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Quantification of corticosteroids in bovine urine using selective solid phase extraction and reversed-phase liquid chromatography/tandem mass spectrometry

Ádám Tölgyesi[a,*], Virender K. Sharma[b], Loréna Kovacsics[a], Jenő Fekete[c]

[a] Central Agricultural Office Food and Feed Safety Directorate, Food Toxicology National Reference Laboratory, Budapest, Mester utca 81, 1095 Hungary
[b] Chemistry Department, Florida Institute of Technology, 150 West University Boulevard, Melbourne, FL 32901, USA
[c] Budapest University of Technology and Economics, Department of Inorganic and Analytical Chemistry, Budapest, Szt. Gellert ter 4, 1111 Hungary

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ABSTRACT

This paper presents the development of a simple liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to determine corticosteroids in bovine urine sample matrices. This method uses a single phase extraction (SPE) for cleaning of the sample with an Oasis MAX cartridge at pH 9.0–9.5 and elution by a neutral organic solvent (acetonitrile/dichloromethane), followed by separation on a GEMINI C18 column in the gradient mode with acetate buffer (pH 4.1)/methanol. A triple quadrupole mass spectrometer equipped with a multimode ion source, set to negative atmospheric pressure chemical ionization (APCI) in the multiple reaction monitoring mode was used for detection. The main advantage of this method over other commonly used methods includes the use of SPE with a low volume cartridge for sample preparation and no ion suppression effects from matrix components of the urine samples in the LC–MS/MS analysis. This allowed a reduction the quantification limits (decision limits, CCα) for the first time to 0.1 μg/L (1 and 0.2 μg/L for triamcinolone and flumethasone, respectively). The developed method was validated in accordance with the European Union Commission Decision 2002/657 EC. The recoveries and within-laboratory reproducibility varied from 77% to 115% and 87% to 107.5%, respectively, at 2, 3, and 4 μg/L levels of corticosteroids. The relative standard deviation (RSD) of the measurements was lower than 30%. The decision limit was calculated by multiplying the signal-to-noise ratio by 3 and the obtained values were in the range of 0.1–1.0 μg/L, confirmed by the analysis of twenty blank samples, which were spiked at the desired concentrations. The detection capability was calculated by the addition of the decision limit and the standard deviation followed by multiplication by 1.64 of the within-laboratory reproducibility at 2 μg/L of corticosteroids. The method was applied to four urine samples, giving concentrations of prednisolone (PRED) residues in the range from 0.3 to 0.9 μg/L.

1. Introduction

Corticosteroids are synthetic variants of natural corticosteroid hormones formed in the cortex of the adrenal gland, which is located above the kidney in mammals. Corticosteroids are transformed by cytochrome P450 enzymes [1] and are known to reduce inflammation, as well as suppress allergic reactions and immune system activity [2,3]. Therefore, corticosteroids are used in both human and animal therapy [4]. Examples of corticosteroids are given in Table 1. Different corticosteroids have similar actions, but their relative strengths and duration of action differ. For example, dexamethasone (DXM) is five times stronger than PRED while triamcinolone (TRIAM) is similar to PRED, but its metabolism is limited and it does not have a mineralocorticoid effect. In the European Union (EU), the therapeutic use of some corticosteroids is restricted by the establishment of maximum residue limits (MRLs) in milk and edible tissues, as given in Table 1 [5,6]. Methylprednisolone (METPRED) has MRLs for only tissues and fat, but must not be used in animals from which milk is produced for human consumption. In urine, only DXM has a minimum required performance limit (MRPL) of 2 μg/L [7]. In Hungary, DXM and PRED are used in animal therapy and have MRLs for milk, fat, and tissues. The present work thus focuses on the quantification of corticosteroids in bovine urine.

Corticosteroids are neutral molecules and their values of log *P* are close to one another (Table 1); therefore, their separation, using chromatographic methods, is relatively difficult. However, several quite successful HPLC or LC–MS/MS methods have been reported in literature [8–31,35,36]. Corticosteroids have been analyzed using either gas chromatography/mass spectrometry (GC–MS) or

* Corresponding author. Tel.: +36 30 9689346.
E-mail address: tolgyesi.adam@t-email.hu (Á. Tölgyesi).

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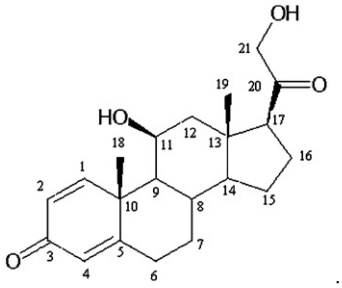
Table 1
Structures, log *P* values, and MRLs of corticosteroids in EU.



