Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 919–928

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Quantitative determination of corticosteroids in bovine milk using mixed-mode polymeric strong cation exchange solid-phase extraction and liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 22 February 2010
Received in revised form 22 June 2010
Accepted 23 June 2010
Available online 1 July 2010

Keywords:
Corticosteroids
LC–MS/MS
Ion suppression
Bovine milk
Jet Stream
Validation

ABSTRACT

A new method was developed to identify and quantify corticosteroids (prednisolone, methylprednisone, flumetasone, dexamethasone, and methylprednisolone) in raw bovine milk by liquid chromatography–tandem mass spectrometry (LC–MS/MS) utilizing mixed-mode polymeric strong cation exchange and reversed-phase (MCX) solid-phase extraction (SPE) to reduce ion effects in a multimode ion (MMI) source. The main advantage of this method over other commonly used methods includes the use of a single SPE cartridge with a low volume for sample preparation and fast separation on the HPLC system with reduced ion suppression. This study is the first to report the determination of methylprednisone, a metabolite of methylprednisolone, in bovine milk.

This method was validated in accordance with the European Union (EU) Commission Decision 2002/657/EC. The recoveries vary between 90% and 105%. The within-laboratory reproducibility (precision) is less than 30%. The decision limits and detection capabilities were calculated along with LODs, which ranged from 0.02 to 0.07 μg/kg.

The method was further enhanced by its successful adaptation to other LC–MS/MS systems equipped with the newly developed ion source, Agilent Jet Stream (AJS). After optimization of the AJS ion source and MS parameters, even lower LOD values were achieved (0.001–0.006 μg/kg) for the corticosteroids. Analytical results obtained with the AJS were characterized by an enhanced area response and similar noise level comparable to those obtained with conventional orthogonal atmospheric ionization (API).

1. Introduction

Glucocorticoids have numerous biomedical effects that make them suitable drugs for a variety of diseases. They affect glucose utilization, fat metabolism, and bone development and are commonly used in the treatment of allergic reactions [1,2]. Additionally, they are also used to reduce inflammation, itching, and redness. Exogenous corticosteroids suppress the body's natural production of corticosteroids by inhibiting the release of the hormone, adrenorticotropic. Structures of the corticosteroids which were analyzed in this study are presented in Table 1. Among the corticosteroids, dexamethasone (DXM) and prednisolone (PRED) have been assigned maximum residue limits (MRLs) for milk in the European Union (EU) [3,4,31] (Table 1). Corticosteroids with no established permitted limits are considered banned substances. There is no minimum required performance limit (MRPL) specifically for corticosteroids in milk [5], but a minimum required performance level (mrpl) has been set as 0.5 μg/kg for banned compounds by the Hungarian National Reference Laboratory (Table 1). The mrpl is either the lowest concentration of the analyte expected to be detected (screening method) or the lowest level at which its identity can be unequivocally confirmed (confirmatory method) [25]. MRLs of different corticosteroids present in biological matrices have been reported in Table 1 [3,4,31]. Although methylprednisolone (METPRED) has an assigned MRL value for tissues and fat, it cannot legally be used with animals that produce milk for human consumption [31]. In Hungary, DXM and PRED are applied for animal therapy. The present work thus focuses on the quantification of corticosteroids in raw bovine milk. Significantly, this is the first study to demonstrate analysis of methylprednisone (METPREDON), a metabolite of MET-

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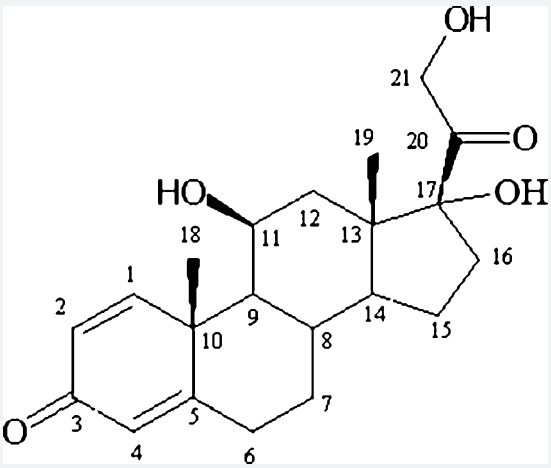
doi:10.1016/j.jpba.2010.06.026

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



Corticosteroid	Abbreviation	C6	C9	C11	C16	log *P*	Animal species	Target tissues	MRLs (μg/kg)	mrpl (μg/kg)
Prednisolone	PRED			–OH		1.64	Bovine	Milk Muscle, fat Liver, kidney	6 4 10	–
Methylprednisone	METPREDON	$-CH_3$		=O		1.24	–	–	–	0.5
Flumetasone	FLU	–F	–F	–OH	$-CH_3$	1.42	–	–	–	0.5
Dexamethasone	DXM		–F	–OH	$-CH_3$	1.83	Bovine Bovine, porcine, equine	Milk Muscle Liver Kidney	0.3 0.75 2 0.75	–
Methylprednisolone	METPRED	$-CH_3$		–OH		1.83	Bovine	Fat, muscle, liver, kidney	10	0.5

PRED. This metabolite is pharmacologically inactive [32], but its appearance in milk suggests the illegal treatment of an animal, so its determination is also necessary.

Corticosteroids have been analyzed in different matrices using a gas chromatography/mass spectrometry (GC–MS) technique. This technique involves a time-consuming derivatization step due to the low volatilities of the corticosteroids, hence is not preferable [7–9,23]. A liquid chromatography method coupled to a diode array detector (LC-DAD) has been applied recently to separate corticosteroids [6]. Because corticosteroids are neutral molecules and their values of log P are close to one another (Table 1); their separation from milk using LC-DAD method is relatively difficult. In recent years, liquid chromatography coupled to different mass spectrometry methods (LC–MS/MS or LC-TOF (time-of-flight)) has been used to determine corticosteroids quantitatively in complex matrixes [10–24,33]. These techniques have good sensitivity and selectivity to analyze corticosteroids.

