Determination of sulbactam in raw bovine milk by isotope dilution–ultra-high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

We developed a sensitive and selective isotope dilution ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method for the determination of sulbactam residue in raw bovine milk. Sulbactam and internal standard, sulbactam-d₅, were extracted from raw bovine milk via liquid-liquid extraction and enriched with strong anion exchange solid-phase extraction cartridges and finally analyzed by using UPLC-MS/MS with multiple reaction monitoring mode. The method was validated according to European regulations. The calibration curve showed good linearity, with a correlation coefficient of 0.9998. Decision limit and detection capability of sulbactam were determined by matrix calibration curve and were 0.0445 and 0.0517 µg/L, respectively. The recoveries of sulbactam in fortified raw bovine milk ranged from 72.1 to 91.5%, with the intra- and interday relative standard deviations ranging from 3.0 to 18.9%. Furthermore, the developed method was applied to analyzing real raw bovine milk samples collected from dairy farms in Beijing, China. Sulbactam was not determined in all samples. The proposed method could ultimately serve as a methodological foundation for the determination of sulbactam in different types of raw milk and dairy products.

Key words: anion exchange, isotope dilution, raw bovine milk, sulbactam, ultra-high-performance liquid chromatography–tandem mass spectrometry

INTRODUCTION

Sulbactam, a β -lactamase inhibitor, is chemically a penicillanic acid sulfone that shows particular activity

against class A enzymes and irreversibly inhibits the hydrolytic activity of β -lactamases (Akova, 2008). In addition, sulbactam has been commercially available in combination with β -lactam antibiotics to extend their spectrum of activity (Akova, 2008; Li et al., 2014). However, when the β -lactam antibiotics were used in excess, β -lactamase, an enzyme able to hydrolyze the β -lactam antibiotics (Frère et al., 1991), might be used in the antibiotic-containing milk to prevent the antibiotics from exceeding the maximum residue limits in bovine milk (European Union, 2010). Meanwhile, sulbactam might also be added to the β -lactamase-containing milk to avoid the determination of β -lactamase. According to the literature (Monaco et al., 2014; Rossolini et al., 2014; World Health Organization, 2014; Docquier and Mangani, 2018), the excessive use of antibiotics can cause a potential hazard for human beings, such as antimicrobial resistance, one of the greatest threats to global health. Thus, to keep β -lactam antibiotics and β -lactamase under regulation and ensure the safety of bovine milk, it is necessary to develop a highly selective and sensitive analytical method for the determination of sulbactam in bovine milk.

Several studies have reported various analytical methods for the determination of sulbactam in pharmaceutical formulations (Mahgoub and Aly, 1998; Aly et al., 2000; Pajchel et al., 2002; Blanco Gomis et al., 2003; Dinç and Baleanu, 2006; Hoang et al., 2014; Marwada et al., 2014) and human plasma and serum (Guillaume et al., 1995; Aly et al., 2000; Carlier et al., 2012); however, few methods have been developed for the determination of sulbactam in foodstuff (Li et al., 2014). The methods reported in previous studies included capillary electrophoresis (Pajchel et al., 2002), UV spectrophotometry (Mahgoub and Aly, 1998; Hoang et al., 2014; Marwada et al., 2014), fractional wavelet transform (Dinç and Baleanu, 2006), chemiluminometric (Aly et al., 2000), HPLC (Guillaume et al., 1995; Blanco Gomis et al., 2003), and ultra-high-performance liquid chromatography–tandem mass

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spectrometry (UPLC-MS/MS; Carrier et al., 2012; Li et al., 2014). Although the method outlined by Li and colleagues (2014) had been reported to determine sulbactam in bovine milk, we still hoped to optimize the mentioned method to achieve better performance by (1) using organic solvents in our study to extract analytes and precipitate proteins without adjusting the pH value of the extraction solution; (2) lowering the method's detection limit than the previous study by replacing high volumes of elute with lower volumes of solvent after an solid-phase extraction (SPE) procedure; or (3) applying the isotope dilution method to the quantitative analysis so that the analysis in a complex matrix could overcome difficulties. In this work, to make extraction easier, methanol/acetonitrile (vol/vol, 1:3) was used to precipitate proteins without adjusting the pH value of the final extraction solution. However, the use of organic solvents for both deproteinization and extraction might result in a high fat-containing extract and other interferences (Li et al., 2014). Toward this end, anion exchange SPE would be performed to further remove the fat and other matrix interferences and to enrich the analytes. Meanwhile, high volumes of elute collected from the SPE procedure would be replaced with lower volumes of solvent so as to obtain a lower detection limit. As mentioned in a previous study (Li et al., 2013), the isotope dilution technique could overcome difficulties with quantification caused by the matrix effect and achieve high accuracy and precision with a lower detection limit. Thus, in our study, the isotope dilution technique would enable the integration of mass spectrometric detection.

