is incorporated into the hair matrix, it may stay there for months [12]. The aim of this study was to develop sample pretreatment protocols for kidney, muscle and hair matrices prior to LC–MS/MS analysis of the

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glucocorticoid residues. After method validation, samples of all these matrices from calves treated with an intramuscular injection of a particular compound were analyzed. The residue concentrations within the different matrices were compared and evaluated to see whether a relationship could be found between them.

2. Experimental

2.1. Reagents and materials

Betamethasone, dexamethasone, flumethasone, prednisone, prednisolone, methylprednisolone, fludrocortisone, triamcinolone, triamcinolone acetonide and beclomethasone were purchased from Sigma (Bornem, Belgium). The internal standard (isoflupredone) was obtained from Steraloids (Newport, USA). HPLC-grade water was prepared by using a Milli-Q purification system (Millipore, Brussels, Belgium). All reagents and solvents were of analytical grade. Acetonitrile, hexane and methanol were purchased from BDH (Poole, Dorset, UK), dichloromethane, diethyl ether and acetone from Acros (Geel, Belgium), formic acid, ethanol, tris-hydroxymethylaminomethane and sodium carbonate from Merck (Darmstadt, Germany). *Helix pomatia* juice, used for enzymatic hydrolysis, was purchased from Boehringer Mannheim (Mannheim, Germany, cat. no. 127698) and proteinase from Fluka (Buchs, Switzerland, art. no. 82528). Disposable C_{18} extraction cartridges with 1 g solid phase were purchased from Varian (Sint-Katelijne-Waver, Belgium).

2.2. Standard solutions

A stock standard solution of each compound was prepared by dissolving 10 mg of the pure compound in 10 ml ethanol. Working solutions were prepared monthly by appropriate dilution of the stock standards in ethanol. All solutions were stored in the dark at −20 °C. The chemical structures of all analyzed compounds are shown in Fig. 1.

2.3. Incurred samples

Twelve calves received a single intramuscular injection of a single dose of a glucocorticoid (dexamethasone, methylprednisolone, flumethasone, prednisolone, triamcinolone and triamcinolone acetonide) at three different levels (2, 5 and 10 mg per 50 kg bodyweight). Another calf was treated with dexamethasone and betamethasone simultaneously. The calves were fed on a mixture of hay and commercial calf feed on an ad-lib basis. The calves were slaughtered 3, 6 or 10 days after treatment. Details of the treatment are presented in Table 1. Liver, kidney, muscle and skin were removed post mortem from each animal and were immediately frozen at −20 °C until analysis.

2.4. Isolation of the compounds

2.4.1. Decontamination procedure of hair samples

The hair was first scraped from the skin by means of a scalpel. The hair samples were cut finely with scissors and approximately 200 mg was placed into a test tube. For the decontamination of the hair samples a wash step with dichloromethane was chosen which has been described in several studies [8,13,14]. The hair was rinsed twice with 5 ml of dichloromethane while the samples were sonicated. After each rinse, the solvent was removed and samples were allowed to dry under a stream of nitrogen in a warm water bath set at 40 °C.

2.4.2. Analyte extraction

2.4.2.1. Hair. A 100 mg aliquot of the dried hair was weighed into a test tube and fortified with 100 μl of a

Table 1
Treatment of animals, day of slaughter after treatment and residue concentration found in the corresponding tissue samples using LC–MS/MS analysis

Calf no.	Drug administered	Dosage (mg 50^{-1} kg^{-1})	Day of slaughter after treatment	Residue concentration ± S.D. (μg/kg), $n = 2$			
				Liver[a]	Kidney	Muscle	Hair
1	Dexamethasone	2	3	1.9 ± 0.1	2.4 ± 0.8	0.1 ± 0.1	[b]
2	Dexamethasone	10	6	0.3 ± 0.0	0.4 ± 0.3	[b]	[b]
3	Dexamethasone	5	10	[b]	0.1 ± 0.0	[b]	[b]
4	Methylprednisolone	5	3	0.5 ± 0.1	[b]	[b]	16.4 ± 1.6
5	Methylprednisolone	10	6	[b]	[b]	[b]	[b]
6	Methylprednisolone	2	10	[b]	[b]	[b]	113.2 ± 2.3
7	Triamcinolone	10	3	7.4 ± 0.1	18.2 ± 1.0	1.1 ± 0.4	9.5 ± 0.1
8	Triamcinolone	2	6	[b]	[b]	[b]	[b]
9	Triamcinolone	5	10	[b]	[b]	[b]	[b]
10	Betamethasone	10	3	14.6 ± 0.1	7.8 ± 0.1	0.9 ± 0.1	29.4 ± 0.1
	Dexamethasone	10	3	3.2 ± 0.2	4.4 ± 0.1	0.2 ± 0.1	[b]
11	Prednisolone	10	3	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	200.0 ± 21.2
12	Flumethasone	10	3	19.6 ± 0.1	13.4 ± 0.3	1.5 ± 0.0	5.9 ± 0.4
13	Triamcinolone acetonide	10	3	4.5 ± 0.2	1.8 ± 0.4	0.4 ± 0.2	347.9 ± 29

