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Simultaneous determination of clenbuterol, chloramphenicol and diethylstilbestrol in bovine milk by isotope dilution ultraperformance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A simple and sensitive analytical method was developed for the simultaneous determination of clenbuterol, chloramphenicol and diethylstilbestrol in bovine milk by isotope dilution ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). Samples were directly purified through HLB cartridge. The organic phase was dried under nitrogen and residues were redissolved in mobile phase. Samples were analyzed by UPLC–MS/MS on an Acquity UPLC® BEH C_{18} column with gradient elution. The samples were quantified using clenbuterol-D_9, chloramphenicol-D_5 and diethylstilbestrol-D_8 as internal standards. The proposed method 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 clenbuterol, chloramphenicol and diethylstilbestrol in bovine milk. The total time required for the analysis of one sample was about 50 min.

1. Introduction

Milk is a child's most important food in the first year of life and is still very important in the next few years. More and more children and adults drink bovine milk or milk products everyday for their health in China now. It is therefore very important to keep milk safe.

Veterinary drugs are administered to food-producing animals in order to prevent and to treat several types of pathologies, to shorten feeding time and to abate the risk of losses. However an illegal or unsuitable drug use can increase the risk of introducing harmful residues into the human food chain. Veterinary drug residues in aquatic products, eggs and milk products, etc. are much more prevalent and risky especially in developing countries. Clenbuterol (CLEN) is β_2-agonists, originally used in the therapeutic treatment of asthma and preterm labor in humans [1]. However, the compound is also misused as nutrient repartitioning agent in livestock by diverting nutrients from fat deposition in animals to the production of muscle tissues [2]. This misuse had caused some severe accidental poisonings in humans [3,4]. CLEN is banned as feed additives for growth promotion in animal production in China and EU [5,6]. Chloramphenicol (CAP) is an effective antibiotic that has widely been used since the 1950s to treat food-producing animals. Because of the well-known risk of aplastic aemia and carcinogenic properties of CAP, its use had been banned in food-producing animals since 1994 in EU [7]. Diethylstilbestrol (DES) was the first synthetic estrogen that was used to prevent miscarriages prior to 1971 and that often is used for clinical therapy. It was also used illegally as a growth promoter in cattle and calves to increase the weight gain of animals [8]. The use of DES has been banned since 1981 in EU because of its teratogenic, mutagenic, and carcinogenic properties [9]. So, it is quite necessary to control their abuse.

To protect consumer health, the EU has established the maximum residue limit (MRL) of 0.05 ng/g in bovine milk for CLEN and the minimum required performance limit (MRPL) of 0.30 ng/g for CAP in food of animal origin and MRPL of 0.50–2.0 ng/g for DES in food of animal origin [10–12]. Therefore, specific and sensitive methods for the identification and quantification of these compounds in milk are required. To identify these compounds in biological samples, gas chromatography–mass spectrometry (GC–MS) [13–20] and liquid chromatography–mass spectrometry (LC–MS or LC–MS–MS) [21–27] methods have been developed. However, GC–MS (MS/MS) requires time-consuming derivatization steps to enhance the volatility of analytes, which might produce interferences and consequently make more difficult quantitation.

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LC–MS/MS has become the main analytical technique for determining β_2-agonists due to its shorter chromatographic run time and without time-consuming derivatization procedures.

Although many methods have been developed for screening and identification of these three compounds in biological samples, there is little literature on simultaneous determination of these three compounds in biological samples. A method for simultaneous detection of CLEN, CAP and 17-beta-estradiol has been developed by Liu et al. with suspension array technology [28]. The limits of detection (LODs) were 0.04, 0.05 and 1.0 ng/mL for CAP, CLEN and 17-beta-estradiol, respectively. The LOD for CLEN is not enough to determine CLEN in milk. Moreover, the method only could be used for screening CAP, CLEN and 17-beta-estradiol. So far, a confirmatory method for simultaneously determine CLEN, CAP and DES in milk by LC–MS/MS with good sensitivity has not been developed.

In this paper, a simple and sensitive UPLC–MS/MS method for simultaneous determination of CLEN, CAP and DES in milk with isotope dilution was developed. 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.