Milk is one of the most complex matrices, requiring extensive testing, and containing many solutes with different properties such as fats, proteins, peptides, neutral lipids (glycerides, phospholipids and sterols), sugars, vitamins and mineral elements, which interfere with various analyses. The removal of these compounds is necessary in methods such as the one described in this application, which determines low concentrations of corticosteroids in biological and environmental samples, in order to avoid ion suppression, which influences the detection limit and reproducibility of the LC–MS/MS method. In the literature, several procedures have been described for preparing samples to analyze corticosteroids for milk samples [6,10–17,24]. Solid-phase extraction (SPE) is generally applied for clean-up procedures to remove matrix solutes from complex fluid samples such as milk or urine and to concentrate the target corticosteroids. The use of simple polymeric cartridges such as the Oasis HLB (hydrophilic–lipophilic balance) as SPE does not offer adequate selectivity for complex fluid matrices, and consequently, low recoveries are obtained for milk samples [16]. To increase the efficiency of a simple polymeric SPE, other post-SPE steps were found to be necessary in order to reduce matrix effects of complex fluid samples [12,14,18–19,24]. Additional steps in sample preparation increased the analysis time and cost [13]. Furthermore, the use of methanol and acetonitrile as solvents in the elution of corticosteroids from the HLB cartridges, result in the co-elution of interfering matrix compounds [16,18–19,21,24].

The present paper describes a simple and efficient extraction procedure for removing interfering ionizable matrix compounds in the analysis of selected neutral corticosteroids. This isolation procedure is based on an enzymatic hydrolysis step followed by one step-SPE clean-up on a mixed-mode polymeric strong ion exchange and reversed phase (MCX). Matrix effects in the MS/MS analysis using multimode ion source (MMI) for corticosteroids were minimized by (i) using the MCX cartridge for sample clean-up at an acidic pH (2.3) and (ii) using acetone for eluting concentrated analytes from the reversed-phase of MCX SPE cartridge. The method for analysis of five corticosteroids (PRED, METPREDON, flumetasone (FLU), DXM, and METPRED) in bovine milk, was validated according to 2002/657 EC Decision standards [25,26].

2. Experimental

2.1. Reagents and samples

The studied corticosteroids were prednisolone (11β,17,21-trihydroxypregna-1,4-diene-3,20-dione), flumetasone (6α,9α-difluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), dexamethasone (9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione), and methylprednisolone (11β,17,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione), which were purchased from Sigma–Aldrich (Budapest, Hungary). A 1 mg/ml stock solution of methylprednisone (17,21-

dihydroxy-6α-methylpregna-1,4-diene-3,11,20-trione) in ethanol was obtained from CRL RIVM (Bilthoven, The Netherlands). This solution was diluted with methanol to 0.4 mg/ml. The testosterone-d5 (17β-hydroxyandrost-4-en-3-one)-d5 used as an internal standard was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). In recent studies testosterone-d5 was successfully applied as an internal standard in the determination of corticosteroids in fluid samples [21,33]. HPLC grade acetonitrile, dichloromethane, acetone, ethanol, 25% ammonia solution, ammonium acetate, and sodium acetate were obtained from Merck (Budapest, Hungary). Methanol and acetic acid were ultrapure and were purchased from Merck (Budapest, Hungary). Helix Pomatia β-glucuronidase (1 MU) was purchased from Calbiochem, San Diego (California, USA).

Stock solutions were prepared by dissolving 10 mg standards (of accurate weight) in 25 ml of methanol to obtain concentrations of 0.4 mg/ml and were stored at −20 °C. These stock solutions can be stored for up to 1 year [19]. For the working standard solutions, 25 μl of the stock solutions were diluted with methanol to 25 ml in volumetric flasks to yield a final concentration of 0.4 μg/ml. Working standard solutions were prepared weekly and were stored at 4 °C. OASIS MCX and MAX (3 ml, 60 mg, 30 μm) SPE cartridges were purchased from Waters Corp. (Budapest, Hungary). The bovine milk samples originated from a Hungarian residue control monitoring program (2009 January to 2009 December) and were stored at −20 °C until analysis.

2.2. Sample hydrolysis and preparation

Samples were hydrolyzed by adding 2 ml of 2 M sodium acetate buffer (pH 5.2) to 5.0 g of milk sample. The pH of the mixture was adjusted to 5.2 ± 0.1 prior to adding 20 μl of 1 MU Helix Pomatia beta-glucuronidase, followed by vortex-mixing and incubation at 37 °C for 16 h. After hydrolysis, 12 ml of acetonitrile was added to the sample and vortex-mixing was applied for 10 s. The sample was then centrifuged at 25 °C for 10 min at 10,000 rpm in order to remove fats and other compounds, which were insoluble in the acetonitrile–water mixture. The upper layer was transferred to a glass tube and evaporated under a gentle nitrogen stream at 55 °C for 50 min. After 50 min, the sample volume was reduced to 1.0–1.5 ml. The sample was then cooled down on ambient temperature.

2.3. Sample concentration and SPE clean-up

Prior to sample cleaning and experimental procedures, both cartridges were conditioned. The MCX cartridge (3 ml, 60 mg) was conditioned by passing two times 3 ml methanol, 3 ml ethanol, 3 ml water, and 3 ml 5% (v/v) acetic acid in water (pH 2.3) through the cartridges. Five ml of 5% (v/v) acetic acid in water at pH: 2.3 was added to the reduced volume samples, which were then vortex-mixed for 30 s. Samples were passed through the MCX cartridge drop wise. The sample cleaning was performed by washing the cartridges two times with 3 ml of 5% (v/v) acetic acid in water (pH: 2.3) and the columns were then dried under vacuum for 20 s. Corticosteroids were eluted with 6 ml acetone. The acetone was evaporated under a gentle nitrogen stream at 45 °C to dryness. The samples were then re-dissolved in a 200 μl of a methanol–water solution (50:50, v/v) and filtered with a 0.45 μm nylon filter (Macherey-Nagel, Düren, Germany).