[a] Results of liver samples analysed in a previous study [6].
[b] Below limit of detection.

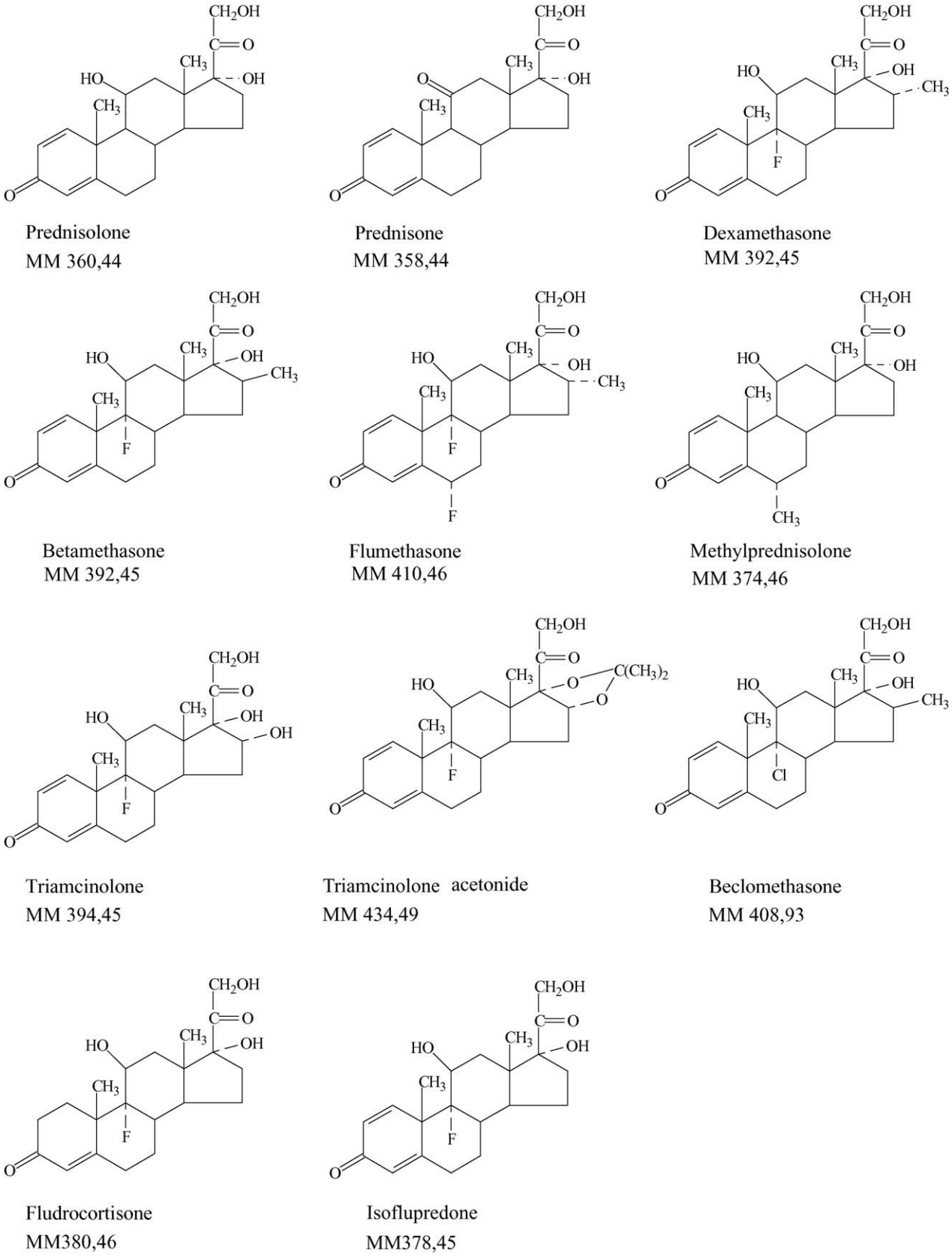

Fig. 1. Chemical structures of the analyzed glucocorticoids.

