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Short communication

Rapid simultaneous determination of dexamethasone and betamethasone in milk by liquid chromatography tandem mass spectrometry with isotope dilution

Li Cun [a], Wu Yinliang [b,*], Yang Ting [b], Zhang Yan [b]

[a] College of Agriculture, Hebei University of Engineering, Handan 056038, China
[b] Ningbo Agriculture Products Quality Detector Center, Ningbo 315040, China

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ABSTRACT

A simple, sensitive and reliable analytical method for the rapid simultaneous determination of dexamethasone and betamethasone in milk by high performance liquid chromatography–negative electrospray ionization tandem mass spectrometry (HPLC–NESI-MS/MS) with isotope dilution was developed. Samples were directly purified through C_{18} cartridge. Then the eluate was dried under nitrogen and residues were dissolved in mobile phase. Samples were analyzed by HPLC–MS/MS on a Hypercarb graphite column with a mixture of acetonitrile–water–formic acid as mobile phase. The samples were quantified using dexamethasone-D_4 as an internal standard. The procedure was validated according to the European Union regulation 2002/657/EC determining specificity, decision limit (CCα), detection capability (CCβ), trueness, precision, linearity and stability. The method is demonstrated to be suitable for the determination of dexamethasone and betamethasone in milk. The total time required for the analysis of one sample was about 35 min.

1. Introduction

Dexamethasone (DM) and betamethasone (BM) are epimeric synthetic glucocorticoids, frequently employed as antipyretic, anti-inflammatory and anti-allergic drugs [1]. They are also widely used in livestock and aquatic animal production as growth promoters because they can improve feed conversion rate [2]. However, these hormones may cause adverse effects on human health including obesity, hypertension, osteoporosis and other diseases. Therefore, the European Union and China have banned these drugs as growth promoters [3–5], and they established maximum residue limits (MRLs) of 2 μg/kg in liver, 0.75 μg/kg in muscle and kidney, and 0.3 μg/kg in milk for DM and BM. Therefore, specific and sensitive methods for the identification and quantification of these two compounds in milk are required.

To identify DM and BM in biological samples, gas chromatography–mass spectrometry (GC–MS) [6,7] and liquid chromatography–mass spectrometry (LC–MS/MS) [8–24] methods have been developed. However, GC–MS requires derivatization to enhance the volatility of DM and BM. Moreover, GC–MS analysis lacks specificity for epimeric compounds such as BM and DM. LC–MS/MS has become the main analytical technique for determining DM and BM due to its shorter chromatographic run time and higher sensitivity and without time-consuming derivatization procedures.

A LC–APCI(+)-MS/MS method for determining DM in bovine milk has been developed by Cherlet et al. with simple sample preparation, but the recovery of 56% was low [15]. Cui et al. have developed an ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method for simultaneous determination of 17 glucocorticoids residues including DM in milk with good sensitivity and accuracy, but the sample preparation involve multiple steps and is very time-consuming [19]. McDonald et al. have described a LC–MS/MS method for determining BM and DM in milk with high sensitivity and accuracy, but the sample preparation is also time-consuming [20]. Recently, although Malone et al. have developed a rapid LC–MS/MS method for determination of 7 anti-inflammatory drugs in bovine milk, the sample preparation still includes complex deproteinization step and liquid–liquid purification. However, none of these methods can simultaneously determine DM and BM in milk by HPLC–MS/MS with dexamethasone-D_4 as internal standard and without deproteinization procedure.

In this paper, we describe a simple and sensitive HPLC–MS/MS method for simultaneous determination of DM and BM in milk with isotope dilution. Solid phase extraction (SPE) is used for sample preparation without complex deproteinization step. Validation parameters tested were specificity, decision limit (CCα), detection capability (CCβ), trueness, precision, linearity and stability.

* Corresponding author at: Ningbo Academy of Agricultural Sciences, Ningbo Agriculture Products Quality Detector Center, Bridge No. 6, Ning Chuan Road, Ningbo 315040, China. Tel.: +86 0574 87928060; fax: +86 057487928026.
E-mail address: wupaddyfield@tom.com (Y. Wu).