Corticosteroids	Abbreviation	C6	C9	C16	C16–17	C17	Log *P*	Animal species	Target tissues	MRLs (μg/kg)
Triamcinolone	TRIAM		–F	–OH		–OH	0.32	–	–	–
Dexamethasone	DXM		–F	$–CH_3$		–OH	1.83	Bovine Bovine Porcine Equidae	Milk Muscle Liver Kidney	0.3 0.75 2 0.75
Prednisolone	PRED					–OH	1.64	Bovine	Muscle, Fat Milk Liver, Kidney	4 6 10
Methylprednisolone	METPRED	$–CH_3$				–OH	1.83	Bovine	Fat, Muscle, Liver, Kidney	10
Triamcinolone acetonid	TRIAM–AC		–F		$–O–C(CH_3)_2–O–$		2.31	–	–	–
Flumethasone	FLU	–F	–F	$–CH_3$		–OH	1.42	–	–	–

high performance liquid chromatography (HPLC) coupled to UV or fluorescence detection [8–15]. The GC–MS method involves a time-consuming derivatization step due to the low volatilities of corticosteroids; hence, it is not preferred [8–12]. The HPLC method requires laborious sample preparation procedures for biological fluids in order to be able to perform fluorescence detection of corticosteroids [15]. In recent years, LC coupled to electrospray/atmospheric pressure chemical ionization (ESI/APCI) mass spectrometry has been used to determine corticosteroids quantitatively in bodily fluids [16–31,35,36]. This technique requires the cleaning of samples for good sensitivity and selectivity prior to the analysis of corticosteroids. One of the main goals of cleaning is to reduce the ion suppression effects of matrix components. Solid phase extraction (SPE) is generally applied to clean urine samples. However, the use of reversed phase octadecyl silica and simple polymeric cartridges in SPE does not have enough selectivity for the complex matrix of a urine sample. Therefore, either two SPE steps or a mixed-mode SPE have been applied in cleaning procedures [18,20,25–29]. Using a mixed-mode SPE, only one study reported optimal conditions for determining corticosteroids in urine samples of pig and cattle [18]. This study used a MAX cartridge (6 mL, 150 mg), which has both strong anion exchange and reversed-phase sorbent phases, for cleaning of samples at pH 5.2. However, at this applied pH and with elution by a neutral solvent, ion suppression effects in MS/MS analysis of corticosteroids was found [18], but when eluting under basic conditions ion suppression was minimized.

The present paper demonstrates that by controlling pH and using selective organic solvents in the sample preparation, reduction in the ion suppression effect can be achieved to a great extent even with the use of a smaller MAX SPE cartridge (3 mL, 60 mg) than that used in a previous study [18]. The cleaning procedure at pH 9.0–9.5 allowed selective adsorption of the matrix components and the corticosteroids of the sample to the anion-exchange cartridge and to the reversed-phase phase of the sorbent, respectively. The use of selective neutral organic solvents in the elution step yielded an eluent which contained only corticosteroids without any matrix components, and hence, no ion suppression effects were observed in the multimode ion source. Importantly, the detection limit could be reduced to 0.1 μg/L for most of the studied corticosteroids, which is about ten times lower than that reported previously [18]. It should be pointed out that not only the condition of MAX SPE method in the present study was different but also the MS/MS instrument and the ionization mode, which also influenced the decision limits. The aims of the present work therefore are (i) to describe the development and validation of an analytical procedure for determining low levels of six corticosteroids in bovine urine; (ii) to apply efficiently a single SPE step, followed by LC separation and subsequent determination by MS/MS; (iii) to meet the requirements of the European Directives [32] in validating the method; and (iv) to apply the method to determine corticosteroids in bovine urine samples obtained under different monitoring programs.

2. Experimental

2.1. Reagents and samples

The studied corticosteroids: triamcinolone (9-Fluoro-11,16,17,21-tetrahydroxypregna-1,4-diene-3,20-dione), dexamethasone [(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], prednisolone [(11β)-11,17,21-Trihydroxypregna-1,4-diene-3,20-dione], methylprednisolone (11,17,21-Trihydroxy-6-methyl-1,4-pregnadiene-3,20-dione), triamcinolone acetonid {9-Fluoro-11,21-dihydroxy-16,17-[1-methylethylidenebis(oxy)]pregna-1,4-diene-3,20-dione}, and flumethasone (6,9-Difluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione) were purchased from Sigma–Aldrich (Budapest, Hungary). The testosterone-d5 ((17β-Hydroxyandrost-4-en-3-one)-d5), was used as an internal standard, was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Stock solutions were prepared by dissolving 10 mg of standards into 10.0 mL of methanol to obtain 1 mg/mL concentration. These solutions were stored at −20 °C. For working standards, 25 μL of the stock solutions was diluted with methanol to 25 mL into volumetric flasks for a final concentration of 1 μg/mL for each component.

Working standard solutions were prepared weekly and were stored at 4 °C.

Acetonitrile, dichloromethane, ethanol, ammonium acetate, and sodium acetate were of HPLC grade and were obtained from Merck (Budapest, Hungary). Methanol and acetic acid were ultrapure and were also obtained from Merck (Budapest, Hungary). Helix Pomatia β-glucuronidase was purchased from Calbiochem, San Diego, (California, USA). OASIS MAX 60 mg (30 μm) 3 mL SPE cartridges for the sample cleaning procedure were purchased from Waters Corporation (Budapest, Hungary). This cartridge has a mixed-mode polymeric strong anion exchange and reversed phase sorbent. The urine samples originated from the Hungarian residue control monitoring program and were collected from January 2008 to July 2009 and were stored at −20 °C until subjected to analysis.

