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Simultaneous determination of amoxicillin and prednisolone in bovine milk using ultra-high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A rapid and sensitive ultra-high performance liquid chromatography tandem mass spectrometric method was developed for simultaneous quantification of amoxicillin and prednisolone in bovine milk. In this method, amoxicillin, prednisolone and the internal standards penicillin G-d_7 (for amoxicillin) and prednisolone-d_6 were extracted from bovine milk using acetonitrile. The C_{18} solid phase extraction cartridges were selected for cleaning-up the extracts. The analytes were determined using a triple quadrupole mass spectrometry in positive electrospray ionization and multiple reaction monitoring mode. Calibration curves were linear over a concentration range of 2–1000 μg/kg for the analytes. The mean recoveries were 89.2–92.3% for amoxicillin and 98.7–102.3% for prednisolone. Limits of detection were 0.5 μg/kg for the analytes, and the limits of quantitation were 2 μg/kg. Decision limit (CCα) and detection capability (CCβ) have also been estimated for each analyte. The method was validated according to the Commission Decision 2002/657/EC and successfully applied to the analysis of amoxicillin and prednisolone in real samples.

1. Introduction

Bovine mastitis is of great economic importance as it associates with decreased milk production, expensive treatment costs, extra labor and an increased rate of culling [1–4]. Compound amoxicillin intramammary infusion (CAIMM) is a combination drug that developed for the treatment of bovine mastitis, comprising amoxicillin (200 mg), sulbactam (50 mg), and prednisolone (10 mg) in 3 g formulation. A fast and robust bioanalytical assay that can simultaneously determine the concentrations of amoxicillin and prednisolone in bovine milk is essential in supporting the clinical development of CAIMM to understand its pharmacokinetics and efficacy.

In the European Union (EU) and China, maximum residue limits (MRLs) of 4 and 6 μg/kg have been established for amoxicillin and prednisolone in milk, respectively [5,6]. Recently, liquid chromatography tandem mass spectrometric (LC/MS) methods using various extraction and deproteinization procedures have been described for the determination of amoxicillin in milk [7–12]. Many analytical methods for the determination of prednisolone have been published, such as gas chromatography (GC) [13], GC/MS [14–16], liquid chromatography method coupled to diode array detector (LC-DAD) [17], LC–MS/MS [18–23]. Up to now, only two papers describe UPLC–MS/MS methods for the determination of amoxicillin or prednisolone in milk [24,25]. However, studies on UPLC–MS/MS for the simultaneous determination of amoxicillin and prednisolone in bovine milk have not been previously described. The objective of this work was to develop a rapid and sensitive analytical method for simultaneous determination of amoxicillin and prednisolone in bovine milk to support the clinical studies of CAIMM.

2. Experimental

2.1. Chemicals and reagents

The reference standards of amoxicillin (>86.6%) and prednisolone (>99%) were purchased from China Institute of Veterinary Drug Control (IVDC, Beijing, China) and Sigma–Aldrich Inc. (St. Louis, MO, USA), respectively. Penicillin G-d_7 (>95%) and prednisolone-d_6 (>99%) were produced at Toronto Research Chemicals Inc. (TRC, Ontario, Canada) and CDN Isotopes (Pointe-Claire, Quebec, Canada), respectively. HPLC-grade acetonitrile and methanol were purchased from Fisher chemicals (Pittsburgh, PA, USA). HPLC-grade formic acid and n-hexane were purchased from Dikma Technologies Inc. (Lake Forest, CA, USA). Sodium dihydrogen phosphate and sodium hydroxide were obtained from Beijing Chemical Co. (Beijing, China). HPLC water was obtained using a Milli-Q Plus water purification system (Millipore, Bedford, MA,

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USA). The C_{18} solid phase extraction (SPE) cartridges (6 cc, 500 mg, Bond Elut) were purchased from Varian (Lake Forest, CA, USA).