2. Materials and methods

2.1. Materials and reagents

Methanol, acetonitrile and ammonium acetate were LC grade. Hexane, ethyl acetate and ammonium hydroxide were analytical grade. Clenbuterol, chloramphenicol, diethylstilbestrol, clenbuterol-D_9 (CLEN-D_9) (100 μg/mL in acetone), and chloramphenicol-D_5 (CAP-D_5) (100 μg/mL in acetone) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Diethylstibestrol-D_8 (DES-D_8) was purchased from Cambridge Isotope Lab. Inc (Andover, MA, USA). The OASIS® HLB SPE cartridges (60 mg, 3 mL) were purchased from Waters (Milford, MA, USA). Water was purified with a Milli-Q reverse osmosis system (Millipore, Milford, MA, USA).

2.2. Standard solutions

Individual stock solutions of CLEN, CAP, DES and DES-D_8 (100 μg/mL) were prepared in methanol. One fortifying mixed standard solution of CLEN (5.0 ng/mL), CAP (30 ng/mL) and DES (50 ng/mL) and one mixed fortifying standard solution for CCβ experiment (5.4 ng/mL for CLEN, 0.70 ng/mL for CAP and 4.0 ng/mL for DES) were prepared by diluting stock standard solution with methanol. An internal working mixed standard solution of CLEN-D_9 (25 ng/mL), CAP-D_5 (100 ng/mL) and DES-D_8 (100 ng/mL) was prepared in methanol. Six individual working standard solutions (1000 ng/mL for CLEN, CLEN-D_9, CAP and CAP-D_5, 5000 ng/mL for DES and DES-D_8) for MS-MS optimization were prepared by diluting each stock solution with 5 mM ammonium acetate solution/acetonitrile (90:10, v/v). Six mixed working standard solutions (0.2–20 ng/mL for CLEN, 1.2–100 ng/mL for CAP and 2.0–200 ng/mL for DES) were prepared by diluting and mixing working standard solutions (1000 ng/mL for CLEN, CLEN-D_9, CAP and CAP-D_5, 5000 ng/mL for DES and DES-D_8) with 5 mM ammonium acetate solution/acetonitrile (90:10, v/v).

2.3. Chromatographic conditions

A Waters Acquity UPLC instrument (Milford, MA, USA) was used in the present study. Separation was carried out on an Acquity BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm) maintained at 30 °C. The mobile phase consisted of solvent A (5 mM ammonium acetate solution) and solvent B (acetonitrile). Initial gradient conditions were set to 10% B and held for 1.5 min before incorporating a linear gradient increasing to 80% B at 4.5 min. At 4.6 min the gradient was programmed to initial conditions to reequilibrate the column for 2.4 min (total run time 7 min). The flow rate was 0.30 mL/min. The injection volume was 10 μL in full loop injection mode.

2.4. Mass spectrometry conditions

Detection was carried out by a Waters Xevo™ TQ triple-quadrupole MS fitted with electrospray ionization (ESI) probe operated in the positive and negative ion mode. The following parameters were optimal: capillary voltage, 3000 V; ion source temperature, 150 °C; desolvation gas temperature, 500 °C; desolvation gas flow rate, 1000 L/h. Detection was carried out in multiple reaction monitoring (MRM) mode. Argon was used as the collision gas, and the collision cell pressure was 4 mBar. For CLEN, $[M+H]^+$ was chosen as precursor ion. For CAP and DES, $[M-H]^-$ was chosen as precursor ion. Their daughter ions and these ions's collision energy and cone voltage were chosen by Waters IntelliStart software. Other parameters are shown in Table 1.

2.5. Sample preparation

After addition of 100 μL of internal standard solution (25, 100 and 100 ng/mL for CLEN-D_9, CAP-D_5 and DES-D_8, respectively) in 10 g milk, the sample was applied to OASIS® HLB 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 15 mL of ethyl acetate. The eluate was evaporated to dryness in a water bath at 40 °C under nitrogen and reconstituted in 500 μL of 5 mM ammonium acetate solution/acetonitrile (90:10, v/v). The resulting solution was filtered through 0.22 μm filter and 10 μL of the filtrate was injected into the UPLC.

2.6. Method validation

The evaluation of the suitability of the method for the determination of CLEN, CAP and DES in milk was carried out according to the European Commission Decision 2002/657/EC [29].