MATERIALS AND METHODS

Materials

Sulbactam standard (>99.4% purity) and sulbactam- d_5 internal standard were obtained from USP-China (Shanghai, China) and Toronto Research Chemicals (Toronto, Canada), respectively. The chemical structure of sulbactam is shown in Figure 1.

Methanol and acetonitrile (LC-MS grade) were obtained from Merck (Darmstadt, Germany). Formic acid, acetic acid, and acetone were purchased from Tedia (Fairfield, OH). Sodium acetate was purchased from Sigma Aldrich (St. Louis, MO). The ultrapure water was deionized and purified to 18.2 M Ω by a Milli-Q system (Millipore, Burlington, MA). The Oasis MAX SPE (150 mg, 6 mL) cartridges were purchased from Waters Corporation (Milford, MA). Alumina-N SPE (2 g, 6 mL) and Florisil SPE (2 g, 6 mL) columns were obtained from Supelco (Bellefonte, PA). Filter mem-

branes (0.22 μ m) used for filtering the extracts were provided by Waters Corporation.

The sodium acetate buffer (50 mM) was prepared by diluting 4.10 g of sodium acetate in 1,000 mL of ultrapure water. The pH was adjusted to 7.0 with acetic acid using an Orion 710A pH/ISE meter (Beverly, MA). The buffer was stored at 4°C and kept stable for 1 wk.

Individual stock solutions of sulbactam (1,000 mg/L) and sulbactam- d_5 (500 mg/L) were prepared in acetonitrile/ultrapure water (vol/vol, 1:1) and stored at -18°C. The analytical working solutions were prepared by diluting individual stock solutions with acetonitrile/ultrapure water (vol/vol, 1:1) before use.

Extraction and Clean-Up

Five milliliters of raw bovine milk sample was added to a 50-mL centrifuge tube combined with sulbactam- d_5 (0.5 μ g/L) and the sample was vortexed for 30 s. Fifteen milliliters of methanol/acetonitrile (vol/vol, 1:3) was then added to the centrifuge tube. The mixture was vigorously shaken and vortexed for 30 s and then centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was then transferred to another centrifuge tube. Afterward, another 10 mL of methanol/acetonitrile (vol/vol, 1:3) was added to the tube and the extraction

Figure 1. The chemical structure of sulbactam.

was repeated accordingly. The supernatant was collected and merged with the previous one. The extraction solution was then concentrated under a gentle nitrogen gas stream at 30°C to approximately 4 to 5 mL.

The concentrates in the centrifuge tube were loaded onto the Oasis MAX SPE cartridges preconditioned with 5 mL of methanol and ultrapure water each. The analyte-containing cartridges were rinsed with 3 mL of 50 mM sodium acetate buffer (pH = 7.0), 3 mL of acetone, and 3 mL methanol in succession and then dried under a vacuum for 2 min. The analytes were eluted with 5 mL of a mixture containing 98% (vol/vol) of methanol and 2% (vol/vol) formic acid. The collected elute was dried under a gentle stream of nitrogen gas at 30°C. Finally, the analytes were dissolved in 200 µL of acetonitrile/ultrapure water (vol/vol, 1:1) and were finally filtered through a 0.22-µm filter membrane before UPLC-MS/MS analysis.

UPLC-MS/MS Analysis

Chromatographic separation was performed on a Waters Acquity UPLC I-Class system consisting of a vacuum degasser, an autosampler, and a binary pump (Waters Corporation) equipped with a 2.1 × 100 mm HSS T3 column, 1.8-µm particle size (Waters Corporation). A gradient program was used with the mobile phase, combining solvent A (ultrapure water and formic acid, 0.1%, vol/vol) and solvent B (acetonitrile) as 10% B (initial), 95 to 50% A (0–3.0 min), 50 to 5% A (3.0–3.1 min), 5% A (3.1–5.0 min), and 5 to 95% B (5.0–5.1 min). The flow rate was 0.4 mL/min and the injection volume was 5 µL. Moreover, the oven and pool temperature were maintained at 40 and 4°C, respectively.

This UPLC system was coupled with a triple quadrupole mass spectrometry (Waters Xevo TQ-S) equipped with an electrospray interface operating in negative ion mode, using the following operation parameters: capillary voltage 3.0 kV, desolvation temperature 450°C, desolvation gas flow 800 L/h, and cone gas flow 150 L/h. The MS detection was operated at multiple reaction monitoring mode and signals were processed by Masslynx 4.1 software (Waters Corporation).