0.1 ng/μl solution of the internal standard isoflupredone. Enzymatic digestion was performed by adding 2 mg of proteinase and 2 ml of Tris–HCl (0.1 M, pH 9.6) and vortex mixing for 30 s. The hair samples were incubated for 2 h at 60 °C. After incubation 3 ml methanol was added for the extraction of the analytes. After vortex mixing and centrifuging for 10 min at 3600 rpm the supernatant was transferred into another test tube. The extraction was repeated with another 3 ml methanol. The combined extracts were evaporated under a nitrogen stream in a water bath at 40 °C.

2.4.2.2. Muscle. Muscle samples were homogenized and a 5 g aliquot was fortified with internal standard isoflupredone (100 μl of a 0.1 ng/μl solution). Enzymatic digestion was carried out as for the hair samples, using however 6 ml of Tris–HCl instead of 2 ml. Proteinase was added in the same amount since it was added to hair samples in abundance. Afterwards an extraction similar to the one for hair samples was carried out with the only difference that two 6 ml methanol volumes were used instead of 3 ml.

2.4.2.3. Kidney. A 5 g aliquot of homogenized kidney tissue was fortified with 100 μl of a 0.1 ng/μl solution of the internal standard (isoflupredone). Ten millilitre of sodium acetate buffer (3 M, pH 5.2) were added and samples were vortex mixed. Enzymatic hydrolysis was executed by adding 100 μl Helix pomatia juice and incubating for 4 h at 40 °C. Afterward, a methanol extraction identical to one of the muscle samples was performed.

Preparation of all samples prior to solid phase extraction (*SPE*). To the dry extracts of all matrices 1 ml ethanol was added and samples were sonicated for approximately 10 min. Just before application onto the extraction cartridges 6 ml water was added to each sample.

2.4.3. Solid phase extraction

The extraction cartridges were conditioned with 2 × 5 ml methanol and 2 × 5 ml water. Then samples were applied slowly and washed with 5 ml of acetone/water (20/80, v/v), 5 ml water and 5 ml hexane. Finally the columns were dried and eluted with 6 ml diethylether. An extra washing step (not necessary for hair samples) was carried out by adding 1 ml of 10% (w/v) sodium carbonate solution, vortex mixing and centrifugating for 1 min at 2000 rpm. The upper diethylether layer was collected and evaporated under a nitrogen stream at 40 °C. The dry residue was dissolved in 50 μl of the HPLC mobile phase (see below) and 10 μl was injected into the HPLC system.

2.5. Liquid chromatography

Liquid chromatographic analyses were carried on an Alliance 2695 HPLC system (Waters, Milford, MA, USA). Chromatographic separation was achieved on a Hypersil

Table 2
Precursor and product ions, cone voltages and collision energies for each transition monitored in MRM (ESI-) analysis

Compound		Precursor ion (*m/z*)	Product ion (*m/z*)	Cone (V)	Collision (eV)
Dexamethasone	$[M + formate]^-$	437	361	40	14
	$[M - H–CH_2O]^-$	361	307	65	20
Betamethasone	$[M + formate]^-$	437	361	40	14
	$[M - H–CH_2O]^-$	361	307	65	20
Flumethasone	$[M + formate]^-$	455	379	20	25
	$[M - H–CH_2O]^-$	379	305	40	30
Prednisone	$[M + formate]^-$	403	327	30	12
	$[M - H–CH_2O]^-$	327	149	70	25
Prednisolone	$[M + formate]^-$	405	329	35	15
	$[M - H–CH_2O]^-$	329	295	45	25
Methylprednisolone	$[M + formate]^-$	419	343	15	25
	$[M - H–CH_2O]^-$	343	309	45	25
Fludrocortisone	$[M + formate]^-$	425	349	40	15
	$[M - H–CH_2O]^-$	349	295	80	20
Triamcinolone	$[M - H]^-$	393	363	60	12
	$[M - H]^-$	393	345	60	15
Triamcinolone acetonide	$[M + formate]^-$	479	413	35	30
	$[M + formate]^-$	479	337	35	25
Beclomethasone	$[M + formate]^-$	453	377	35	14
	$[M + formate]^-$	453	341	35	20
I.S. Isoflupredone	$[M + formate]^-$	423	347	45	15
	$[M + formate]^-$	423	293	45	30

Hypercarb column (100 mm × 2.1 mm, 5 μm particle size) preceded by a guard column (Hypercarb, 20 mm × 2 mm, 5 μm). The mobile phase consisted of acetonitrile/water (90/10, v/v) + 0.3% (v/v) formic acid. Elution was isocratic at a flow rate of 0.22 ml/min. A chromatographic run took 30 min.