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 0.2, 0.5, 1.0, 5.0, 10 and 20 ng/mL for CLEN, 1.2, 3.0, 6.0, 20, 50 and 100 ng/L for CAP and 2.0, 5.0, 10, 25, 100 and 200 ng/mL for DES (only *trans*-DES was calculated). The concentration of internal standard was 5.0 ng/mL for CLEN-D_9 and 20 ng/L for CAP-D_5 and DES-D_8 (only *trans*-DES-D_8 was calculated).

For CLEN, the CCα was established by analyzing 24 blank materials per matrix fortified with CLEN at 0.05 ng/g. The CCβ was established by analyzing 20 blank materials per matrix fortified with CLEN at their CCα.

For CAP and DES, the CCα was established by analyzing 24 blank materials per matrix to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise (S/N) ratio was used as CCα (for DES, only S/N ratio of *trans*-DES was calculated). The CCβ was established by analyzing 20 blank materials per matrix fortified with the analyte at their CCα.

Recovery (trueness) of CLEN, CAP and DES were measured in blank milk that was fortified at MRL level of CLEN (0.05 ng/g), MRPL level of CAP (0.30 ng/g) and MRPL level of DES (0.50 ng/g). The forti-

Table 1
LC–ESI-MS/MS parameters for CLEN, CAP, DES, CLEN-D_9, CAP-D_5 and DES-D_8.

Analyte	Precursor ion (*m*/*z*)	Daughter ion (*m*/*z*)	Dwell time (s)	Collision energy (eV)	Cone voltage (V)
CLEN	277.0	202.8[a], 131.7	0.05	16, 28	22
CAP	321.0	151.8, 257.0[a]	0.25	18, 12	24
DES	267.2	222.1, 237[a]	0.50	35, 28	43
CLEN-D_9	286.0	204.0	0.05	16	22
CAP-D_5	326.0	257.0	0.25	12	24
DES-D_8	275.2	245.0	0.50	28	43

[a] Ion for quantification.

fied samples were analyzed and the recoveries were calculated by comparing the measured concentration to the fortified concentrations. The repeatability and within-laboratory reproducibility were measured on the same 18 fortified blank milk samples (analyzed in three independent analytical runs) and expressed by coefficient of variation (CV_r and CV_R, respectively).

The stability was determined in two different ways: (a) in solvent (stock solutions) by UPLC with photodiode array detector at 243 nm and (b) in matrix (fortified milk at 0.05 ng/g for CLEN, 0.30 ng/g for CAP and 0.50 ng/g for DES).

3. Results and discussion

3.1. Sample preparation

For the determination of veterinary drug residue in milk, the sample pretreatment procedures usually include labor-intensive deproteinization procedure [30]. 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 our previous experiment for determination of dexamethasone (DM) and betamethasone (BM) in bovine milk [31].

SPE is the popular technique currently available for rapid and selective sample preparation. For CLEN, reversed SPE cartridges (C_{18} and HLB) and ion exchange SPE cartridges (MCX and SCX) were usually used for sample preparation. For CAP and DES, reversed SPE cartridges (C_{18} and HLB) and normal SPE cartridge (Si) were often used for sample preparation. So, C_{18} (Supelco) and HLB (Waters) cartridges were selected for optimization of SPE procedures in this study. According to the previous developed method for determination of DM and BM, ethyl acetate was used as eluent. It was found that good recoveries (above 90%) can be acquired for DES and CAP when the volume of ethyl acetate was 5 mL for two kinds of cartridges. For C_{18} cartridge, the recoveries for CLEN only were 0, 2–3, 15–17 and 31–34% for 5, 10, 15 and 20 mL of ethyl acetate, respectively. For HLB cartridge, the recoveries for CLEN were 62–65, 87–90, 93–97 and 94–97% for 5, 10, 15 and 20 mL of ethyl acetate, respectively. To acquire good recoveries for CLEN, CAP and DES, 15 mL of ethyl acetate and HLB cartridge were chosen in this study.

3.2. Optimization of UPLC conditions

For LC–MS/MS method, the choice of mobile phase must be concerned based on the consideration of ionization efficiency. It was found that the mixture of acetonitrile and formic acid solution was not suitable as mobile phase for CAP and DES because they could not generate high responses under the ESI^- mode. Moreover, about half of *trans*-DES will transformed into *cis*-DES when the mixture of acetonitrile and the high concentration of ammonium acetate solution (above 40 mM) as mobile phase. After our careful investigation, the mixture of 5 mM ammonium acetate solution and acetonitrile was chosen as mobile phase because all of three kinds of drug could generate high responses under ESI^+ (CLEN) or ESI^- (CAP and DES) mode.