Amoxicillin (1 mg/mL) stock solution was prepared in water and prednisolone (1 mg/mL), penicillin G-d_7 (1000 μg/mL) and prednisolone-d_6 (500 μg/mL) stock solutions were prepared in methanol. The stock solutions were stored in the dark at −20 °C. Working standard solutions and mixed internal standard working solutions in acetonitrile:water (50:50, v/v) were used for spiking samples. 0.05 M phosphate buffer solution was prepared by diluting 7.80 *g* NaH_2PO_4 in 1 L of HPLC water. The pH was adjusted to 8.5 with 10 M NaOH solution.

2.2. Sample preparation

Milk samples (2.00 ± 0.02 g) were weighed and fortified with mixed internal standard working solutions at levels corresponding to 10 μg/kg. Acetonitrile (6 mL) was added and the samples were shaken vigorously for 30 s, and centrifuged at 8603 × *g* for 10 min (Sigma 2K15, Germany). Then the samples were extracted again following the procedure mentioned above. Eight milliliters of acetonitrile saturated n-hexane was added and the n-hexane layer was discarded. The extracts were evaporated at 37 °C under nitrogen to approximately 1 mL (ca.1 h) and then mixed with 10 mL 0.05 M phosphate buffer at pH 8.5. After mixing, the pH of the extracts were re-adjusted to pH 8.5 using 0.2 M sodium hydroxide solution. The extracts were loaded onto the C_{18} cartridges pre-conditioned with 5 mL methanol, water, and 0.05 M phosphate buffer (pH 8.5). The cartridges were washed subsequently with 3 mL 0.05 M phosphate buffer (pH 8.5) and 2 mL water. The analytes were eluted with 3 mL acetonitrile and were evaporated to dryness under a gentle stream of nitrogen at 37 °C. The residue was dissolved in 1 mL of 0.1% formic acid–water:0.1% formic acid–acetonitrile (98:2, v/v) and vortexed for 1 min. The solution was filtered through a 0.2 μm filter (PALL, Washington, USA) and 10 μL solution was injected into the UPLC–MS/MS system.

2.3. UPLC–MS/MS analysis

Chromatographic analysis was performed using an Acquity UPLC system (Waters, Milford, MA, USA) and separations were achieved using an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm, Waters) at 30 °C. The analytes were separated with a mobile phase consisting of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) at a flow rate of 0.3 mL/min. The UPLC gradient conditions were optimized as follows: 0–1 min, 98% A; 1–1.5 min, 98–15% A; 1.5–2.5 min, 15% A; 2.5–3.0 min, 15–2% A; 3.0–3.5 min, 2–98% A; 3.5–5.5 min, 98% A.

Mass spectrometric analysis was carried out using a Quattro LC triple quadrupole tandem mass spectrometry (QuattroMicromass API, Manchester, UK) in positive electrospray ionization mode (ESI+). ESI parameters were as follows: capillary voltage, 2.8 kV; source temperature, 80 °C; desolvation temperature, 300 °C; cone gas flow, 25 L/h; desolvation gas flow, 460 L/h. Collision-induced dissociation was performed using argon as the collision gas at the pressure of 2.5×10^{-3} mbar in the collision cell. Optimized MS/MS parameters for amoxicillin and prednisolone were shown in Table 1.