2.6. Mass spectrometry

Mass spectrometry was carried out in the negative electrospra ionization mode (ESI). ESI multiple reaction monitoring (MRM) mass spectra were acquired on a Quattro Micro mass spectrometer (Waters, Milford, MA, USA). Cone voltages and collision energies were optimized during tuning. They were adjusted for each transition specifically and varied between 15 and 70 V (cone voltage) and 12 and 35 eV (collision energy). The capillary voltage was set at 3.0 kV. Source and desolvation temperatures were set at 120 and 350 °C, respectively. The flow rates of nitrogen used as nebulizing and desolvation gas, were 130 and 500 l/h respectively. In the collision cell, argon was used as collision gas at a pressure of 2.4×10^{-3} mbar. *m/z* values of the precursor and product ions for each analyte, cone voltages and collision energies are shown in Table 2.

2.7. Method validation

For the validation, the rules of the European Commission Decision 2002/657/EC [15] were followed. This implicates that for a quantitative confirmatory method detection capabilities (CCβ) and decision limits (CCα) needed to be determined, together with the trueness, precision and specificity. Detection and decision limits were established by the calibration curve procedure according to ISO 11843 [16]. To obtain these calibration curves, blank kidney, muscle and hair sample material was fortified at five different levels. As an approximation of the trueness, blank samples were fortified at three nominal levels and the estimated amounts were correlated with the nominal concentrations and expressed as % bias. Precision was determined at three different levels, on the same day (within-day repeatability) and over separate days (between-day repeatability). In order to test the specificity of the method, at least 10 blank samples were analyzed and checked for interfering compounds in the regions of interest where the target analyte is expected to elute.

3. Results and discussion

3.1. Isolation of the compounds

3.1.1. Analyte extraction

First of all glucocorticoids need to be released from the hair matrix. In general methods to achieve this can be divided into solvent extraction, acidic, basic and enzymatic digestion. For the solvent extraction methanol was used and samples were sonicated for 6 h. Alkaline hydrolysis was performed with 2 ml NaOH (1 M) and samples were incubated 30 min at 90 °C while for acidic digestion 2 ml HCl (1 M) was added, followed by an incubation of 16 h at 50 °C. The enzymatic digestion of the hair was carried out by adding Tris–HCl and proteinase to the samples. The methods were evaluated by comparing the signal-to-noise (S/N) ratios for each compound. After alkaline digestion no analyte could be detected. Methanol and HCl extraction yielded comparable results, but average S/N ratios were only 40% and 27%, respectively of the S/N ratios obtained after enzymatic digestion. Chromatograms after enzymatic digestion showed very little interferences and therefore this extraction method was selected.

This digestion using proteinase was also applied to the muscle samples, since previous experiments in our laboratory proved the necessity of muscle digestion prior to further extraction [17].

The necessity of a preliminary deconjugation step in grown kidney samples was evaluated. Hydrolysis conditions as previously optimized in liver samples using experimental design [6], were used. Kidney tissues of the calf treated simultaneously with beta- and dexamethasone were analyzed with and without a preliminary hydrolysis step. The betamethasone residue concentration found was 7.8 μg/kg after hydrolysis, while it was only 5.2 μg/kg (66%) without. In the same samples 5.5 μg/kg dexamethasone was found when hydrolysed and 5 μg/kg (91%) when not. Given that glucocorticoids have either very low MRLs (0.75 μg/kg for beta- and dexamethasone in kidney) or are completely forbidden, the use of a deconjugation step can be crucial in the decision whether a sample is compliant or non compliant. Taking this into consideration a preliminary enzymatic hydrolysis step for kidney samples was opted for.

3.1.2. Solid-phase extraction

The extra sodium carbonate wash step was added to remove interfering compounds, like hydrophilic or ionic compounds or salts, out of the organic phase and pass them on to the aqeous layer. Hair extracts were, after the methanol extraction, relatively clean and did not need the extra sodium carbonate washing step after SPE.