For the choice of gradient elution program, the main task was to determine the ratio of ammonium acetate solution (solvent A) and acetonitrile (solvent B) as the initial mobile phase. The ratio candidates were designated as 95:5, 90:10, 80:20, 70:30 and 60:40 (v/v) of A:B. Results of multiple injections (matrix standard solution and standard solution) showed that nice peak shape could be achieved with the increase of acetonitrile content in initial mobile phase. Nevertheless, when the initial mobile phase was 80:20, 70:30 and 60:40 (A:B), a relatively low separation efficiency and strong matrix suppression effect occurred because of the over-fast peak elution. Considering separation efficiency and peak shape, 90:10 (A:B) of initial mobile phase was a reasonable choice. Fig. 1 shows the MRM chromatograms of mixed standard solution of CLEN, CAP and DES.

3.3. MS-MS optimization

Six individual working standard solutions (1000 ng/mL for CLEN, CLEN-D_9, CAP and CAP-D_5, 5000 ng/mL for DES and DES-D_8) were infused to optimize the MS–MS parameters of the six compounds and to select the appropriate diagnostic ions. The infusion process was carried out with the same chromatographic conditions as those used during analysis.

For the confirmation of CLEN, CAP and DES, which were included in Group A of Annex I of Council Directive 96/23/EC [32], a minimum of four identification points are required. In this experiment, four identification points were obtained by monitoring one parent ion (1 point) and two transitions (each 1.5 points) were monitored. The selected transitions for CLEN, CAP and DES and the optimal MS–MS conditions are shown in Table 1.

Three isotope compounds (CLEN-D_9, CAP-D_5 and DES-D_8) were added as internal standards for quantitation purposes. CLEN-D_9, CAP-D_5 and DES-D_8 were used as internal standards for quantitation of CLEN, CAP and DES, respectively. Although only the *trans* isomer was found in standard solution, *cis* isomer will be produced during the procedure of sample preparation for DES and DES-D_8. It is well known only *trans* DES is effective to increase the weight gain of animals [33], and only about 10% *trans* DES have been transformed into *cis* DES in this study. So only the *trans* isomer was used to quantify for DES as Schmidt et al. had done [34].

3.4. Method validation

3.4.1. 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 0.2–20 ng/mL (CLEN), 1.2–100 ng/mL (CAP) and 2.0–200 ng/mL (DES). The results of the linearity are reported in Table 2. The correlation coefficients (*r*) of the calibration curves were above 0.9992. Using these curves, recoveries can be calculated at each fortification level. The MRM chromatograms of the standard solution are shown in Fig. 1.

3.4.2. Specificity

The specificity was evaluated by analyzing 20 blank milk samples. Fig. 2a and b indicates that there were no interfering peaks