2.2. Sample hydrolysis and cleaning

Samples were hydrolyzed by adding 2 mL of 2 M sodium acetate to 5.0 mL of a urine sample. The pH of the mixture was adjusted to 5.2 before adding 20 μL of 1 MU Helix Pomatia β-glucuronidase (Calbiochem). This addition was followed by vortex-mixing and incubation at 37 °C for 16 h. The pH of the hydrolyzed sample was adjusted to obtain values between 9.0 and 9.5 by adding drops of 1 M NaOH.

Before the concentration and cleaning procedure, the SPE column was well-conditioned by passing 3 mL of methanol and 3 mL of ethanol through the column two times, and followed by rinsing two times with 3 mL of water. The hydrolyzed sample was slowly passed through the SPE cartridge (3 mL and 60 mg). The SPE column was later washed three times with 3 mL of water and then dried under vacuum for 20 s. Corticosteroids were eluted first with 5 mL of acetonitrile and then with 2 mL of dichloromethane. The eluted samples were evaporated to volumes of 50–100 μL under a gentle nitrogen stream at 45 °C and then dissolved in methanol–water (50:50, v/v) and adjusted to a final volume of 1.0 mL.

2.3. HPLC conditions

Corticosteroids were separated on a GEMINI C-18 (150 mm × 4.6 mm, 5 μm) (Phenomenex, Torrance, USA) column equipped with a GEMINI C-18 guard column (4 mm × 3.0 mm) by the use of gradient elution. Gradient elution started with 50% (v/v) B. Mobile phase B increased from 50% to 100% (v/v) over 10 min, followed by 100% (v/v) B for 4 min. After 14 min, mobile phase B was decreased to 50% (v/v) over 0.5 min. Mobile phase A was a mixture of 5 mM ammonium acetate and 0.05% (v/v) acetic acid in water (pH 4.1) while mobile phase B was 100% methanol. This approach minimized the time for separating corticosteroids. The flow rate was 0.8 mL/min and the analysis time was 21 min. The injection volume was 10 μL and the column thermostat was 30 °C.

2.4. Instruments and mass spectrometry conditions

SPE vacuum manifolds were obtained from Merck (Budapest, Hungary). The nitrogen evaporator was a Caliper TurboVap LV. The LC–MS system was an Agilent 6410 Triple Quad equipped with an Agilent 1200 Binary pump LC and Agilent 6410A mass selective detector with Agilent multimode ion source (G1978B) (Santa Clara, USA). Data analysis was performed using Agilent Mass Hunter B 01.04 software.

The mass selective detector was used in the MRM mode for the highest selectivity and sensitivity, as well as the lowest detection limit. The multimode ion source was in negative APCI mode. Nitrogen gas was used for drying and collision. The MS detector settings were as follows: gas temperature, 300 °C; gas flow, 5 L/min; vaporizer, 160 °C; nebulizer pressure, 413.7 kPa; capillary voltage, 2000 V; and capillary current, 4 μA.

2.5. Quantification

Six point spiked standard curves (including zero) were constructed for the quantification of the blank and spiked samples. The MS software was used to obtain regressions, weighted with relative concentrations^{-1}. The internal standard was 8 μg/L testosterone-d5, which was added to the prepared samples.

The analytical method was validated according to the 2002/657 EC Decision [32] and the parameters assessed were selectivity, linearity, recovery, within-laboratory reproducibility, decision limit (CCα), and detection capability (CCβ). The decision limit was confirmed by twenty blank samples spiked at the calculated CCα concentrations.

3. Results and discussion

3.1. Optimization of mass spectrometry

Four identification points were obtained using the MRM mode with one precursor ion and two product ions. The steroids produced precursor ions in both negative and positive mode (Table 2). The multimode ion source of the MS produced more intensive precursor ions in negative APCI mode than in the positive APCI mode for corticosteroids. The precursor ions were acetate adducts, $[M+CH_3COO]^-$, for all corticosteroids, except triamcinolone, $[M-H]^-$. An internal standard (ISTD), testosterone-d5, was measured as $[M+H]^+$ in a positive APCI mode. Although testosterone-d5 has an opposite polarity, however, in a recent study, it was shown that it can be used to determine low levels (ng/L) of corticosteroids by applying the negative adduct mode [35]. Initially, cortisol-d4 as an internal standard was used in the negative mode, which was applied previously [36]. However, results were not good because an endogenous corticosteroid, tetrahydrocortisol interfered during the chromatography separation in the Gemini C-18 column. The testosterone-d5 in the current study was appropriate for detection in the negative mode for all of the tested steroids.

First, the precursor ions were optimize-scanned with the third quadrupole. The mass spectra of the molecules were recorded on seven fragment potencies between 90 and 150 V. After choosing the optimized fragment potency for the found precursor ions, the collision energies of the ion transitions were optimized between 0 and 30 V with a product ion scan. The intensity of MRM transitions was maximal on vaporizer temperature of 160 °C.

3.2. General conditions for liquid chromatography

Urine is a complex matrix with several unknowns of different concentrations; therefore, a relatively long Gemini C-18 column (150 mm × 4.6 mm) was selected to avoid any interference. Using methanol as the mobile phase, the sensitivity of the method was much higher than that of acetonitrile as a mobile phase. The internal standard was added to the samples at the end of sample preparation to facilitate the quantitative determination of the components [34].