2.4. HPLC conditions

Corticosteroids were separated on an Ascentis Express C-18 (150 mm × 4.6 mm, 2.7 μm) (Sigma–Aldrich, Budapest, Hungary) column, using gradient elution with mobile phases A and B. Mobile phase A was a mixture of 5 mM ammonium acetate and 0.05% (v/v) acetic acid in water (pH 4.1), and mobile phase B was 100% methanol. Gradient elution started using 50% (v/v) mobile phase B which was increased linearly from 50 to 100% (v/v) over 5 min, followed by 100% (v/v) B for 4 min. After 9 min, mobile phase B was decreased to 50% (v/v) over a period of 0.5 min. The flow rate was 0.7 ml/min and the analysis time was 15 min. The injection volume was 10 μl and the thermostat of the analytical column was set at 30 °C.

2.5. Instruments and mass spectrometry conditions

The vacuum manifold for the SPE was obtained from Merck (Budapest, Hungary). The nitrogen evaporator was a Caliper TurboVap LV. Vortex-mixing was done on a WhirliMixer (Fisons Scientific, UK) and centrifugations were performed on a Sigma 3-18K centrifuge (Sigma, Osterode am Harz, Germany). For chromatographic separations, an Agilent 1200 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A auto sampler, G1316A column thermostat) was used (Agilent Technologies, Palo Alto, CA, USA), which was connected to an Agilent 6410A triple quadrupole equipped with an Agilent multimode ion source (G1978B) (Agilent Technologies, Palo Alto, CA, USA). Data acquisition was performed using the Agilent Mass Hunter B.01.04 software.

The mass selective detector was used in the MRM (multiple reaction monitoring) mode for the highest possible selectivity and sensitivity. The multimode ion (MMI) source was operated in the negative APCI mode. 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.

For the adaptation study, an Agilent 6460 Triple Quad LC–MS equipped with an Agilent 1200 binary pump LC and an Agilent 6460 mass selective detector with an Agilent Jet Stream (AJS) (G1958-65138) ion source (Agilent Technologies, Palo Alto, CA, USA) was used. Data analysis was performed using Agilent Mass Hunter B.02.01 software. The AJS with thermal gradient focusing increases the ion density in front of the MS sampling orifice, and consequently, more ions enter the MS system. Thermal gradient focusing technology uses super-heated nitrogen as a sheath gas to improve ion production and desolvation. This novel technique (introduced in July 2008) may result in a limit of detection (LOD) twenty times lower, than that obtained with LC–MS/MS using either an ESI or APCI ion source. However, this lower LOD strongly depends on sheath gas temperature and compound thermal stability [30].

Instrumental settings for the Agilent 6460 mass selective detector include: gas temperature: 350 °C, gas flow: 4 l/min, sheath gas temperature: 350 °C, sheath gas flow: 12 l/min, nebulizer pressure: 344.8 kPa, capillary voltage: 3500 V, nozzle voltage: 0 V in negative mode and 500 V in positive mode. Nitrogen gas was used as the drying and collision gas for all LC–MS/MS instruments.

2.6. Quantification

An internal standard (2 μg/kg testosterone-d5) was added to the samples at the end of the sample preparation procedure. Six-point (including zero) standard curves were constructed for quantification. The Mass Hunter Quantitative software was used to obtain regressions, weighted with relative concentrations^{-1}. The analytical method was validated according to 2002/657/EC Decision [25,26] standards and the parameters assessed included selectivity, linearity, recovery, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ), limit of detection (LOD), and limit of quantification (LOQ). The decision limit and limit of detec-

Table 2
Agilent 6410A MS/MS equipped with multimode ion source: settings and ion ratios of two ion transition reactions of the 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	0–5.3		APCI	Negative	0	Data not stored								
						PRED	$[M+CH_3COO]^-$	**329.4**	75 ms	110 V	20 V	6.7 ± 0.3%	3.4–10.1%	6.7–7.6%
							419.3	280.1	75 ms		25 V			
						METPREDON	$[M+CH_3COO]^-$	**342.2**	75 ms	90 V	5 V	24.0 ± 0.5%	18.0–30.0%	18.8–26.0%
							431.2	313.2	75 ms		15 V			
2	5.3–6.3	MRM	APCI	Negative	350	FLU	$[M+CH_3COO]^-$	**379.2**	75 ms	140 V	10 V	13.0 ± 0.5%	9.1–16.9%	10.6–16.3%
							469.3	305.3	75 ms		30 V			
						DXM	$[M+CH_3COO]^-$	**361.1**	75 ms	90 V	15 V	32.5 ± 0.6%	24.4–40.6%	25.9–36.5%
							451.3	307.2	75 ms		30 V			
						METPRED	$[M+CH_3COO]^-$	**343.2**	75 ms	110 V	15 V	16.5 ± 0.3%	11.6–21.5%	15.3–18.1%
							433.2	309.2	75 ms		25 V			
3	6.3–9.0		APCI	Positive	200	Testosterone-d5	$[M+H]^+$ 294.3	**100.3**	200 ms	120 V	30 V			
4	9.0–15		APCI	Negative	0	Data not stored								

Ion ratios of compounds in standard solutions were calculated as an average of the five calibration points and the ratios of spiked samples were calculated from the 18 fortified samples (3 levels and 6 parallel).
ΔEMV = Delta Electron Multiplier Voltage.

tion were checked by twenty blank samples which had been spiked to attain the desired 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 2 product ions. The steroids produced precursor ions in both negative and positive modes (Table 2). The MMI source resulted in more intense acetate adduct $[M+CH_3COO]^-$ precursor ions in the negative APCI mode than $[M+H]^+$ ions in the positive APCI mode for the analyzed corticosteroids. The internal standard, testosterone-d5, was measured as $[M+H]^+$ using the positive APCI mode.

During the first step, the precursor ions were optimize-scanned with the second quadrupole for the best fragmentor voltage. The mass spectra of the molecules were recorded by the employment of seven fragmentor potentials between 90 and 150 V. After choosing the most intense fragmentor voltage for the found precursor ions, the collision energies of the ion transitions were optimized between 0 and 30 V using a product ion scan. The MMI source maintained a vaporizer temperature of 160 °C.