2.4. Method validation

The validation procedure for the developed method was carried out according to the Commission Decision 2002/657/EC [26]. To evaluate the linearity, matrix-matched calibration curves were constructed at the concentrations of 2, 5, 10, 50, 100, 500 and 1000 μg/kg in blank milk samples on three different days. The correlation coefficient (*r*) was determined and had to fall within the range specified ($r \geq 0.99$). Limit of quantification (LOQ) was defined as the lowest drug concentration on the calibration curves. The limit of detection (LOD) was defined as the lowest measured concentration from which it was possible to deduce the presence of the analyte with reasonable statistical certainty. The criterion of the signal to noise (S/N) ratio of 3/1 was used in our study. The selectivity of the method was evaluated by comparing chromatograms of 20 blank matrix with spiking samples at the MRL level. The recovery study was estimated by spiking the samples in six replicates at each concentration level (0.5, 1, and 1.5 MRL). Responses of the spiked samples were compared with the response obtained for a blank matrix spiked after clean-up. The trueness, expressed as the difference between the measured concentration and the spiked concentration, had to be within −20% to +10%. The intra- and inter-day precision was evaluated at three concentration levels of the recovery study on a single day and on three different days, respectively. Commission Decision 2002/657/EC stated that the precision for quantitative methods lower than 100 μg/kg should be as low as possible. Adjusting the pH to 8.5 was evaluated in the perspective of robustness. The aqueous spiking sample extracts (1 μg/g) with three replicates under different pH conditions (4, 6, 7.5, 8.5, 9, and 10) were investigated their effect on analytes recovery.

Decision limit (CCα) was calculated by analyzing 20 blank milk samples fortified at the MRLs over three days, and using the concentration at the MRLs level plus 1.64 times the within-laboratory standard deviation (inter-day) obtained. Detection capability (CCβ) was calculated as CCα plus 1.64 times the corresponding standard deviation.

Stability of the analytes in matrix was evaluated at three concentration levels of the recovery study. The spiking milk samples were stored at ambient temperature for 1, 6, 12, and 24 h and at −20 °C freezer for at least six months. The mean concentration of each concentration level was compared to the corresponding concentration determined in the initial testing.

3. Results and discussion

3.1. Sample preparation

Simultaneous determination of amoxicillin, prednisolone, and sulbactam in bovine milk was designed at the beginning of the experiment. There was a problem when optimizing sample cleaning-up that was a necessary step to minimize the matrix effect. Experiments using cartridges such as SAX (Strong Anion Exchange), PSA (Primary Secondary Amine), C_{18} from Varian, Oasis HLB from Waters and Amino cartridges from Agela (Bonna-Agela Technologies, Tianjin, China) were investigated but had not obtained good effect. At last, sulbactam was confirmed to have a good retention on MAX cartridges from Waters. But they were not suitable for amoxicillin and prednisolone.

Sample preparation was the most critical section because amoxicillin and prednisolone possessed different physicochemical properties ($\log K_{ow}$, pK_a, etc.). The amphoteric, instability and high polarity characteristics of amoxicillin make its analysis difficult, which pK_a value was 2.4, 7.4 and 9.6 [27]. As prednisolone was a neutral analyte not affected by pH adjustment, it was extracted together with amoxicillin into the organic phase. Different pretreatment steps were studied, in an attempt to find the most appropriate extractant. Sulfuric acid and sodium tungstate were initially selected for deproteinization, but this technique resulted in the pH instability of the extracts so that it affected the retention of the analytes on C_{18} cartridges. Then simple deproteination by trichloroacetic acid and trifluoroacetic acid was found to be used for prednisolone, but the recovery of amoxicillin was only about

Table 1
Optimized MS/MS parameters for amoxicillin and prednisolone.

Analyte	Precursor ion	Product ions	Dwell time (s)	Cone voltage (eV)	Collision energy (V)
Amoxicillin	366.1	349.1[a]	0.10	18	10
		208.2	0.10	18	9
Prednisolone	361.1	343.1[a]	0.10	20	13
		147.1	0.10	20	15
Penicillin G-d_7	342.4	160.1[a]	0.10	20	12
Prednisolone-d_6	367.5	150.3[a]	0.10	16	20

[a] Ion used for quantification.