3.3. Method development of sample preparation

Urine contains several acidic compounds (pK_a = 3–7). At pH 5.2, used in a previous study [18], the acidic matrix compounds were only partially ionized and the SPE cartridge could not absorb all of them on the anion exchange portion. With an increase in the sample pH to pK_a+2, the acidic matrix solutes of the samples were

Table 2
MS/MS detector settings and ion ratios of two ion transition reactions of the six analytes in standard solutions and spiked samples.

Segment	Time (min)	Scan type	Ion mode	Ion polarity	ΔEMV	Compound	Precursor ion	Product ions	Dwell time	Fragmentor	CE	Ion ratios of standard solutions	Maximum permitted tolerances	Ion ratios of spiked samples
1	0–5.0		APCI	Negative	0	Data not stored								
2	5.0–6.5		APCI	Negative	300	TRIAM	$[M-H]^-$ 393.1	**345.1** 325.1	200 ms	120 V	10 V	10 V	31%	23–39%	25–39%
									200 ms						
		MRM				DXM	$[M+CH_3COO]^-$ 451.3	**361.1** 307.2	75 ms	90 V	15 V	30 V	27%	20–34%	22–28%
									75 ms						
3	6.5–9.0		APCI	Negative	150	PRED	$[M+CH_3COO]^-$ 419.3	**329.4** 280.1	75 ms	110 V	20 V	25 V	11%	8–14%	9–13%
									75 ms						
						MET–PRED	$[M+CH_3COO]^-$ 433.2	**343.2** 309.2	75 ms	110 V	15 V	25 V	20%	15–25%	16–21%
									75 ms						
						TRIAM–AC	$[M+CH_3COO]^-$ 493.3	**413.3** 337	75 ms	150 V	20 V	20 V	41%	31–51%	38–50%
									75 ms						
						FLU	$[M+CH_3COO]^-$ 469.3	**379.2** 305.3	75 ms	140 V	10 V	30 V	12%	8–16%	11–13%
									75 ms						
4	9.0–21.0		APCI	Positive	150	Testosterone-d5	$[M+H]^+$ 294.3	**100.3**	200 ms	120 V	30 V				

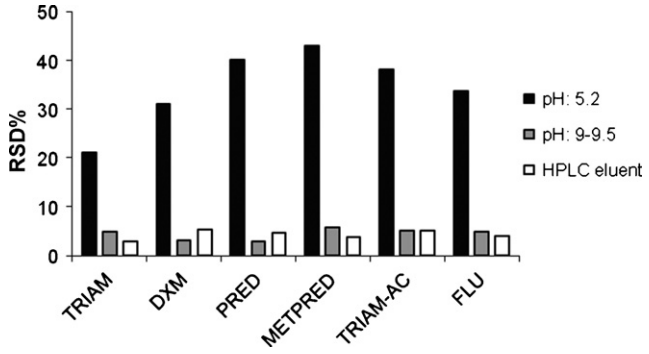


Fig. 1. Effects of pH on ion suppression and system suitability. Different blank bovine urines were cleaned at pH: 5.2 and pH: 9.0–9.5 in six series. After the sample preparation, 2 μg/L standard and 8 μg/L ISTD were added to the samples.

fully ionized, which then interacted strongly with the ion exchange phase of the sample preparation cartridge. Under this condition, the acidic matrix solutes did not wash out with the neutral eluent solvents, acetonitrile and dichloromethane. With this modification, the ion suppression effect was reduced and both the sensitivity and reproducibility were increased. Hence, one of the main goals of the LC–MS technique was achieved. The experiments conducted are explained below.

Six different blank bovine urine samples at pH 5.2 were cleaned on MAX cartridges and were evaporated to 50–100 μL after elution. A 2 μg/L standard mixture and 8 μg/L internal standard were dissolved in cleaned blank samples and the volume was adjusted to 1.00 mL with methanol–water (50:50, v/v). Samples were homogenized by vortexing for 30 min. The six independently diluted 2 μg/L standard mixtures and the 8 μg/L internal standard in methanol–water (50:50, v/v) HPLC solvents were also prepared. Twelve solutions were injected into the LC–MS system. Peak areas were integrated to calculate the relative areas [area of target corticosteroid, (areas of internal standard)$^{-1}$]. Signal values (relative areas of corticosteroids and area of internal standard) are summarized in Table 3. The RSD% of the relative areas in the spiked blank urine samples ranged from 21.0% to 43.1% (Fig. 1), which were much higher than the RSD of the relative areas of the steroid mixture in the HPLC solvent (2.8–5.4%). Since corticosteroids could not be degraded during sample preparation, the loss could only be due to ion suppression in the ion source. The relative areas of compounds were different due to the variation in ion suppression effects in different bovine urine samples; therefore, the RSD% of areas was also high. This is consistent with the lack of any observed ion suppression in the standard mixture. Interestingly, no ion suppression effect was observed in the internal standard's signal for spiked urine samples and HPLC solvent (Table 3).

Six different blank bovine urine samples were also cleaned on MAX cartridges at pH 9.0–9.5 and were subjected to a similar procedure. After injecting samples into the LC–MS, the RSD% of relative areas of the compounds were 2.8–5.7% and no ion suppression effects were observed. The sample preparation was then performed at pH 9.0–9.5 in order to eliminate ion suppression effects.