3.2. General conditions for liquid chromatography

Milk is a complex mixture of substances of variable concentrations; therefore, a relatively long column was selected to avoid potential interferences. An Ascentis Express C-18 (150 mm × 4.6 mm, 2.7 μm) analytical column was employed to achieve high resolution between steroids and neutral interfering compounds and to avoid column overload, as compared to a short column which is more widely used in HPLC-MS analyses. This shell-type column introduced in 2007 has a high peak capacity and a relatively low pressure drop in both gradient and isocratic modes [29]. The Ascentis Express has a high efficiency (high plate number) for the separation of neutral steroids. In a recent study of steroid analysis, a peak capacity of approximately 150 was attainable on a small (50 mm × 2.1 mm, 2.7 μm) Ascentis Express C-18 column with an analysis time of 25 min [29]. This column is very different from fully porous columns. The core–shell particles contain 1.7 μm non-porous core and 0.5 μm porous shells. The chromatographic peaks are narrower and both the peak capacity and sensitivity is improved using this type of column. The Ascentis and Halo columns are the same but are sold under different names.

This column, used in gradient elution method, allowed the same analysis time to the Zorbax Eclipse XDB (100 mm × 2.1 mm, 1.8 μm) shorter column, which was used in isocratic method for determination of corticosteroids in another study [20]. Although analysis time of a gradient elution method is always longer compared to isocratic one, the effectiveness of Ascentis express enables fast separation. Although the Ascentis Express is longer and wider (150 mm × 4.6 mm), compared to Zorbax Eclipse XDB used in other study, it led to fast and efficient separation in this study.

This method allows one to perform four sample injections per hour. The Ascentis Express with a small pore size frit (2 μm) also provides a longer column life compared to other analytical columns.

Methanol was used in the mobile phase and gave higher sensitivity than acetonitrile. Using methanol–water (acetate buffer) as the mobile phase, the pressure drop was high due to the 2.7 μm porous particles of the column. We had to use a lower flow rate of 0.7 ml/min. This flow rate was appropriate for ionization because of the low vaporizer temperature (160 °C) that resulted in a high intensity for ion traces in the MMI source.

The internal standard was added to the samples at the end of sample preparation to improve the accuracy of the quantitative determinations [28]. Testosterone-d5 was used as an internal standard for all corticosteroids, which were detected in opposite polarity. We have used this substance as internal standard for matrices such as urine or water [21,33]. Cortisol-d4, detected in negative acetate adduct form, was also tried as internal standard but an endogenous steroid, tetrahydrocortisol interferenced with it during the chromatographic separation.

3.3. Method development of solid-phase extraction

Polymeric Oasis SPE cartridges are frequently used for the determination of corticosteroids from different matrices [12,14,16,18–21,24,33]. Oasis HLB is often applied for corticosteroid clean-up procedures from fluid matrices such as water, urine, and milk [12,14,16,18–19,21,24]. Since the HLB phase is unable to adsorb ionic matrices and neutral corticosteroids selectively, other SPE steps are necessary to clean the complex fluid samples of basic or acidic compounds [12,14,18–19]. In a method where a single-step HLB clean-up was used for milk, the recoveries were very low (15.8–27.2%) [16]. Cui et al. and Yang et al. used silica, ENVI-carb and amino-propyl SPE in addition to HLB extraction to reduce the matrix effects of a milk sample [12,14]. These steps increase the analysis time substantially. In a recent study of urine samples, a single MAX SPE cartridge was successfully used for cleaning in the determination of corticosteroid from bovine and pig urine samples [20]. Although in one paper milk sample preparation without a deproteinization procedure is described [17], this is necessary because commonly used SPE cartridges can easily clog from precipitated proteins. During the optimization of our SPE method, deproteinized, spiked (MRL or mrpl level) milk samples were subjected to the cleaning procedure at different pH values (2.3 or 11) with the use of MCX or MAX cartridges.

Three different raw bovine milk samples in three series were spiked to MRL or mrpl concentrations at the beginning of clean-up procedures and before hydrolysis and subsequently cleaned using MCX and acidic control (pH 2.3) with different elution conditions. Three samples were eluted with 6 ml of dichloromethane (DCM) and another three with 4 ml acetonitrile (ACN) followed by 2 ml of dichloromethane (DCM), while the last three samples were eluted with 6 ml of acetone. After injection into the LC–MS/MS, results were compared with the results of the standard solutions prepared in methanol–water (50/50, v/v) solution. For PRED, DXM and METPRED, using acetone as the sample solvent yielded the highest recoveries (106%, 94% and 95%) while the results with DCM and ACN followed by DCM were significantly lower (43–66%). For METPREDON, the use of dichloromethane and acetone yielded better recoveries (97% and 106%) as compared to ACN followed by DCM (80%). For FLU, elution with ACN followed by DCM yielded the best recovery (89%) compared to acetone and DCM (113% and 72%). Overall, acetone proved to be the most efficient solvent (94–113%) for the elution of corticosteroids from the MCX cartridge.

Three different bovine milk samples, spiked to attain MRL or mrpl concentrations, were also subjected to MAX SPE cleansing under basic (pH 11) conditions. Before the MAX clean-up, 5 ml 0.1 M ammonia in water at pH 11 was added to the reduced volume milk samples, followed by vortex-mixing for 30 s. The cartridge was conditioned by passing 3 ml methanol, 3 ml ethanol, 3 ml water, and 3 ml 0.1 M ammonia in water (pH 11) twice through the cartridges. Samples were then passed through the MAX cartridge drop wise and the columns were washed two times with 3 ml 0.1 M ammonia in water (pH 11) and subsequently dried under a vacuum for 20 s. Corticosteroids were eluted with 6 ml of acetone. The acetone was evaporated under a gentle nitrogen stream at 45 °C to dryness and the samples were then re-dissolved in a 200 μl of a methanol–water solution (50:50, v/v) and filtered with a 0.45 μm nylon filter (Macherey-Nagel, Düren, Germany). Samples were injected into LC–MS/MS. The average recovery values were compared to those obtained by using the MCX with acidic pH conditions and acetone elution. It was found that the MCX SPE clean-up and elution yielded higher recoveries (94–113%) than those obtained with the MAX SPE (56–73%).