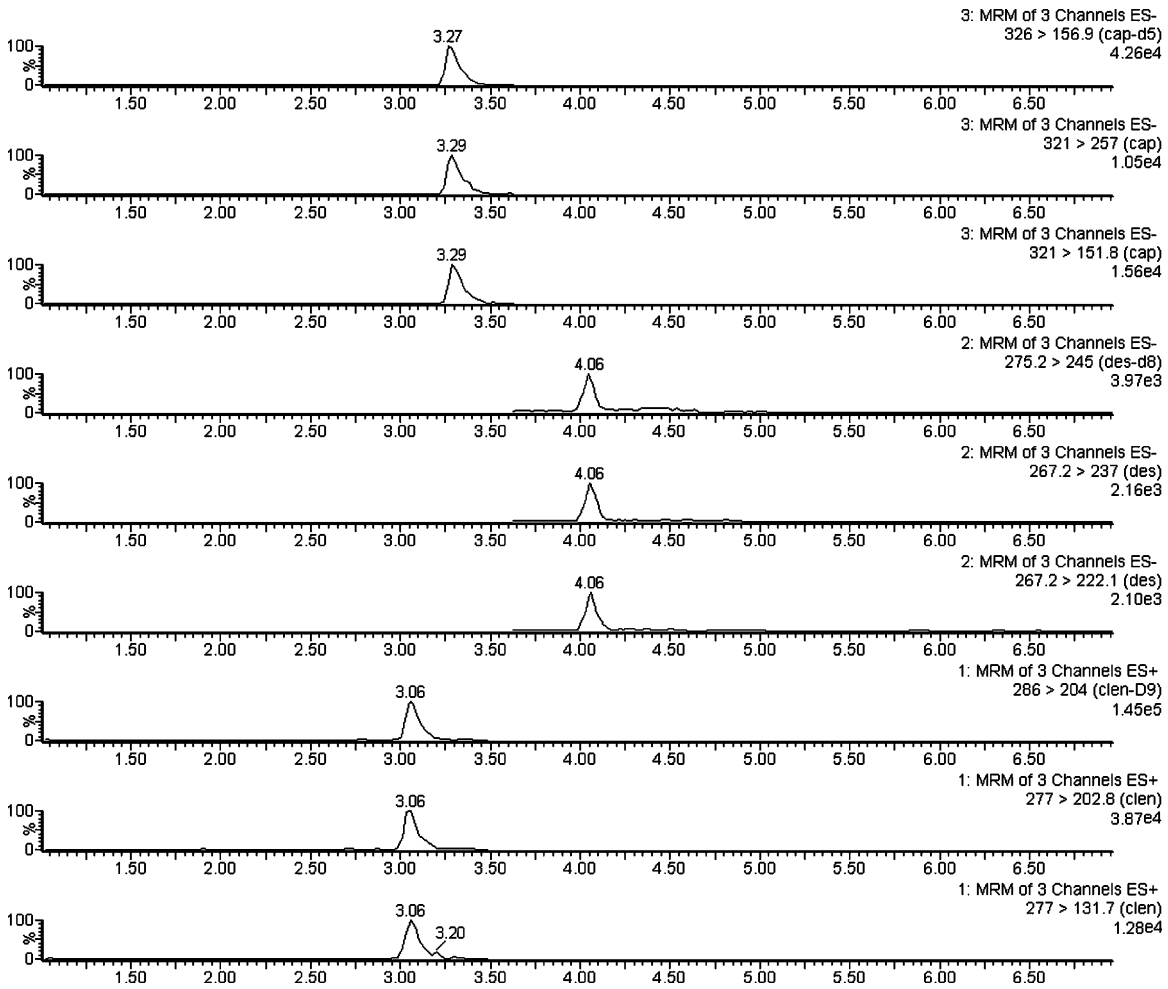

Fig. 1. The MRM chromatogram of CLEN (1.0 μg/L), CAP (6.0 μg/L), DES (10.0 μg/L), CLEN-D_9 (5.0 μg/L), CAP-D_5 (20.0 μg/L) and DES-D_8 (20.0 μg/L) in standard solution. The MRM transitions from top to bottom correspond to CAP-D_5 (326 > 156.9), CAP (321 > 257 and 321 > 151.8), DES-D_8 (275.2 > 245), DES (267.2 > 237 and 267.2 > 222.1), CLEN-D_9 (286 > 204) and CLEN (277 > 202.8 and 277 > 131.7).

Table 2
The linearity and regression coefficients of standard curves of CLEN, CAP and DES by UPLC–MS/MS.

Analyte	b^a	a^b	r
CLEN	0.2164 ± 0.0025	0.0121 ± 0.0038	0.9992
CAP	0.0361 ± 0.0006	0.0293 ± 0.0050	0.9994
DES	0.0559 ± 0.0017	0.0671 ± 0.0049	0.9996

[a] b = slope(±SD of slope).
[b] a = intercept(±SD of intercept).

from endogenous compounds at the retention times of CLEN, CAP and DES.

3.4.3. Recovery and precision

The recovery and reproducibility of the method were measured by analyzing six blank samples fortified with CLEN, SAL and RAC at 0.05 ng/g (CLEN), 0.30 ng/g (CAP) and 0.50 ng/g (DES) on three separate occasions. The results are shown in Table 3. The average recoveries, repeatability, and reproducibility varied from 94.5 to 106.0%, from 4.7 to 5.3% (CV_r), and from 5.0 to 5.6% (CV_R), respectively. These recoveries and CVs for CLEN, CAP and DES are better than many previously developed methods for milk and other biological samples by LC–MS/MS [21–27], possibly due to the use of isotope compounds as internal standard for quantification in the present study.