3.4. Selectivity

A 5 mL sample was obtained from a pre-analyzed mixture of blank samples that contained 15 different bovine urine samples and one spiked sample, which were subjected to analysis. There was no signal observed for the solute in the blank urine MRM chromatograms (Fig. 2a and b). It should be pointed out that a matrix peak appeared on an MRM chromatogram of PRED with an ion trace 419.2 ≫ 329.2 at a retention time of 6.8 min. This peak did not interfere with the separation of PRED at a retention time of 7.4 min. A

Table 3
Signal values of ion suppression experiment.

1. Day	5.0 mL urine + 2 μg/L standard mix + ISTD								
pH: 5.2	1	2	3	4	5	6	Average	S	RSD%
TRIAM	0.553	0.626	0.638	0.415	0.604	0.377	0.535	0.112	21.0
DXM	3.078	3.792	4.153	1.859	4.282	2.286	3.242	1.007	31.1
PRED	3.355	4.228	4.315	1.901	4.531	2.160	3.415	1.148	33.6
METPRED	2.354	2.945	2.811	0.611	2.726	1.454	2.150	0.927	43.1
TRIAM–AC	1.860	1.710	2.031	0.708	1.989	0.776	1.512	0.608	40.2
FLU	1.207	1.307	1.303	0.450	1.249	0.595	1.018	0.389	38.2
ISTD	1258	1364	1411	1419	1443	1525	1441.8	83.3	5.8
	2 μg/L standard mix in HPLC solvent + ISTD								
TRIAM	0.703	0.671	0.656	0.677	0.679	0.649	0.672	0.019	2.8
DXM	4.134	4.645	4.538	4.624	4.325	4.811	4.513	0.244	5.4
PRED	4.867	5.333	5.001	5.189	4.904	5.299	5.099	0.202	4.0
METPRED	2.954	3.243	3.044	3.032	3.060	3.225	3.093	0.115	3.7
TRIAM–AC	1.982	1.919	1.808	1.938	1.817	2.033	1.916	0.089	4.7
FLU	1.504	1.695	1.607	1.606	1.666	1.740	1.636	0.083	5.1
ISTD	1502	1526	1503	1479	1550	1525	1552.6	53.9	3.5
2. Day	5.0 mL urine + 2 μg/L standard mix + ISTD								
pH: 9–9.5	1	2	3	4	5	6	Average	S	RSD%
TRIAM	3.579	3.549	3.726	3.964	3.898	3.938	3.776	0.184	4.9
DXM	21.432	20.174	22.138	20.691	21.069	20.735	21.040	0.682	3.2
PRED	17.999	17.231	19.771	18.641	19.260	18.476	18.563	0.900	4.8
METPRED	13.988	12.426	14.820	14.040	13.516	13.619	13.735	0.789	5.7
TRIAM–AC	11.427	11.413	11.297	10.611	11.302	11.021	11.179	0.314	2.8
FLU	4.092	4.236	4.647	4.386	4.278	4.022	4.277	0.224	5.2
ISTD	401	419	375	410	422	420	407.8	17.9	4.4
	2 μg/L standard mix in HPLC solvent + ISTD								
TRIAM	4.066	3.926	4.225	4.130	4.545	4.325	4.2	0.216	5.1
DXM	21.592	22.655	22.719	22.450	22.496	20.841	21.1	0.75	3.4
PRED	19.222	19.289	19.659	19.238	20.024	21.115	19.8	0.735	3.7
METPRED	15.412	16.834	16.507	16.445	16.308	17.575	16.5	0.706	4.3
TRIAM–AC	8.982	9.308	9.366	9.600	9.320	10.077	9.4	0.368	3.9
FLU	6.046	6.022	6.348	5.711	5.489	6.054	5.9	0.301	5.1
ISTD	407	404	406	421	418	406	410.3	7.2	1.8

secondary ion trace of PRED (419.2 ≫ 280.1) was not observed in the blank sample, but the trace was seen in the chromatogram of the spiked sample. Therefore, the separation of steroids could be observed without interference.

3.5. Identification

MRM chromatograms are shown in Fig. 2a and b. The quantified ion trace is shown at the upper left corner while the qualified ion trace is next to it. When the two ion traces appeared with the same defined ion ratios and expected retention times, the compound was positively identified. Confirmation of the substance was conducted using a minimum of four identification points [32]. A precursor ion was one identification point. One ion trace is defined as 1.5 points; therefore, two ion traces were the other three identification points used to satisfy the condition for identification of the substance. The ion ratios in the standard solution and in the samples during the validation were applied. As shown in Table 2, the ion ratios of each spiked sample fell within the maximum permitted tolerances for positive identifications.

3.6. Linearity

Calibration was based on a matrix spiked curve. The calibration curves were formed by spiking 5.0 mL blank urine samples with standard solutions of different concentrations. A six-point calibration (including zero) was performed at levels of 1, 2, 3, 4 and 5 μg/L. Correlation coefficients (r^2) were between 0.967 and 0.989. Residues spread randomly around zero.