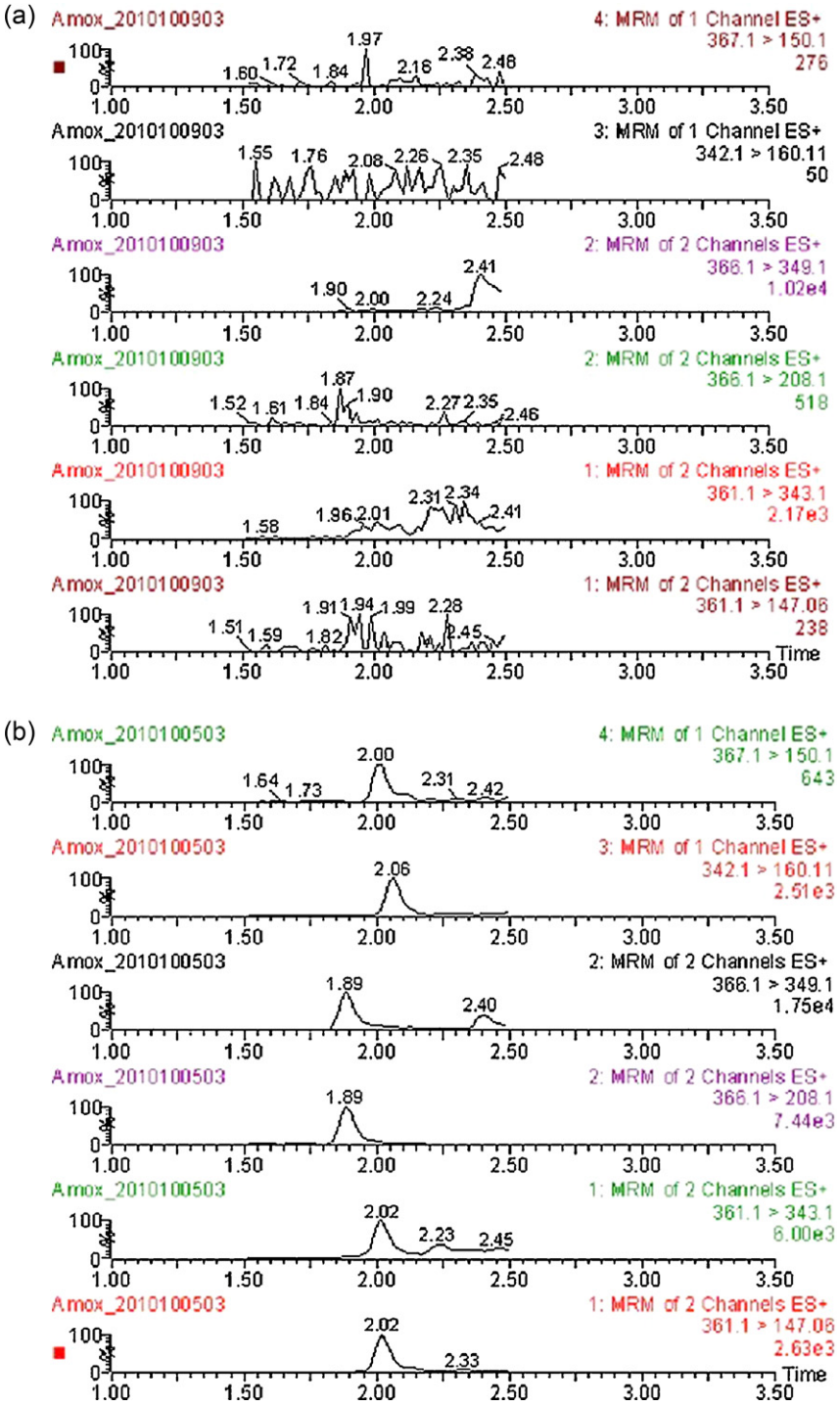

Fig. 1. MRM chromatograms of amoxicillin and prednisolone in bovine milk. (a) Blank milk sample; (b) Spiking milk sample with amoxicillin (2 μg/kg), prednisolone (2 μg/kg), penicillin G-d_7 (10 μg/kg), and prednisolone-d_6 (10 μg/kg).