Both MCX and MAX SPE cartridges have the same reverse-phase surface which is essential for the retention of neutral corticosteroids. However, the surface of the MCX also contains anionic groups which can be identified and which can interact with protonated amino groups present in proteins and peptides and which are unable to be eluted by neutral solvents, such as acetone. The amount of interfering matrices in the effluent can be reduced to a low level without ion suppression. Acetone has high elution strength for the elution of neutral steroids. On the surface of the MAX sorbent, some cationic groups can be determined. Under basic conditions, acidic groups in the proteins and peptides are in ionic form; therefore, ionic interactions occur between the surface and some matrix components. However, results have shown that recovery and matrix removal were better using the MCX cartridge and acidic pH conditions. Co-elution of interfering polar compounds, such as peptides, was avoided since they were retained by anionic groups of the MCX cartridge and acetone was unable to elute them together with the target analytes. Neutral corticosteroids occurring in milk were probably co-eluted by acetone, but the adjustment of an adequate gradient elution for performing chromatographic separation prevented them from suppressing analyte response, as demonstrated by the experiments performed for studying ion suppression and described in Section 3.4.

3.4. Experiment on ion effect

Ion suppression effects between different milk samples were studied in recent articles [33,35]. Five different blank raw bovine milk samples were cleaned on MCX cartridges under acidic conditions. Samples were eluted with acetone into receiving vials which contained 6 μg/kg PRED, 0.5 μg/kg METPREDON, 0.5 μg/kg FLU, 0.3 μg/kg DXM, 0.5 μg/kg METPRED and 2 μg/kg testosterone-d5 (ISTD), respectively (MRL or mrpl values). After elution, samples were evaporated to dryness at 45 °C under a gentle nitrogen stream and re-dissolved in a 200 μl methanol–water (50:50, v/v) solution. Samples were homogenized by vortexing for 30 s and they were filtered with a 0.45 μm nylon filters into HPLC vials. Five standard solutions which contained 6 μg/kg PRED, 0.5 μg/kg METPREDON, 0.5 μg/kg FLU, 0.3 μg/kg DXM, 0.5 μg/kg METPRED and 2 μg/kg testosterone-d5 (ISTD), respectively (MRL or mrpl values), were evaporated to dryness at 45 °C in a gentle nitrogen stream and re-dissolved in a 200 μl methanol–water (50:50, v/v) solution. Samples were homogenized by vortexing for 30 sec and were stored in HPLC vials.

Ten solutions were injected into the LC–MS/MS system, randomly. Peak areas were integrated to calculate the relative areas [area of target corticosteroid × (areas of internal standard)$^{-1}$]. Signal values (relative areas of corticosteroids) are summarized in Table 3. The RSD% of the relative areas in the spiked blank milk samples ranged from 5.5% to 8.1% (Table 3), which were a little lower than the RSD% of the relative areas of the corticosteroid mixture prepared in a clean methanol–water solvent (6.1–11.6%). There was not a relative matrix effect, since RSD for standards in solvent and in matrix was comparable. Evaporation to dryness may have affected the slightly high RSD% of solutions. Comparing the average signal values of spiked samples to standards in HPLC grade solvent (Table 3), we can conclude that ion enhancement is observed. The value of the matrix effect is 117–127% evaluating the results with evaporated standards.

Table 3
Signal values of ion affect experiment. Ion enrichment was calculated as (average standard in matrix/(average standard in HPLC solution) − 1) × 100.

	PRED	METPREDON	FLU	DXM	METPRED
MRL or mrpl standard + 2 ppb ISTD in HPLC solvent					
1	17.227	1.614	3.630	2.702	2.058
2	16.454	1.542	2.934	2.084	1.793
3	15.218	1.454	2.878	2.306	1.779
4	17.983	1.763	3.697	2.599	2.090
5	16.771	1.792	3.389	2.639	1.914
Average	16.730	1.633	3.306	2.466	1.927
S	1.022	0.144	0.383	0.262	0.145
RSD%	6.1	8.8	11.6	10.6	7.5
MRL or mrpl standard + 2 ppb ISTD in cleaned blank sample					
1	20.391	1.977	3.829	2.984	2.432
2	17.319	1.861	3.488	2.731	2.181
3	20.459	2.102	4.052	3.157	2.718
4	20.794	2.145	4.103	3.065	2.393
5	19.671	2.010	3.855	2.771	2.546
Average	19.727	2.019	3.865	2.942	2.454
S	1.407	0.111	0.242	0.185	0.198
RSD%	7.1	5.5	6.3	6.3	8.1
Ion enhancement					
	18%	22.7%	17%	19%	27%

Ion enhancement was tested by comparing three calibration curves that were constructed from analysis of samples which had been prepared in different ways. One curve was based on standards prepared from evaporating a methanolic standard at 45 °C in a gentle nitrogen stream and re-dissolving it in 200 μl of a methanol–water (50/50, v/v) mixture. A second curve was based on standards prepared by dilution in volumetric flasks with methanol–water (50/50, v/v) using HPLC grade solvent. The last curve was based on standards which had been added to the cleaned matrix after sample preparation. The curves were constructed using standard concentrations of 0.5–2 MRL and of 0.5–2 mrpl, respectively. Signal values and slopes are summarized in Table 4. The calculated slopes show that some ion enhancement was observed (slopes of standards in cleaned matrix were calculated to 110–125% of the slope for standards in methanol/water) while some loss during evaporation was observed (slopes of evaporated/reconstituted standards were calculated to 76–88% of the slope without evaporation). These two effects, each within acceptable ranges, may have counteracted in our results.

3.5. Selectivity

Blank and spiked samples at MRL or mrpl levels were also analyzed. A signal was not observed at the retention times of the investigated solutes on MRM chromatograms of the blank milk (Fig. 1b), and hence, the separated steroids were measured without interference.

3.6. Identification

MRM (multiple reaction monitoring) chromatograms of the individual compounds are shown in Fig. 1a. Confirmation of the analytes was carried out using four identification points [26]. One precursor ion refers to one identification point and two transitions together represent three points. The most abundant MS/MS transition of each compound was used for quantification, while the second most abundant transition was used for identification. During the identification, the value of the qualifier ratio was studied, which is the intensity ratio of the quantifier and qualifier transitions. The qualifier ratio was found to have a constant value throughout the whole concentration range for each compound, allowing for correct identification and quantification in the concentration range studied, as shown in Table 2. The maximum permitted tolerance under 10% is ±50%, while between 10% and 20% it is ±30% and between 20% and 50% it is ±25% [26]. The ion ratios of each spiked sample fell within the maximum permitted tolerances for positive identifications.