3.7. Recovery, within-laboratory reproducibility

Recovery was estimated by spiking the blank samples at three different levels in six series. Validation at levels 1.0, 1.5, and 2.0 MRPL (2, 3 and 4 μg/L) was performed to meet the EU guidelines. Within-laboratory reproducibility was also evaluated by repeating the recovery test with different operators, solvents, and laboratory temperatures (20, 25 and 30 °C), and employing the same method on two different days in six and seven series. Within-laboratory reproducibility was calculated from the 19 results for each level. According to the 2002/657 EC Decision, the recovery and the within-laboratory reproducibility must be 70–110% and the RSD% as low as possible on a 1–10 μg/L level. A previous analytical strategy resulted in a 30% precision for this level [33]. These conditions were observed for all corticosteroids, except TRIAM, which had a 34.8% RSD in the recovery test (Table 4).

3.8. Decision limit (CCα) and detection capability (CCβ)

Twenty blank urine samples were analyzed to calculate the signal-to-noise ratio at the expected time window for the corticosteroids. Three times the signal-to-noise ratio was the estimated decision limit. Because the noise ratios were zero for DXM and TRIAM for both primary and secondary ion traces, their decision limits were estimated from the signal-to-noise ratio as the lowest calibration point (1 μg/L). The detection capability was calculated as the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility on the 2 μg/L level. According to the 2002/657 EC, the decision limit of a substance should be lower

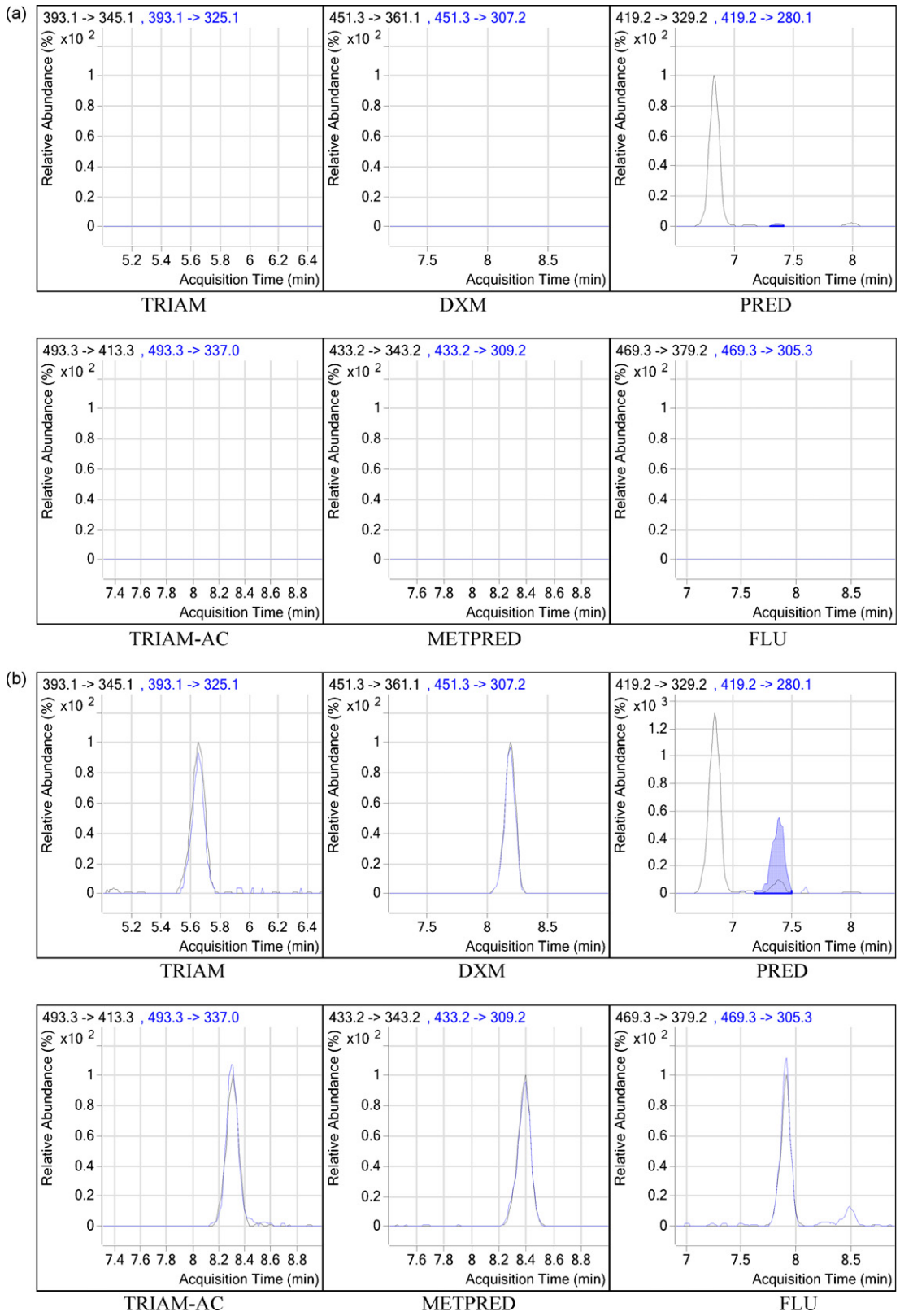


Fig. 2. MRM chromatograms of blank bovine urine sample (a) and of 2 μg/L spiked bovine urine sample (b). The quantify ion trace is in the left upper corner. The qualify ion trace is next to the quantify ion trace.