RESULTS AND DISCUSSION

Optimization of UPLC-MS/MS Conditions

First, the effects of different UPLC columns on the target analytes retentions were studied. Two kinds of columns, namely Waters Acquity BEH C18 (100 mm × 2.1 mm, 1.7 µm) and Waters Acquity HSS T3 (100 mm × 2.1 mm, 1.8 µm), were investigated. The HSS T3 particle technology is derived from C18 ligands and proprietary end-capping to achieve optimum characteristics suited for highly polar compound retention; therefore, Waters Acquity HSS T3 column enabled strong retention of highly polar compounds, such as sulbactam. We compared the retention performances and ionization efficiency for sulbactam [10 µg/L in acetonitrile/ultrapure water (vol/vol, 1:1)] between the 2 columns with a mobile phase consisting of acetonitrile and formic acid (0.1%, vol/vol) buffer. Compared with Waters Acquity BEH C18, Waters Acquity HSS T3 column showed excellent retention, peak shape (Supplemental Figure S1A; <https://doi.org/10.3168/jds.2018-16187>) and peak area (Supplemental Figure S1B; <https://doi.org/10.3168/jds.2018-16187>) of sulbactam. Therefore, the HSS T3 column was used for further studies.

Given that the mobile phase also played a critical role in retention and ionization efficiency, the influence of different aqueous mobile phases, ultrapure water and formic acid (0.1%, vol/vol) buffer, was studied. Formic acid (0.1%, vol/vol) buffer showed better performance for peak shape (Supplemental Figure S2A; <https://doi.org/10.3168/jds.2018-16187>) and peak area (Supplemental Figure S2B; <https://doi.org/10.3168/jds.2018-16187>) of sulbactam [10 µg/L in acetonitrile/ultrapure water (vol/vol, 1:1)] than ultrapure water. Thus, formic acid (0.1%, vol/vol) buffer was selected as the aqueous mobile phase in our study.

The parent ions $[M-H]^-$, listed in Table 1, were observed by injecting 0.5 µg/mL of individual standard solutions. Based on the confirmation of analytes, more than 2 daughter ions for each precursor ion should be obtained for the low-resolution MS analysis according to the guideline of Commission Decision 2002/657/EC (European Commission, 2002). The optimum values

Table 1. Multiple reaction monitoring parameters of sulbactam and sulbactam-d₅ in ultra-high-performance liquid chromatography–tandem mass spectrometry

Compound	Retention time (min)	Molecular weight (Da)	Precursor ion (<i>m/z</i>)	Collision energy (eV)	Daughter ions (<i>m/z</i>)
Sulbactam	1.67	233	232	24, 14	140 ^a , 64
Sulbactam-d ₅	1.67	238	237	11	193 ^a

^aMeans ions used for quantification.

Table 2. The performance characteristics of the developed method for the determination of sulbactam in raw bovine milk

Compound	Concentration range ($\mu\text{g/L}$)	Regression equation	r	$\text{CC}\alpha^1$ ($\mu\text{g/L}$)	$\text{CC}\beta^2$ ($\mu\text{g/L}$)
Sulbactam	0.5–10.0	$y = 1.9395x + 0.0142$	0.9996	0.0445	0.0517

¹Decision limit.²Detection capability.

of daughter ions and their collision energies were also shown in Table 1.

Optimization of SPE Clean-Up

The SPE was used to further remove the matrix interferences and to enrich the extracts. Several kinds of SPE cartridges commonly used were evaluated, including Alumina-N, Florisil, and Oasis MAX. Figure 2 shows that, after clean-up by the Alumina-N and Florisil cartridges, approximately 5% sulbactam [10 $\mu\text{g/L}$ in acetonitrile/ultrapure water (vol/vol, 1:1)] was detected in the eluate from the eluting procedure. Due to the fact that both Alumina and Florisil are polar sorbents, the poor recoveries might result from the highly polar nature of acetonitrile, which interferes with the retention of polar analytes, such as sulbactam, on the polar sorbents of cartridges. However, in the previous study (Campone et al., 2018), good recoveries of ochratoxin A, an acidic compound, were observed by the use of Oasis MAX cartridges (mixed-phase SPE sorbents containing reversed phase and anion exchange material). Therefore, Oasis MAX cartridges were selected to improve the recovery in this work. As we expected, Oasis MAX cartridge obtained good recoveries ($\geq 93.5\%$) of sulbactam at all fortified concentration levels in Figure 2.

Figure 2. Comparison of recoveries of sulbactam with 3 different candidate cartridges: Oasis MAX (150 mg, 6 mL; Waters Corp., Milford, MA), Alumina-N (2 g, 6 mL; Supelco, Bellefonte, PA), and Florisil (2 g, 6 mL; Supelco). Error bars represent the average recovery.