3.4.4. 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 values of the CCα were 0.054, 0.007 and 0.04 ng/g for CLEN, CAP and DES, respectively. The values of the CCβ were 0.058 ng/g, 0.010 and 0.055 ng/g for CLEN, CAP and DES, respectively. Additionally, limits of detection (LODs) were 0.009, 0.007 and 0.04 ng/g for CLEN, CAP and DES, respectively, based on

Table 3
Average recoveries and variation coefficients (CV_r and CV_R) of CLEN, CAP and DES from bovine milk by UPLC–MS/MS.

Drug	Fortified concentration (ng/g)	Average recovery (%, n = 6)			CV_r (%)	CV_R (%)
		Day 1	Day 2	Day 3		
CLB	0.05	98.1 ± 2.8	102.7 ± 2.6	104.5 ± 3.7	4.7	5.2
CAP	0.30	103.6 ± 3.4	94.9 ± 3.6	97.3 ± 2.1	4.9	5.6
DES	0.50	94.5 ± 3.1	103.9 ± 2.9	106.0 ± 2.7	5.3	5.0

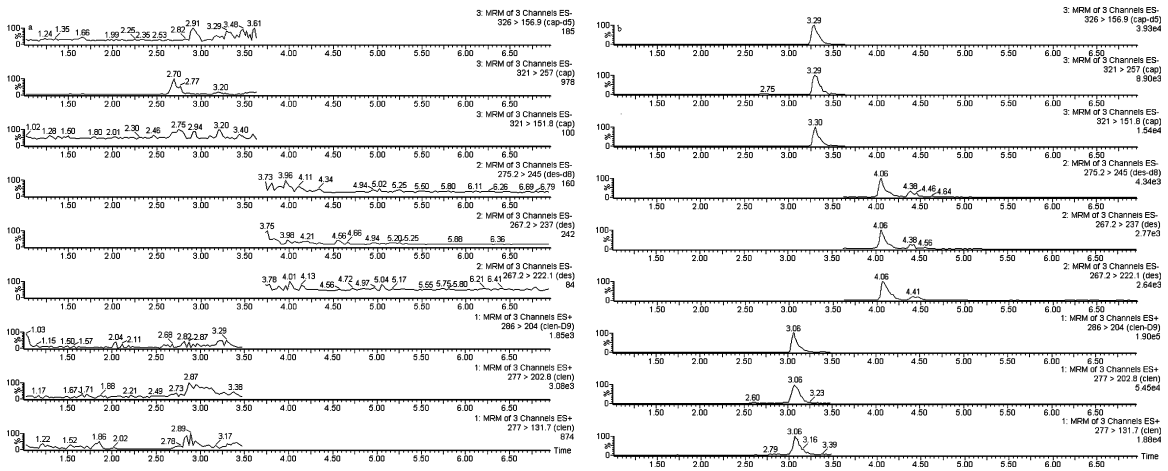

Fig. 2. (a) The MRM chromatogram of blank milk. (b)The MRM chromatogram of blank milk fortified with CLEN (0.05 ng/g), CAP (0.30 ng/g), DES (0.50 ng/g), CLEN-D_9 (0.25 ng/g), CAP-D_5 (1.0 ng/g) and DES-D_8 (1.0 ng/g).

three times signal to noise ratio. LOQs were 0.03, 0.02 and 0.13 ng/g for CLEN, CAP and DES, respectively, based on 10 times signal to noise ratio. The LOD of this method is similar or better than those of previously published MS methods for these three kinds of drug in biological samples [13–20].

3.4.5. Stability

The stock standard solutions in methanol were stored for at least 6 months at −20 °C. The stock solutions were analyzed every 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 [35]. Fortified milk samples of CLEN (0.05 ng/g), CAP (0.30 ng/g) and DES (0.50 ng/g) stored at −20 °C were analyzed after 3, 7 and 14 days. It was found that the recoveries of CLEN, CAP and DES had no obvious change.

3.5. Applications of the method

Sixty milk samples commercially available from the local market were analyzed for CLEN, CAP and DES using the above method. No CLEN and DES were found in these samples. CAP was found in two samples with the concentration of 0.03 and 0.17 ng/g.

4. Conclusion

In the present study, a rapid and sensitive method for the simultaneous determination of CLEN, CAP and DES in milk by UPLC–MS/MS with isotope dilution 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 CLEN, CAP and DES in milk.

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