than MRPL and the detection capability must be lower or equal to MRPL. The MRPL of 2 μg/L has been set only for DXM and this value was also selected in the present study for other corticosteroids. As shown in Table 4, the results of both the decision limit and the detection capability met the conditions of the 2002/657 EC Directive. Decision limits were confirmed by analyzing twenty blank samples which had been spiked to the individually calculated CCα concentrations (see Table 4). Decision limit was accepted

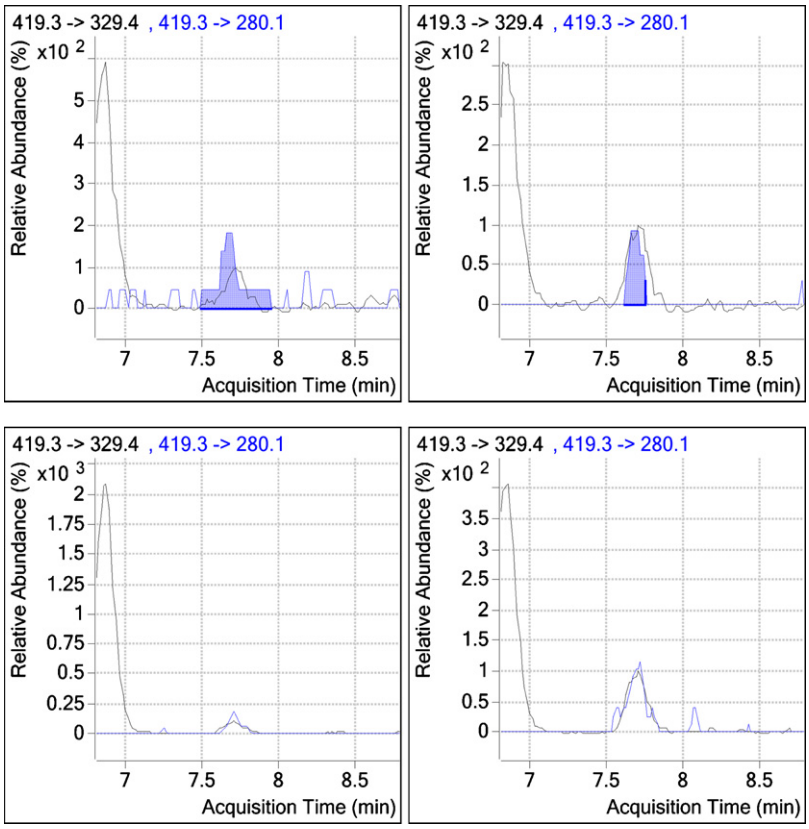


Fig. 3. MRM chromatograms of PRED in bovine urine samples.

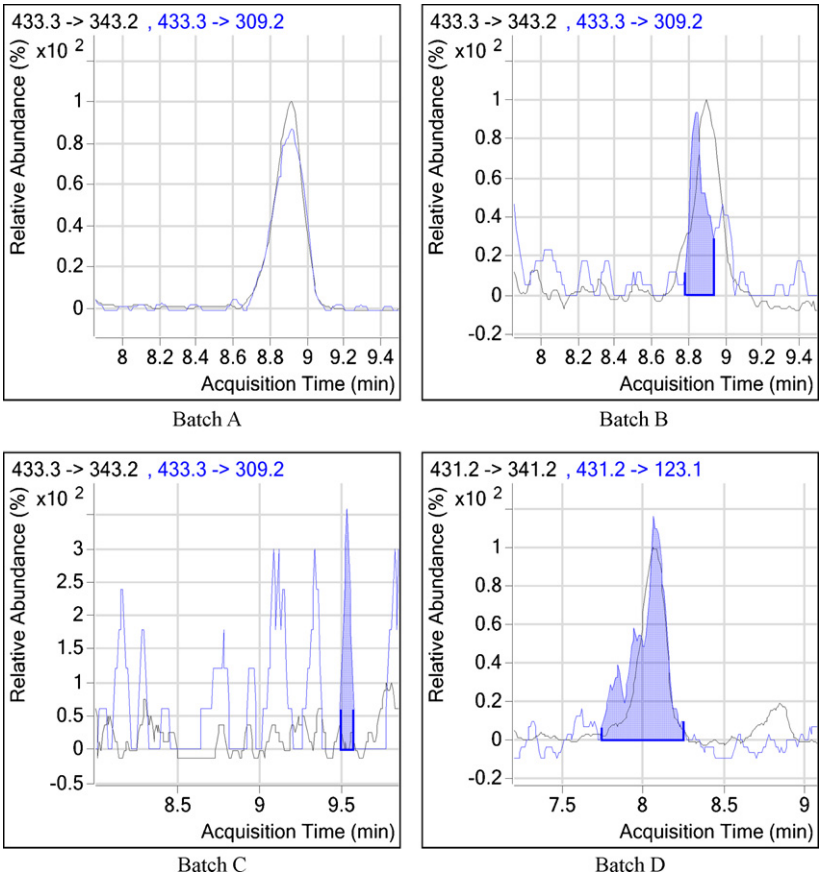


Fig. 4. MRM chromatograms of Batch A, B, C, and D of proficiency test.