3.2. LC–MS/MS analysis

According to the Commission Decision 2002/657/EC, in LC–MS/MS analysis two transitions need to be followed for each analyte (one precursor ion with two product ions or two precursor ions each with one product ion), yielding the required four identification points. With formic acid in the mobile phase, the conjugated base of this acid ionized the compounds, yielding adductions. This $[M + \text{formate}]^-$ ion was the most abundant ion in the MS spectra of all analytes except in that of triamcinolone, where it was $[M - H]^-$ (see Table 2). Thus, this ion was selected as a precursor ion in the first and most important transition (used for quantification). The major fragmentation step is the loss of formaldehyde

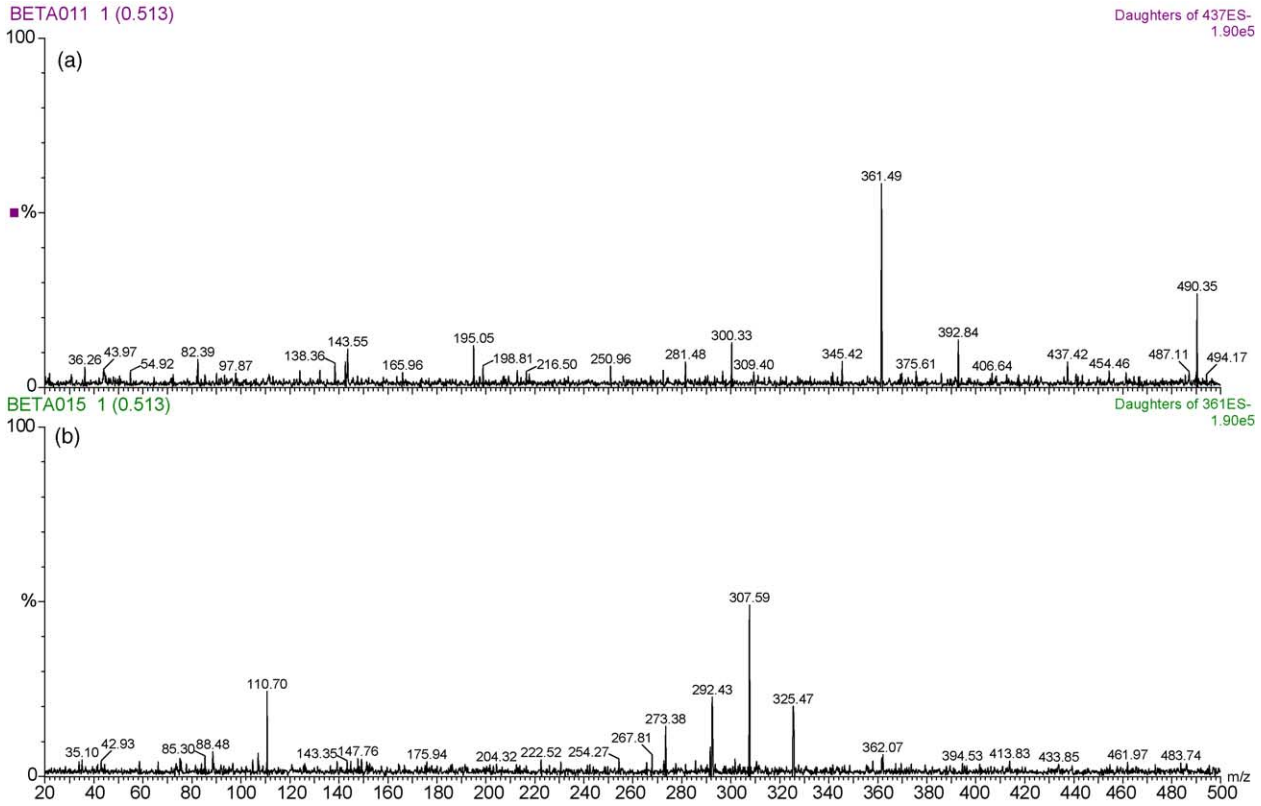

Fig. 2. ESI-MS/MS product ion spectra of betamethasone (*m/z* 392) with the $[M + formate]^-$ ion (*m/z* 437) selected as the precursor ion (a) for a cone voltage set at 40 V and a collision energy at 14 eV, and the $[M - H–CH_2O]^-$ ion (*m/z* 361) selected as precursor ion (b) at a cone voltage set at 65 V and a collision energy at 20 eV.