Method Validation

The isotope dilution method could serve as an effective method to compensate for the loss of analytes during the extraction and clean-up procedure and to overcome difficulties with quantification caused by the matrix interference during trace-level analysis. Thus, isotope dilution-UPLC-MS/MS was applied for the quantitative analysis in this work. Linearity of the analytical method was verified using the matrix calibration curves for sulbactam at 5 different concentration levels (0.5, 1, 2, 5, and 10 $\mu\text{g/L}$). Standard solutions were prepared in sulbactam-free milk samples and 10 $\mu\text{g/L}$ of sulbactam- d_5 was added. Table 2 shows the matrix calibration parameters, including linear range, linear equation, and correlation coefficient; we found good linearity of the analytical response, with a correlation coefficient of 0.9998. Figure 3 shows the typical chromatogram of the blank raw milk sample fortified at the lowest level (0.5 $\mu\text{g/L}$) of sulbactam.

Method sensitivity was established by measuring the values of the decision limit ($\text{CC}\alpha$) and detection capability ($\text{CC}\beta$) of sulbactam, which were determined by

Figure 3. Chromatograms of the blank raw bovine milk sample spiked at lowest level (0.5 $\mu\text{g/L}$) of sulbactam.

the matrix calibration curve procedure according to ISO 11843 (International Organization of Standardization, 2000). Briefly, the blank milk samples were fortified at 5 levels, ranging from 0.05 to 0.15 $\mu\text{g/L}$ (Table 3), and 0.5 $\mu\text{g/L}$ of sulbactam- d_5 solution were also added into the samples. After analysis and identification, the matrix calibration curves were developed to calculate $\text{CC}\alpha$ and $\text{CC}\beta$ values. The $\text{CC}\alpha$ was equal to the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory repeatability of the intercept. The $\text{CC}\beta$ was equal to the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory repeatability of the mean measured content at the decision limit (European Commission, 2002). Table 2 showed $\text{CC}\alpha$ and $\text{CC}\beta$ values for sulbactam (0.0445 and 0.0517 $\mu\text{g/L}$, respectively). These values were lower than the method developed in the previous work (0.3 $\mu\text{g/L}$ for sulbactam) for analyzing sulbactam in bovine milk samples (Li et al., 2014). Compared with the previous study, we noted 2 reasons for the good performance of the method developed in our study with lower $\text{CC}\alpha$ and $\text{CC}\beta$ values. First, after the extraction and clean-up procedures, the extracts were dissolved in 200 μL of acetonitrile/ultrapure water (vol/vol, 1:1), which was smaller than previously reported method (1 mL). Due to the smaller final volume, we obtained better sensitivity. Second, to achieve good accuracy and precision with the smaller final volume in our study, the isotope dilution technique was introduced to compensate for the variation of the final volumes and check the instrumental performance through use of relative response. Therefore, the robustness of proposed method was validated and could be used to determine low levels of sulbactam in raw bovine milk samples.

The accuracy and precision of the method were evaluated by analyzing the raw bovine milk samples ($n = 5$ for intraday test, $n = 3$ for interday test) fortified at 5 levels ranging from 0.05 to 0.15 $\mu\text{g/L}$ (Table 3). As shown in Table 3, the average recoveries for sulbactam ranged from 72.1 to 91.5% and the repeatability given as intra- and interday relative SD ranged from 3.0 to

18.9%, indicating the good accuracy and precision of the proposed method.

Finally, the developed method was applied for the determination of sulbactam in 10 raw bovine milk samples (2 replicates for each sample) that were randomly collected from 2 dairy farms in Beijing, China. However, sulbactam was not determined in all samples.

CONCLUSIONS

We developed a more sensitive isotope dilution-UPLC-MS/MS method for the determination of sulbactam in raw bovine milk samples compared with previous studies. Isotope dilution and anion exchange SPE techniques were applied to achieve good performance using trace-level sample matrices. To the best of our knowledge, this was the first report for the analysis of sulbactam in raw bovine milk by anion exchange SPE-isotope dilution-UPLC-MS/MS. The proposed method was verified by $\text{CC}\alpha$, $\text{CC}\beta$, accuracies, precisions, and recoveries according to the European Commission Decision 2002/657/EC. The $\text{CC}\alpha$ and $\text{CC}\beta$ values for sulbactam were 0.0445 and 0.0517 $\mu\text{g/L}$, respectively, which were lower than the previous method. Furthermore, our findings will be useful for the analysis of sulbactam in different types of raw milk and dairy products.

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Table 3. Recoveries and precision of sulbactam in fortified raw bovine milk

Fortified levels of sulbactam ($\mu\text{g/L}$)	Intraday ($n = 5$)		Interday ($n = 3$)	
	Recovery (%)	Relative SD (%)	Recovery (%)	Relative SD (%)
0.050	91.5	5.1	83.9	8.2
0.075	77.5	15.4	75.4	6.1
0.100	82.5	10.7	80.6	3.0
0.125	74.8	12.6	74.8	3.1
0.150	72.1	18.9	76.2	8.2

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