Table 2
Trueness, mean recovery, intra- and inter-day precision, CCα and CCβ of amoxicillin and prednisolone in bovine milk.

Analyte	CCα (μg/kg)	CCβ (μg/kg)	Fortification level (μg/kg)	Trueness (%)	Mean recovery (%)	Intra-day precision (RSD%)[a]	Inter-day precision (RSD%)[b]
Amoxicillin	4.2	5.4	2	93.4	92.3	3.8	10.8
			4	90.1	91.6	2.6	6.5
			6	88.3	89.2	6.4	7.3
Prednisolone	7.8	9.6	3	101.2	102.3	3.4	12.1
			6	98.3	99.2	4.1	9.6
			9	96.4	98.7	4.3	8.2

[a] RSD values of intra-day precision are calculated at each concentration level ($n = 6$).
[b] RSD values of inter-day precision are calculated at each concentration level on three different days ($n = 18$).

35%, probably due to the degradation of amoxicillin in the low pH condition. Nevertheless, deproteination and extraction by acetonitrile gave satisfactory results for both amoxicillin and prednisolone.

3.2. MS/MS conditions

MS/MS parameters were obtained by infusing a standard solution of 1 μg/mL in a mixture of acetonitrile:water (50:50, v/v). Then the response of the analytes was estimated at different source temperature conditions (75, 80, 85, 90, 100, and 110 °C) to optimize tuning parameter. Source temperature was mainly affected by the sample solvent. Results indicated that low source temperature could lead to incomplete solvent evaporation, thus affect the electrospray ionization efficiency of the analytes. In contrary, when the ions were from atmospheric pressure into the vacuum at higher source temperature condition, the decreased temperature would result in endothermic expansion of the ions. Hence, source temperature was set at 80 °C.

3.3. Method validation

Twenty blank milk samples (collected from local dairy farms) were analyzed to verify the selectivity of the proposed methods. Selectivity was found to be satisfactory, with no endogenous interference was observed (Fig. 1). To calibrate the curves, good linearity ($r > 0.9993$) was observed over the range of 2–1000 μg/kg for the analytes. The LOQs and LODs were 2 μg/kg and 0.5 μg/kg for the analytes. The trueness, mean recovery, intra- and inter-day precision, CCα and CCβ obtained from spiking samples at three fortification levels (0.5, 1, and 1.5 MRL) were summarized in Table 2. Effect of different pH of the aqueous sample extracts spiking 1 μg/g working standard solutions on the recovery of the analytes were evaluated in the perspective of robustness, as shown in Fig. 2. It was concluded that a good retention for amoxicillin was obtained at pH 8.5, while pH conditions had little influence on the recovery of prednisolone.

Stability of the analytes in milk samples at ambient temperature and at −20 °C freezer was summarized in Table 3. With regard to the stock solution stability, there was no little loss for amoxicillin after storage at room temperature for 8 h or at 4 °C for 20 days [28]. And acceptable stability (≥95%) was obtained for prednisolone in methanol at both ambient temperatures and −10 to −30 °C conditions [18].

3.4. Sample analysis

The developed analytical method was successfully applied to the determination of amoxicillin and prednisolone in 20 bovine milk samples obtained from local dairy farms. Six samples presented

Table 3
Stability of amoxicillin and prednisolone in matrix ($n = 6$).