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2. Materials and methods

2.1. Materials and reagents

Acetonitrile, methanol and formic acid were LC grade. Hexane and ethyl acetate were analytical grade. Dexamethasone and betamethasone were purchased from U.S. Pharmacopeia (Maryland, USA). Dexamethasone-D_4 was purchased from Medical Isotopes Inc. (Massachusetts, USA). The C_{18} SPE cartridges (500 mg, 6 mL) were purchased from Supelco (Bellefonte, PA, USA). Water was purified with a Milli-Q reverse osmosis system (Millipore, Milford, MA, USA).

2.2. Standard solutions

Individual stock solutions of three standards (100 μg/mL for DM and BM, 20 μg/mL for dexamethsone-D_4) were prepared in methanol. Three fortifying standard solutions of DM and BM (15, 30 and 45 ng/mL) and one fortifying standard solution (32 ng/mL for DM and 33 ng/mL for BM) were prepared by diluting and mixing each stock standard solution with methanol. Fortifying standard solution of dexamethsone-D_4 (30 ng/mL) was prepared in methanol. Mixed working standard solutions (3–500 ng/mL) were prepared by diluting and mixing each stock solution with 4 mM ammonium formate/methanol (40:60, v/v).

2.3. Chromatographic conditions

A Waters 2695 HPLC instrument was used in the present study. Separation was carried out on a Hypercarb column (30 mm × 2.1 mm, 5 μm) maintained at 30 °C. The LC mobile phase consisted of acetonitrile/water/formic acid (95:5:0.5, v/v/v). The flow rate was 0.2 mL/min. The injection volume was 10 μL. Run time was 4 min.

2.4. Detection conditions

Detection was carried out by a Waters Quattro Micro™ API triple-quadrupole MS fitted with electrospray ionization (ESI) probe operated in the electrospray negative ion mode. The following parameters were optimal: capillary voltage, 3200 V; ion source temperature, 110 °C; and desolvation gas temperature, 350 °C at a flow rate of 600 L/h. Detection was carried out in multiple reaction monitoring (MRM) mode. Argon was used as the collision gas, and its pressure was regulated to 4 mbar. Other parameters of analytes are shown in Table 1.

2.5. Sample preparation

After addition of 50 μL of 30 ng/mL internal standard solution in 5 g milk, the sample was applied to SPE cartridge which was activated with 5 mL of methanol followed by 5 mL of water. The cartridge was washed with 5 mL of water and dried with strong vacuum for 5 min, then washed with 5 mL of hexane. The analytes were eluted with 5 mL ethyl acetate. The eluate was evaporated dry under a stream of nitrogen at 40 °C. The residue was reconstituted in 200 μL of mobile phase. The resulting solution was filtered through 0.45 μm nylon membrane filter and 10 μL of the filtrate was injected into the HPLC.

2.6. Method validation

The evaluation of the suitability of the method for the determination of DM and BM in milk was carried out according to the European Commission Decision 2002/657/EC [25].

To verify the absence of interfering substances around the retention time of analytes, 20 blank milk samples were analyzed.

Calibration curves were constructed using mixed working standard solutions by plotting the peak area ratio of quantitative ion pair of each standard to internal standard at concentrations of 3, 7.5, 20, 50, 100, 200 and 500 ng/L. The concentration of internal standard was 7.5 ng/L.

The CCα was established by analyzing 24 blank materials per matrix fortified with DM and BM at 0.3 μg/kg. The CCβ was established by analyzing 24 blank materials per matrix fortified with DM and BM at their CCα.

Reproducibility (precision) and recovery (trueness) of DM and BM were measured in blank milk that was spiked at MRL level (0.3 μg/kg), at half of the MRL level (0.15 μg/kg) and one and a half of the MRL level (0.45 μg/kg). The spiked samples were analyzed and the recoveries were calculated by comparing the measured concentration to the spiked concentrations.

The stability was determined in two different ways: (a) in solvent (stock solutions) and (b) in matrix (spiked milk at 0.3 μg/kg).

3. Results and discussion

3.1. Sample preparation

For the determination of BM and DM in milk, the sample pretreatment procedures that were previously published included labor-intensive deproteinization procedure [15,19,20]. However, the liquid milk samples may be directly purified through SPE column, and thus make the sample preparation easier and faster. Moreover, the sample preparation without deproteinization procedure was feasible through above specificity trueness and precision experiment.