$[M - H–CH_2O]^-$ from the hydroxymethyl group (C21). By raising the cone voltage for the second transition (see Table 2), the $[M - H–CH_2O]^-$ ion can be generated in the source by an in-source collision induced dissociation. This ion can thus be selected in the first mass spectrometer as a precursor ion and further fragmented in the collision cell. Further dissociations include consecutive losses of H_2O, CH_4, HF and HCl depending on the analyte. Fig. 2a presents the ESI-MS/MS spectrum of betamethasone; were the $[M + formate]^-$ ion at *m/z* 437 is selected as the precursor ion in the first mass spectrometer at a cone voltage of 40 V and collision energy at 14 eV, while the second mass spectrometer is used in the scanning mode. Fig. 2b shows the MS/MS spectrum of betamethasone where the $[M - H–CH_2O]^-$ is formed in the source due the higher cone voltage (65 V) and selected as precursor ion in the first mass spectrometer. The second mass spectrometer is used in the scanning mode yielding the product ion at *m/z* 307 as most abundant ion. By operating in the MRM mode, co-eluting analytes did not interfere with each other unless they had the same precursor and product ions. This was the case for beta- and dexamethasone, epimers with identical mass spectra. For this purpose the Hypercarb column was used, which has the ability to distinguish between closely related isomers. Thus, beta- and dexamethasone could be separated adequately. ESI-MRM chromatograms of the first transition followed for betamethasone and dexamethasone are presented in Figs. 3 and 4a and show a baseline separation between these analytes.

3.3. Method validation

To determine CCα and CCβ, at least six calibration curves with five data points ranging from 0 to 10 μg/kg in muscle and kidney tissue and from 0 to 200 μg/kg in hair were obtained. The peak area ratios (compound/IS) were plotted against the corresponding concentrations and the calculated correlation coefficients were at least 0.99 for all analytes in the different matrices. In Table 3. decision limits and detection capabilities of all three matrices are presented. For substances with an MRL, CCα was obtained by fortifying blank material at this MRL, whereas for banned substances, blank material was fortified at 0.25 μg/kg. More details on these calculations can be found in the Commission Decision 2002/657/EC [15]. MRLs as defined by EC legislation are also included in Table 3. CCα and CCβ were higher in hair than in the other two matrices since the amount of hair sample to begin with was 100 mg versus 5 g for the other tissues.

The trueness expressed as % bias was determined at three levels (1, 2 and 4 μg/kg for kidney and muscle and 50, 100 and 200 μg/kg for hair samples). % Bias lay between −3% and +5% for kidney, between −9% and +8% for muscle and between −8% and +6% for hair samples.