3.7. Linearity

Calibration standards were prepared in a methanol–water (50/50, v/v) solution. Six-point calibrations (including zero) were performed between 0 and 12.0 μg/kg for PRED and 0–0.60 μg/kg for DXM. In the case of banned substances (METPREDON, FLU, and METPRED), calibrations ranged from 0 to 2.0 μg/kg. Correlation coefficients (r^2) ranged between 0.9765 and 0.9946.

3.8. Recovery, within-laboratory reproducibility

The intraday recovery was estimated by spiking blank samples at three different concentrations in six series [26]. The validation levels for PRED were 3, 6 and 9 μg/kg; and for DXM, were 0.15, 0.30 and 0.45 μg/kg (0.5 MRL, MRL and 1.5 MRL). The validation levels for METPREDON, FLU and METPRED were performed on 0.5, 0.75, and 1.0 μg/kg (mrpl, 1.5 mrpl and 2 mrpl). These concentrations meet the EU guidelines (Table 5). Intraday recoveries were also evaluated using external standard method. As can be seen in Table 5, both recovery and precision were better for all corticosteroids using testosterone-d5 as an ISTD in evaluating the results. Within-laboratory reproducibility was also evaluated by repeating the recovery test with different operators, solvents, and batches of MCX. The same method was employed on two different days in

Table 4
Matrix effect on signal values.

		PRED	METPREDON	FLU	DXM	METPRED
Evaporated std	0.5 MRL/0.5 mrpl	0.781	1.765	0.438	0.446	3.173
	MRL/mrpl	3.317	5.818	1.353	1.265	8.411
	2 MRL/2 mrpl	6.566	12.021	2.676	2.505	17.957
	Slope	87%	76%	83%	88%	82%
Std in volumetric flash	0.5 MRL/0.5 mrpl	1.652	2.823	0.771	0.576	4.501
	MRL/mrpl	3.188	6.119	1.484	1.172	8.328
	2 MRL/2 mrpl	7.894	16.068	3.305	2.910	22.215
	Slope	100%	100%	100%	100%	100%
Std in cleaned matrix	0.5 MRL/0.5 mrpl	1.663	3.154	0.754	0.607	4.342
	MRL/mrpl	3.569	7.142	1.742	1.409	10.817
	2 MRL/2 mrpl	8.817	17.602	4.067	3.390	24.705
	Slope	1125	110%	125%	117%	113%
Evaporated × cleaned slope		98%	84%	104%	103%	93%

six series. Recoveries fulfilled the 2002/657 EC Decision guidelines (Table 6). Each compound had a recovery value determined from 54 results (3 day, 3 levels and 6 parallel, $3 \times 3 \times 6$) which spanned the range from low to high concentrations. Within-laboratory reproducibility was calculated as the precision. Precision is expressed as the relative standard deviation (RSD%) of the method [34]. For calculation of within-laboratory reproducibility the mean concentrations were calculated from 18 results per level for 3 days (54 results per compound). These concentrations include the interday recoveries.

The RSD% should be as low as possible, under 100 μg/kg [26]. Previous analytical strategies have attained 30% precision at these levels [27]. These conditions were observed for all corticosteroids (Tables 5 and 6).

3.9. Decision limit (CCα) and detection capability (CCβ), limit of detection (LOD) and limit of quantification (LOQ)

The MRL substance decision limit was calculated as the permitted limit plus 1.64 times the standard deviation of the within-laboratory reproducibility at the MRL level [26]. The substances which did not contain a permitted limit were analyzed with twenty different blank samples to calculate the signal-to-noise ratio at the time window in which the corticosteroids were eluted. The decision limit was estimated as three times the signal-to-noise ratio [26]. The detection capability was calculated as the value of the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility at decision limit [26].

According to 2002/657 EC, the decision limit of a banned substance should be lower than the mrpl and the detection capability