3.2. Separation conditions

Luo et al. used a Hypercarb column with an isocratic elution and a time-programmed flow rate [17] for successful separation of DM and BM. We also acquired successful separation using the same column and mobile phase when the flow rate was fixed to 0.2 mL/min.

For glucocorticoids included in group A of Annex I, Council Directive 96/23/EC [26], a minimum of four identification points are required. In this experiment, four identification points, one parent (1 point) and two transitions (each 1.5 points) were monitored. Previous developed LC–ESI-MS/MS methods of DM and BM residues have reported three ways to chose precursor ions including $[M+HCOO]^-$, $[M+H]^+$ and $[M-H]^-$. We chose $[M+HCOO]^-$ as the precursor ion because the ion was the most abundant peak

Table 1
LC–ESI-MS/MS parameters for DM, BM and dexamethasone-D_4.

Analyte	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Dwell time (s)	Collision energy (eV)	Cone voltage (V)
DM	437.00	361.00[a], 307.40	0.5	20.35	35
BM	437.00	361.00[a], 307.40	0.5	20.35	35
Dexamethasone-D_4	441.00	363.00	0.5	20	35

[a] Ion for quantification.

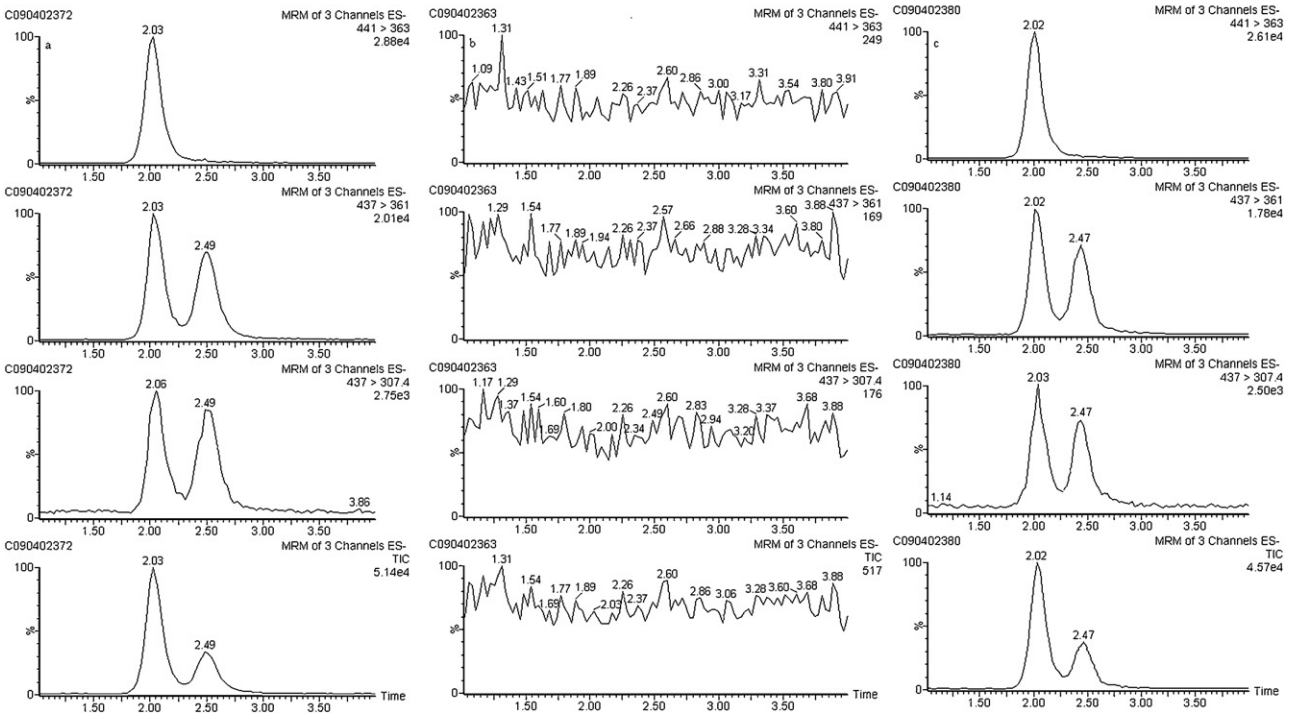

Fig. 1. (a) The MRM chromatogram of DM and BM in standard solution (7.5 μg/L). (b) The MRM chromatogram of blank milk. (c) The MRM chromatogram of blank milk spiked with DM and BM at 0.3 μg/kg.