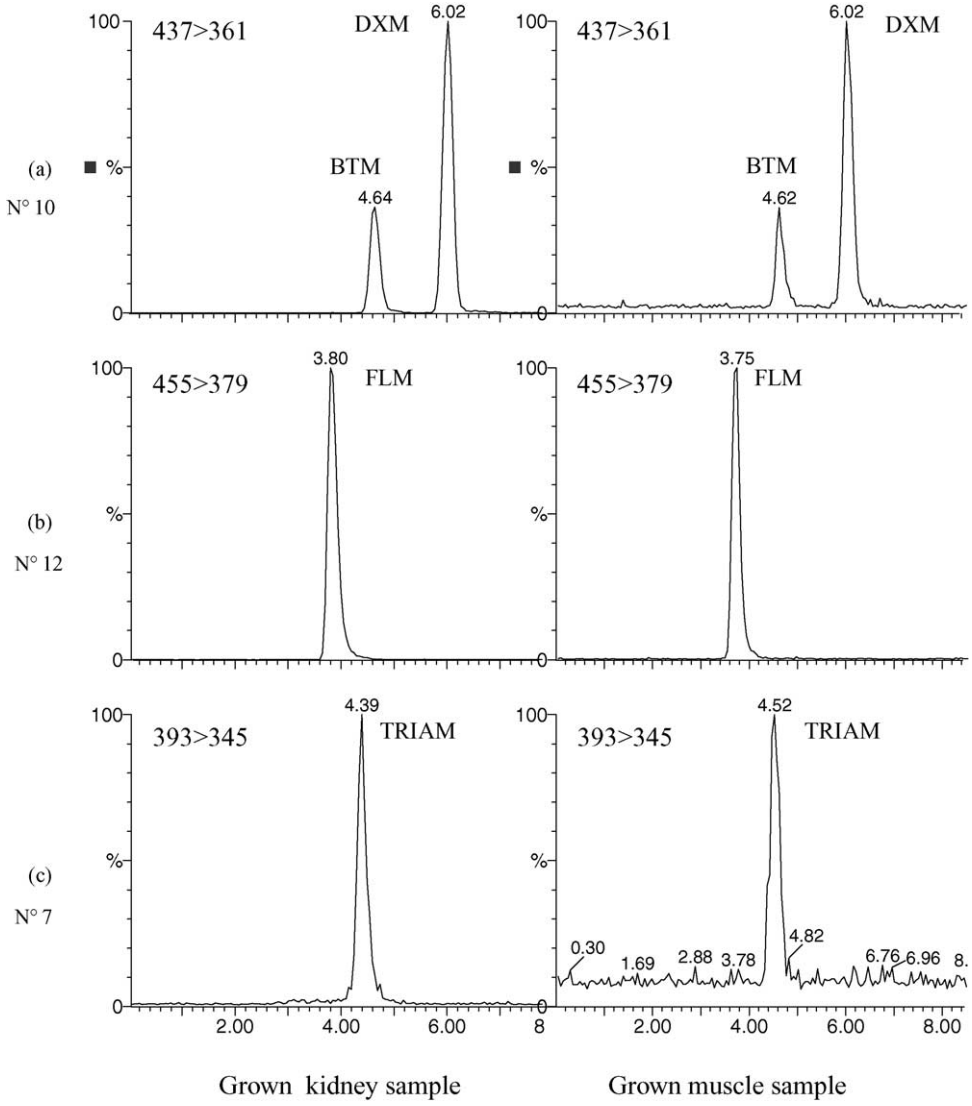

Fig. 3. LC–MS/MS (MRM) chromatograms following the transition of betamethasone (BTM) and dexamethasone (DXM) (a), flumethasone (FLM) (b) and triamcinolone (TRIAM) (c) of the kidney and muscle sample extracts of calves no. 10, 12, 7.

Table 3
Maximum residue limits (MRLs) as defined by EC legislation, decision limits (CCα) and detection capabilities (CCβ) in kidney, muscle and hair samples of all glucocorticoids analyzed

Analyte	MRL (μg/kg)		Kidney (μg/kg)		Muscle (μg/kg)		Hair (μg/kg)	
	Kidney	Muscle	CCα	CCβ	CCα	CCβ	CCα	CCβ
Flumethasone	–	–	0.1	0.3	0.3	0.3	7.8	15.6
Betamethasone	0.75	0.75	1.0	1.3	0.9	1.1	12.3	23.7
Dexamethasone	0.75	0.75	1.0	1.4	0.9	1.0	6.1	17.6
Prednisolone	10	4	10.6	10.8	4.2	4.5	6.6	10.6
Prednisone	–	–	0.4	1.0	0.4	0.8	10.6	19.7
Methylprednisolone	10	10	10.6	11.3	10.3	10.5	13.8	23.7
Fludrocortisone	–	–	0.4	0.6	0.3	0.6	14.8	30.1
Triamcinolone	–	–	0.6	1.1	0.5	0.9	11.4	17.5
Triamcinolone acetonide	–	–	0.3	0.6	0.4	0.6	14.8	22.1
Beclomethasone	–	–	0.4	0.5	0.4	0.8	26.0	42.3