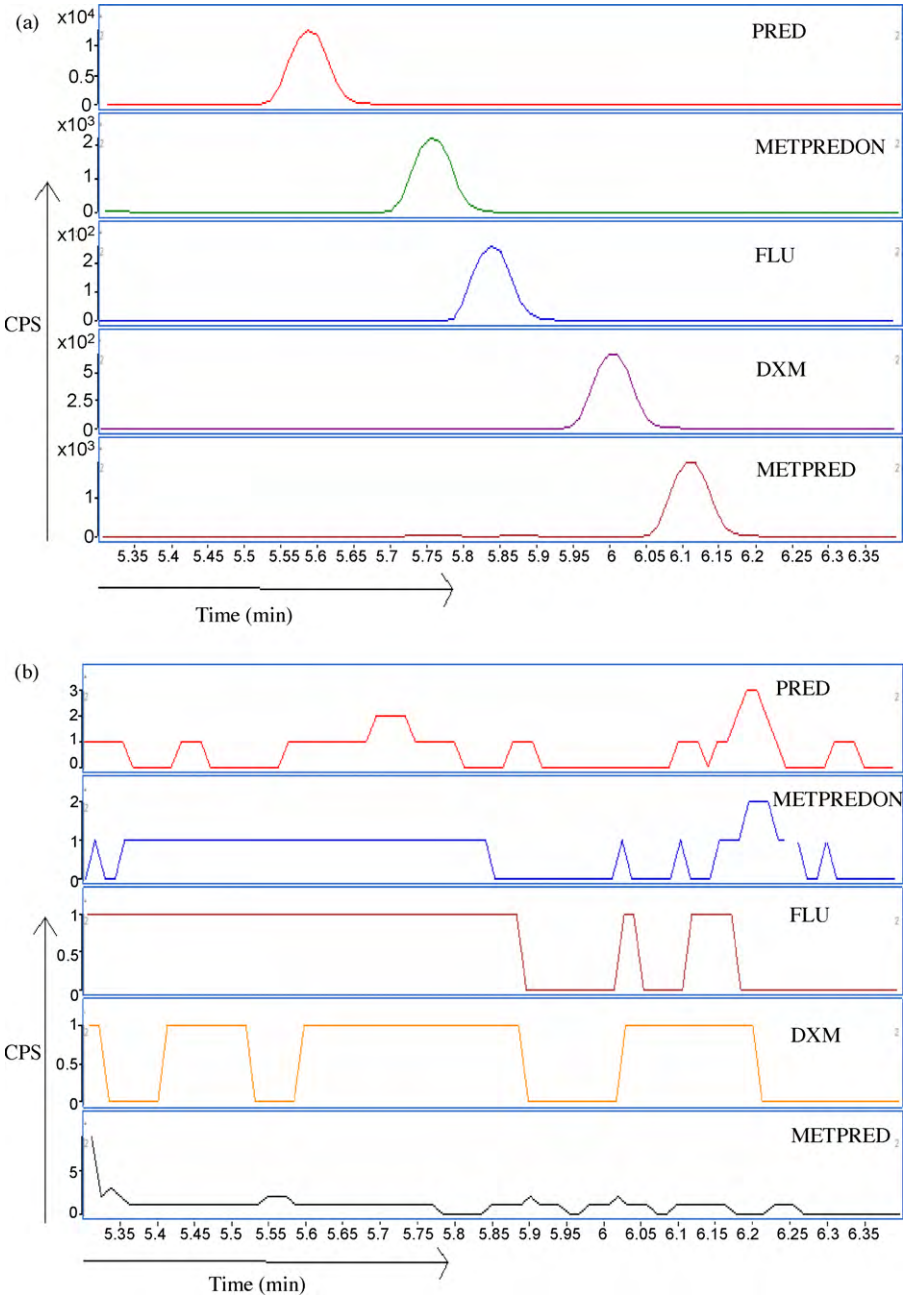


Fig. 1. Quantitative MRM chromatograms of spiked bovine milk sample (a) and blank bovine milk sample (b). Spiking concentrations were 6 μg/kg for PRED, 0.5 μg/kg METPREDON, 0.5 μg/kg FLU, 0.3 μg/kg DXM and 0.5 μg/kg METPRED.

Table 5
Intraday recovery (mean and range, $n = 6$) and precision at 0.5 MRL, MRL, 1.5 MRL and mrpl, 1.5 mrpl, 2 mrpl. Results were evaluated with both internal standard and external standard methods.

Compound	0.5 MRL			MRL/mrpl			1.5 MRL/1.5 mrpl			2 mrpl		
	Mean recovery%	Range%	RSD%	Mean recovery%	Range%	RSD%	Mean recovery%	Range%	RSD%	Mean recovery%	Range%	RSD%
Internal standard method												
PRED	99	95–103	3.3	90	77–99	9.8	80	73–91	8.2			
METPREDON				89	82–98	5.8	90	81–95	5.5	86	76–98	9.2
FLU				96	82–106	9.7	100	89–109	7.6	98	82–107	8.6
DXM	104	93–120	8.7	93	80–103	8.5	83	76–91	7.7			
METPRED				91	76–110	14.3	95	85–101	6.4	91	76–99	9.2
External standard method												
PRED	77	79–97	13.5	83	72–106	14.3	77	65–102	17.3			
METPREDON				66	48–104	31.3	85	77–103	11	86	75–110	14.4
FLU				78	62–106	21.1	94	85–108	8.8	92	83–120	14.4
DXM	83	76–93	7.7	86	75–101	11.3	79	70–102	14.6			
METPRED				69	58–96	20.5	81	73–99	11.1	82	73–109	16.6

must be lower or equal to the mrpl [25,26]. As shown in Table 6, the results for both the decision limit and the detection capability meet the conditions of the 2002/657 EC directive.

The LOD was determined as the lowest concentration of a compound at which the ion ratio of the two ion transitions was still acceptable. LODs were calculated as 3 times the signal-to-noise ratio while the LOQs were determined as 3.33 times the LOD value (Table 7). For the banned substances, the LOD was equal to the decision limit. Decision limits and LODs were confirmed by analyzing twenty different blank milk samples which had been spiked to the individually calculated concentrations. Values were accepted for a compound when the signal-to-noise ratios for spiked samples were higher than three and the ion ratios were in an acceptable range.

4. Future developments

The development of LC–MS/MS hardware is in progress. To obtain a higher sensitivity, the efficiency of the ion source is one of the key questions. One vendor offers a different ion source in which the ion producing process is similar, but the geometry and solvent evaporation steps are different, and which improve the ion formation efficiency. This is the current situation with the Agilent 6460 triple quadrupole MS system equipped with an Agilent Jet Stream (AJS) ion source. The major difference between AJS and ESI is the employment of thermal gradient focusing, which uses heated nitrogen to improve desolvatation and ion generation. The heated nitrogen sheath gas surrounds the nebulizer spray, which increases the desolvation efficiency and delivers more ions to the mass spectrometer, while also reducing the number of neutral solvent clusters [30].

For regulation purposes, this is a positive step towards accurate analysis of banned drug residues where a zero tolerance level is in place. On the other hand, some ambiguities still exist. The new ion source was tested with respect to sensitivity. The optimized parameters and settings can be found below.

The optimization of the AJS was carried out using flow injection analysis with a 0.4 μg/ml mix of the corticosteroids, similar to MMI. Experiments were performed at different sheath gas temperatures and flows, which indicate an influence on intensity. The sheath gas temperature was changed from 100 to 400 °C in increments of 50 °C with three parallel measurements, while the sheath gas flow was altered from 7 to 12 l/min in 1 l/min increments with three parallel measurements. A value of 12 l/min and 350 °C was selected for further experiments. The optimized AJS MS/MS ion traces are shown in Table 8.

The intensity difference between the AJS coupled to 6460 and MMI coupled to 6410A MS/MS was significant for the measured corticosteroid MRM transitions. To evaluate the difference in sen-

Table 6
Recovery (17–18 sample per level) and within-laboratory reproducibility (precision, RSD%) at 0.5 MRL, MRL, 1.5 MRL and mrpl, 1.5 mrpl, 2 mrpl. Decision limit (CCα) and detection capability (CCβ).