Table 2
The linear equations and regression coefficients of standard curves of DM and BM by HPLC–MS/MS.

Analyte	Liner equations	R^2
DM	$Y = 0.0804X + 0.2182$	0.9997
BM	$Y = 0.0749X + 0.1609$	0.9998

in the mass spectra when the mobile phase consisted of acetonitrile/water/formic acid (95:5:0.5, v/v/v).

3.3. Method validation

3.3.1. Linearity

The calibration graphs were obtained by plotting the peak area ratio of the quantitative ion pair of each standard to internal standard versus drug concentration in 3–500 ng/mL. The results of the linearity are reported in Table 2. The correlation coefficients of the calibration curves were above 0.999. Using these curves, recoveries can be calculated at each fortification level.

3.3.2. Specificity

The specificity was evaluated by analyzing 20 blank milk samples. Fig. 1b and c indicates that there were no interfering peaks from endogenous compounds at the retention times of DM and BM.

3.3.3. Trueness and precision

The recovery and reproducibility of the method were measured by analyzing six blank samples fortified with DM and BM at each of three concentrations (0.15, 0.30 and 0.45 μg/kg) on three separate occasions. The results are shown in Table 3. The mean recoveries were between 89.6 and 103.4%, and the intra-day CVs and inter-day CVs ranged from 1.98 to 3.88% and 3.21 to 4.75%, respectively. These recoveries and RSD for DM are better than many previously developed methods for milk by LC–MS/MS [15,19,20], possibly due to the use of dexamethasone-D_4 as internal standard to quantify DM in the present study.

3.3.4. CCα and CCβ

According to the concept of the European Commission Decision 2002/657/EC, the CCα (decision limit) and CCβ (detection limit) have been estimated. The results of the CCα were 0.32 and 0.33 μg/kg for DM and BM, respectively. The results of the CCβ were 0.34 and 0.36 μg/kg for DM and BM, respectively. Additionally, limits of detection (LODs) were 0.010 and 0.013 μg/kg for DM and BM, respectively. The LOD of this method is similar to or less than those of previously published methods for DM and BM in milk [15,19,20].

3.3.5. Stability

The stability of the stock standard solutions in methanol was at least 6 months at 4 °C. The stock solutions were analyzed every

Table 3
Mean recoveries (MR) and variation coefficients (CV) of DM and BM from milk by HPLC–MS/MS.

Drug	Fortified concentration (μg/kg)	Average recovery (%, $n = 6$)			Intra-assay CV (%)			Inter-assay CV (%)
DM	0.15	96.8	99.4	103.2	2.10	2.45	2.70	3.52
	0.30	98.5	97.6	99.8	2.39	2.46	2.20	3.21
	0.45	99.3	103.4	97.7	2.75	2.23	1.98	3.45
BM	0.15	92.1	93.6	97.8	3.17	2.68	3.26	4.78
	0.30	90.5	93.1	91.6	3.42	3.10	2.67	4.72
	0.45	92.8	89.6	94.1	3.03	3.88	3.56	4.43

month and the instrumental responses were compared with the peak areas obtained at the moment of solution preparation ($t = 0$). The acceptance criterion was a response comprised between 95 and 105% of the initial one [27]. Spiked milk samples of DM and BM at 0.30 μg/kg stored at −20 °C were analyzed after 5, 10 and 15 days. It was found that the recoveries of DM and BM had no obvious change.

3.4. Applications of the method

Fifteen milk samples commercially available from the local market were analyzed for DM and BM using the above method. DM was found in one sample with the concentration of 0.06 μg/kg.

4. Conclusion

In the present study, a rapid and sensitive method for the simultaneous determination of DM and BM in milk by HPLC–MS/MS with dexamethasone-D_4 as internal standard was developed. This method was validated with fortified milk samples and good recoveries with excellent CVs were obtained. The CCα and CCβ were found to be sufficiently low to determine the residues of DM and BM in milk.

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