Table 4
Recovery, within-laboratory reproducibility, decision limit (CCα), and detection capability (CCβ).

Recovery (n = 6)									
Analytes	Spiking level	Recovered concentration (μg/L)	RSD%	Spiking level	Recovered concentration (μg/L)	RSD%	Spiking level	Recovered concentration (μg/L)	RSD%
TRIAM	2 μg/L	1.6 ± 0.4	25.0	3 μg/L	2.3 ± 0.8	34.8	4 μg/L	3.6 ± 1.0	27.8
DXM		2.2 ± 0.3	13.6		3.0 ± 0.4	13.3		4.5 ± 0.3	6.7
PRED		2.3 ± 0.2	8.7		2.8 ± 0.2	7.1		3.9 ± 0.2	5.1
METPRED		1.8 ± 0.1	5.5		2.6 ± 0.3	11.5		3.8 ± 0.3	7.9
TRIAM–AC		1.9 ± 0.2	10.5		2.7 ± 0.2	7.4		4.0 ± 0.2	5.0
FLU		2.1 ± 0.3	14.3		3.0 ± 0.3	10.0		4.5 ± 0.4	8.9

Within-laboratory reproducibility (n = 19)										
Analytes	Spiking level	Recovered concentration (μg/L)	RSD%	Spiking level	Recovered concentration (μg/L)	RSD%	Spiking level	Recovered concentration (μg/L)	RSD%	CCα (μg/L)	CCβ (μg/L)
TRIAM	2 μg/L	2.1 ± 0.6	28.5	3 μg/L	2.6 ± 0.7	26.9	4 μg/L	4.3 ± 1.2	27.9	1.0	2.0
DXM		1.9 ± 0.3	15.8		2.7 ± 0.3	11.1		3.6 ± 0.8	22.2	0.1	0.6
PRED		2.0 ± 0.4	20.0		2.7 ± 0.3	11.1		3.6 ± 0.8	22.2	0.1	0.8
METPRED		1.8 ± 0.2	11.1		2.6 ± 0.2	7.7		3.5 ± 0.5	14.3	0.1	0.4
TRIAM–AC		1.8 ± 0.3	16.7		2.8 ± 0.3	10.7		3.7 ± 0.6	16.2	0.1	0.6
FLU		1.9 ± 0.3	15.8		2.7 ± 0.3	11.1		3.6 ± 0.9	25.0	0.2	0.7

Table 5
Formal results of proficiency test.

Batch		Our results (μg/L)	CRL results (μg/L)
A	High level methylprednisolone	0.67	0.71
B	Low level methylprednisolone	0.12	0.11
C	Blank	<CCα	<CCα
D	High level methylprednisone	0.84	0.83

for a compound when all spiked sample's signal-to-noise ratio was higher than three and the ion ratios were in the acceptable range.

4. Application of method

Four bovine urine samples, originating from the Hungarian residue control monitoring program of 2008, were subjected to the analysis of corticosteroids using the developed method (Fig. 3). PRED was the only coroticosteroid detected in the samples and had concentrations ranging from 0.3 to 0.9 μg/L.

In the summer of 2009, the proficiency test with a theme "Methylprednisolone and Metabolites" in bovine urine was organized by the Community Reference Laboratory (CRL RIVM) in The Netherlands. There were four batches of incurred bovine urine samples, which were lyophilized. Of the four batches, A, B, C, and D, batch C was the blank sample (Fig. 4). Significantly, separation of corticosteroids in the performance test was conducted before the method was fully validated. An earlier study used an old GEMINI analytical column (S/N: 456767-7, B/N: 5520-77), but results with the new GEMINI (S/N: 490792-14, B/N: 5520-82) column gave shifts of 0.3–0.4 min for the retention times of corticosteroids in MRM chromatograms, which was used in the validation study. Batches A and B had high and low concentrations of methylprednisolone. Batch D contained a high concentration of methylprednisone as shown in the MRM chromatogram (Fig. 4). The concentration levels were provided to all participants at the beginning of the proficiency test and included two independent series of two analyses performed on two different days (2 × 2). The averages of the four results per batch are given in Table 5. The results show that this method provides accurate determination of corticosteroids from spiked samples, as well as incurred ones (Table 5).

5. Conclusions

A modified method was developed for the determination of cortisteroids in bovine urine. The main advantage of the new method over the previous method in the literature is the elimination of ion suppression in the LC–APCI–MS/MS analysis. This was proven by using a mixed mode solid-phase cartridge with a pH adjustment to 9.0–9.5, which gave increased sensitivity and reproducibility of the measurements, but the relative standard deviations were quite comparable to an earlier method [18]. Additionally, the method was validated successfully, based on the 2002/657 EC Decision. Selectivity, linearity, recovery, and within-laboratory reproducibility conditions met the conditions of the EC Decision. The decision limits were 0.1–1.0 μg/L, which fulfilled one of the requirements of EU. The RSD was under 30% and within-laboratory reproducibility was in the range from 87% to 107.5%. These values were also in agreement with limits set by the EU. The method was successfully applied to the proficiency test and it has now been accredited by the National Accreditation Body. This method was also applied to screening of corticosteroids in urine samples. Prednisolone was the only corticosteroids detected in four bovine urine samples.

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