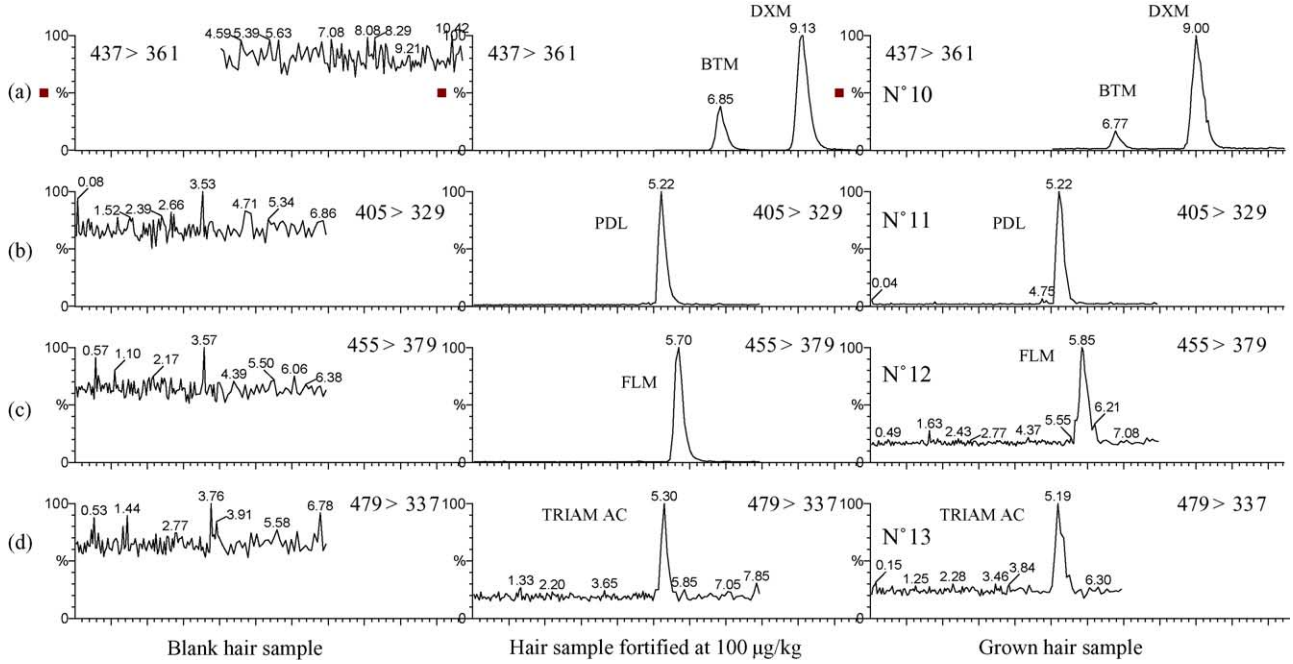

Fig. 4. LC–MS/MS (MRM) chromatograms following the transitions of betamethasone (BTM) and dexamethasone (DXM) (a), prednisolone (PDL) (b), flumethasone (FLM) (c) and triamcinolone acetonide (TRIAM AC) (d) of blank hair sample extracts, extracts of hair samples fortified at 100 μg/kg and hair samples of calves no. 10, 11, 12, 13, respectively.

The within day ($n = 4$) and between day ($n = 3$) repeatabilities were determined at the same levels as the trueness. The coefficients of variation ranged from 0.9% to 10.5% for kidney samples, from 2.0% to a maximum of 13.3% for muscle samples and from 0.5% to 15.8% for hair samples.

With regard to specificity no interfering compounds were observed in the region of interest where the target analyte is expected to elute.

3.4. Analysis of incurred samples

The usefulness of the developed methods was demonstrated by analyzing the hair, muscle and kidney samples of 13 calves treated with synthetic glucocorticoids. Each sample was analyzed twice and the data are shown in Table 1. Residue concentrations found in liver, analyzed in a previous study [6] and kidney tissues were comparable and relatively high compared to the residue concentrations found in muscle samples. Those did not exceed 1.5 μg/kg and were not higher than 19% of the corresponding liver or kidney residue concentrations (with one exception calf no. 11). Dexamethasone treatment did not yield any detectable residues in the hair samples. When animals were slaughtered more than 3 days after dexamethasone treatment no residues could be detected in muscle tissue, while in liver residues of dexamethasone could be detected till day 6 and in kidney even till day 10. No methylprednisolone residues were found in kidney and muscle tissues. Triamcinolone residues were detected in all matrices when animals were slaughtered three days after treatment, when the slaughter happened later than that nothing was detected. In general, the residue concentrations detected in muscle samples are only 5% of those found in liver or kidney. Fig. 3 shows MRM chromatograms of kidney and muscle sample extracts of some of the treated calves. As for the hair samples, after a single dose administration, glucocorticoid residues could be detected at highly varying concentrations ranging from below the detection limit to 348 μg/kg. MRM chromatograms of blank, fortified and grown hair sample extracts are represented in Fig. 4. When comparing residue concentrations in the different matrices, no correlation could be found between residue concentrations found in hair samples and the concentrations found in the other matrices. In this point of view, the location of the hair sample on the body might be critical. Further studies would be needed to determine whether a hair/tissue ratio could be found and whether hair could serve as a predictor for the residue concentrations in edible tissues.

4. Conclusions

In the development of extraction and sample clean up procedures for kidney, muscle and hair samples, the objective was to obtain a method that had the same frame for all three matrices with minor adaptations according to the matrix. This frame consists of a liquid methanol extraction followed by a SPE clean up. Extra steps added are a deconjugation step in the case of kidney samples and an enzymatic digestion step for hair and muscle samples. An alkaline wash step after SPE was necessary for muscle and kidney samples, but not for hair.

All these extracts were then analyzed using LC–MS/MS in the MRM mode. All protocols were successfully validated according to European criteria and real grown sample material was analyzed.

Residue concentrations in muscle tissue were only 5% of the residue concentrations found in liver and kidney, which were comparable. Hair sample residue concentrations could be up to 348 μg/kg, therefore, hair can be an interesting and suitable matrix for detection of synthetic glucocorticoids misused in livestock farming.

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