		Analyte					
		Amoxicillin			Prednisolone		
Fortifical concentration (μg/kg)		2	4	6	3	6	9
Ambient temperature (18 °C)							
1 h	Accuracy[a] (%)	101.2	102.1	100.5	102.1	99.4	100.3
	RSD[a] (%)	3.4	2.8	1.6	2.6	3.1	2.2
6 h	Accuracy (%)	99.1	97.5	98.4	101.4	101.2	100.1
	RSD (%)	1.8	1.6	2.9	1.5	3.1	3.4
12 h	Accuracy (%)	95.3	94.2	93.7	100.3	99.3	99.8
	RSD (%)	1.2	2.4	3.1	1.6	2.4	3.1
24 h	Accuracy (%)	90.4	89.1	89.6	99.8	99.1	99.4
	RSD (%)	1.1	2.5	2.1	3.1	2.5	1.0
−20 °C freezer							
1 weeks	Accuracy (%)	102.1	103.6	99.7	101.9	100.6	99.9
	RSD (%)	2.6	4.5	2.2	3.1	3.2	1.5
2 weeks	Accuracy (%)	101.1	101.3	99.1	100.3	99.9	99.6
	RSD (%)	1.7	1.1	2.1	2.2	2.3	2.8
3 weeks	Accuracy (%)	97.3	96.4	95.3	99.9	99.6	99.1
	RSD (%)	3.1	2	2.7	2.2	1.8	1.9
4 weeks	Accuracy (%)	95.6	95.2	93.4	99.3	99.5	101.1
	RSD (%)	1.6	1.7	2.1	3.1	2.6	2.9
12 weeks	Accuracy (%)	85.4	88.3	82.1	98.4	99.1	97.3
	RSD (%)	3.2	2.6	1.8	1.5	2.8	3.1
24 weeks	Accuracy (%)	78.2	75.1	74.1	97.5	98.2	98.1
	RSD (%)	2.1	1.6	1.8	1.7	2.3	2.2

[a] Accuracy and RSD are calculated at each concentration level ($n = 6$).

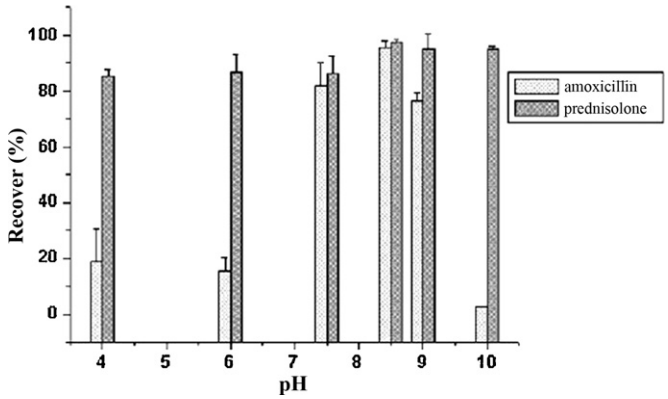

Fig. 2. Effect of different pH of the aqueous sample extracts spiking 1 μg/g standard solutions on the recovery of the analytes in the perspective of robustness. Error bars represent the standard deviation ($n = 3$).

traces of amoxicillin and only one sample was found to be positive with respect to prednisolone. The concentration of prednisolone in the positive sample was 2.56 μg/kg, which did not exceed the MRL (6 μg/kg) set by China. Three positive amoxicillin samples, whose concentrations were 21.86, 13.61, and 10.34 μg/kg, respectively, exceeded the MRL (4 μg/kg) set by China. The concentrations of the other three incurred samples were lower than the corresponding MRL. After investigation, we found that some dairy cows were intramammary administered Synulox LC (Pfizer, USA) three days ago before milk samples were collected. Synulox LC, a combination drug that comprising amoxicillin (200 mg), clavulanic acid (50 mg), and prednisolone (10 mg) in a 3 g syringe, was widely used for treatment of bovine mastitis in China's dairy farms in recent years.

4. Conclusions

The developed UPLC–MS/MS method was applied to the simultaneous quantitative analysis of amoxicillin and prednisolone in bovine milk samples. The extraction method is based on simple liquid-liquid extraction with acetonitrile and n-hexane defatting, followed by C_{18} cartridges clean-up minimizing the matrix effect. Furthermore, the use of UPLC–MS/MS reduces analytical time with short total run time (5.5 min), and improves sensitivity and resolution. The method is rapid and sensitive with LOQs of 2 μg/kg and LODs of 0.5 μg/kg for amoxicillin and prednisolone.

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