Compound	0.5 MRL			MRL/mrpl			1.5 MRL/1.5 mrpl			2 mrpl			CCα (μg/kg)	CCβ (μg/kg)
	Mean recovery%	Range%	RSD%	Mean recovery%	Range%	RSD%	Mean recovery%	Range%	RSD%	Mean recovery%	Range%	RSD%		
PRED	100	58–131	18.9	99	50–142	23.3	93	73–121	16.5				8.3	11.5
METPREDON				90	28–138	33.7	93	51–131	25.2	100	76–134	20.3	0.06	0.09
FLU				95	50–128	23.9	99	49–133	25.1	105	76–133	16.3	0.02	0.03
DXM	96	40–127	29.2	97	37–130	26.8	95	76–120	15.6				0.43	0.62
METPRED				91	44–126	26.4	99	47–127	19.4	101	76–130	15.2	0.07	0.10

Table 7
LODs and LOQs obtained with two instruments.

	6410A equipped with MMI used in APCI mode		6460 equipped with AJS	
	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
PRED	0.06	0.20	0.002	0.007
METPREDON	0.06	0.20	0.003	0.010
FLU	0.02	0.07	0.001	0.003
DXM	0.02	0.07	0.001	0.003
METPRED	0.07	0.23	0.006	0.020

Table 8
Agilent 6460 MS/MS equipped with Jet Stream ion source: settings and ion ratios of two ion transition reactions of the 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	0–5.3			Negative		Data not stored								
						PRED	$[M+CH_3COO]^-$ 419.3	**329.1** 280.0	20 ms 20 ms	104 V	12 V 40 V	19.9%	13.9–25.9%	18.3–20.6%
						METPREDON	$[M+CH_3COO]^-$ 431.2	371.1 **341.1**	20 ms 20 ms	94 V	0 V 8 V	29.3%	22.0–36.6%	22.5–26.9%
2	5.3–6.3	MRM	Jet Stream	Negative	350	FLU	$[M+CH_3COO]^-$ 469.3	**379.1** 305.1	20 ms 20 ms	111 V	12 V 40 V	23.5%	17.6–29.4%	21.6–24.4%
						DXM	$[M+CH_3COO]^-$ 451.3	**361.1** 307.1	20 ms 20 ms	101 V	12 V 32 V	24.9%	18.7–31.1%	23.2–25.8%
						METPRED	$[M+CH_3COO]^-$ 433.2	**343.2** 309.1	20 ms 20 ms	111 V	12 V 36 V	23.2%	17.4–29.0%	23.6–25.2%
3	6.3–9.0			Positive	400	Testosterone-d5	$[M+H]^+$ 294.3	**113**	200 ms	138 V	28 V			
4	9.0–15			Negative		Data not stored								

sitivity between the two MS systems, 20 different blank samples were spiked to determined LOD values using the Agilent 6410A triple quadruple instrument with MMI (Table 7). After sample preparation, the samples were injected into the 6460 LC–MS/MS equipped with AJS. LODs and LOQs were then calculated (Table 7). The LOD was established as 3 times the signal-to-noise ratio, while the LOQ was 3.33 times the LOD. Results obtained with the AJS with 6460 LC–MS/MS had LOD values which were more than 10 times lower for METPRED. In the case of METPREDON, FLU and DXM, LOD values were twenty times lower. The LOD of PRED was determined to be thirty times lower compared to the MMI source with the 6410A instrument (Table 7). In view of the results presented above, this method is shown to offer superior detection at levels required by the 2002/657 EC Decision.

5. Conclusions

A fast, rapid and inexpensive SPE method was developed to identify and quantify corticosteroid residues in bovine milk samples by LC–MS/MS. The deproteinzed milk sample was cleaned at pH 2.3 which enabled the selective interaction of basic proteins and peptides with the cation exchange part of the MCX SPE column. Acetone was found to elute only the corticosteroid compounds from the SPE cartridge, reducing ion suppression of the matrix compounds. Acetone would also elute the neutral lipids from the reversed-phase of the MCX cartridge, but during the chromatographic separation they might not have eluted along with corticosteroids and hence would not affect ion suppression in the MMI source. Interfering fats were removed by centrifugation of the sample at high rpm. The main advantage of the developed method over the previous methods found in the literature is the reduction of the ion suppression effects with a single-step SPE procedure in conjunction with LC–MS/MS analysis. Additionally, the method was validated successfully for the 6410A instrument, based on the 2002/657/EC Decision requirements. Selectivity, linearity, recovery, and within-laboratory reproducibility conditions met the conditions of the EC Decision. The intraday and interday recoveries vary between 83–104% and 90–105%, respectively. The within-laboratory reproducibility (precision) of the measurements is less than 30%. Decision limits of 8.3 and 0.43 μg/kg for PRED and DXM varied between 0.02 and 0.07 μg/kg for banned substances. LODs using the 6410A ranged from 0.02 to 0.07 μg/kg for the measured corticosteroids which is lower than the earlier reported LODs of 0.02–0.16 μg/kg using electrospray ionization (ESI) [12]. This is the first report of corticosteroid analyses at such low LOD values in milk samples. This method was also successfully applied using the 6460 LC–MS/MS equipped with an Agilent Jet Stream ion source. The regulatory level of banned substances (amounts which are prohibited) should be reevaluated because the analytical sensitivity of LC–MS/MS measurements varies from instrument to instrument.

Finally, the Agilent Jet Stream (AJS) ion source was coupled to another MS/MS detector with the aim of lowering LODs compared to the use of the MMI source. After optimizing conditions for the AJS source, LODs of the six corticosteroids used in this study were found to decrease by 10–30 times to 0.001–0.006 μg/kg.

The validated method was applied to determine concentrations of selected corticosteroids in milk samples, obtained under a Hungarian residue control monitoring program, but as yet no corticosteroid residues have been identified in the milk samples.

Acknowledgment

We wish to thank the reviewers of this paper for their comments, which greatly improved the paper.

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