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Quantitative analysis of lincomycin in animal tissues and bovine milk by liquid chromatography electrospray ionization tandem mass spectrometry

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Abstract

A sensitive method for determining lincomycin in bovine milk, animal muscles and organs using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) is presented. Milk and homogenized animal tissues were extracted with acetonitrile twice after addition of an appropriate amount of clindamycin, a lincosamide analogue as the internal standard. The combined extracts were finally made up to 10 ml with distilled water and partitioned with hexane to remove the animal fats prior to analysis. Analytes in the extracts were separated on a reversed phase C18 column (250 mm × 2.1 mm, 5 μm) using a mobile phase of a 3:7 (v/v) mixture of 0.1% formic acid in acetonitrile and an ammonium formate buffer (ammonium formate:formic acid:acetonitrile:water, 1:5:50:950, v/v/v/v) running at a flow rate of 0.2 ml min^{-1}. Presence of lincomycin was confirmed by the presence of two characteristic product ions at *m*/*z* 126.1 and 359.2 within a defined retention time window from the precursor ion at *m*/*z* 407.2, whilst quantification was based on the relative ratio of the sum of the peak areas at *m*/*z* 126.1 and 359.2 for lincomycin to that of the internal standard (peaks at *m*/*z* 126.1 and 377.2) with reference to the respective ratios of the calibration standards. The validated method that was found to have linear responses in the calibration range from 25 to 3000 μg kg^{-1} and satisfactory intra-day and inter-day accuracy (94.4–107.8%) and precision (1.3–7.8%) at concentrations ranging from 100 to 1500 μg kg^{-1} has been applied to real samples and matrix spiked samples. It is considered robust and suitable for analysis of lincomycin in milk and animal tissues.

Keywords: Lincomycin; Antibiotic; Residue analysis; LC–MS/MS

1. Introduction

Lincomycin, a member of the lincosamide family, is a moderately broad spectrum antibiotic that has been used as a veterinary medicine. It is commonly administered to animal via oral or intramuscular routes; typical oral doses for poultry are up to 50 mg kg^{-1} per day for up to 7 days and intramuscular doses for cattle are up to 15 mg kg^{-1} per day for up to 4 days, respectively. The amount of residue in various tissues will vary between individual animals and will depend upon the time taken after the last dose. Commercial

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lincomycin products usually contain impurities such as lincomycin B and 7-epilincomycin but in negligible quantities (<1%) [1]. Once introduced into the body, lincomycin is bio-transformed to a number of metabolites, none of which would be present in a concentration sufficient enough to serve as a residue marker [2]. As a result, analyses of lincomycin rely wholly on the monitoring of the parent compound.

Determination of lincomycin in pharmaceutical products by capillary electrophoresis [3] and high performance liquid chromatography (HPLC) with ultra-violet detection at wavelength range of 200–210 nm [1,4]; and HPLC with electrochemical detection [5] has been reported. However, the absence of a suitable chromophore in lincomycin and presence of matrix interferences limit the usefulness of the above applications in biological samples. Other quantitative measurements of lincomycin residues in animal kidneys [6] and salmon tissues [7] by gas chromatography; and in milk and animal tissues by HPLC [8] are either not sensitive enough or involve labour intensive sample treatment and cleanup procedures. These drawbacks restrict the versatility of these reported methods for measuring trace amounts of lincomycin in the demanding high throughput residue analysis required nowadays. Although liquid chromatography–mass spectrometry (LC–MS) [9] and the more selective LC–MS/MS [10] have been applied to the analysis of clindamycin (a lincosamide analogue) in plasma, there is only one LC–MS method recently reported for the analysis of lincomycin in milk samples in the literature [11]. The described method did not employ an internal standard for correction and the presence of an intense co-eluting interfering peak at *m/z* 126 prevented accurate quantification.

In this paper, the development and validation of a sensitive and selective LC-ESI/MS/MS method for determination of lincomycin, at or below the maximum residue limits (MRLs), in bovine milk (150 $\mu g\,kg^{-1}$) and edible tissues (muscle: 100 $\mu g\,kg^{-1}$; kidney: 1500 $\mu g\,kg^{-1}$; liver: 500 $\mu g\,kg^{-1}$ in bovine, porcine and poultry) as stated under the Harmful Substances in Food Regulation and Public Health (Animals and Birds) (Chemical Residues) Regulations in the Laws of Hong Kong is presented. These MRLs are comparable to those established by the European Union [12]. From a risk assessment and analysis approach [13], the amount of residues of veterinary chemicals that would cause adverse effects was estimated from the MRLs which were based upon the consumer intake of not exceeding 64% of the average daily intake (ADI). ADI for lincomycin was calculated as 600 μg for a 60 kg person from microbiological tests. Therefore, the method is capable of detecting and quantifying lincomycin residues in food at levels posing health hazards to humans upon consumption of the concerned foodstuff.

2. Experimental

2.1. Chemical standards and reagents

Lincomycin hydrochloride and clindamycin hydrochloride were purchased from Fluka (Buchs, Switzerland). To confirm the validity and purity of the standard, an additional source of lincomycin hydrochloride was acquired from Sigma (St. Louis, USA). HPLC grade acetonitrile and methanol and analytical grade *n*-hexane were purchased from Labscan Asia (Bangkok, Thailand). Analytical grade formic acid and ammonia formate were acquired from Riedel-de Haën (Seelze, Germany). A water purifier from Fistreem Cyclon (Loughborough, UK) was used to purify distilled water.

2.2. Instrumentation

An HPLC (Hewlett-Packard HP 1100 series, Rockville, USA) integrated system consisting of a 100-well auto-sampler, a 10 μl sample loop, a degasser, a quaternary pump and a thermostated column oven set at 25 °C were used. Chromatographic separation was performed by delivering a 3:7 (v/v) mixture of 0.1% formic acid in acetonitrile and an ammonium formate buffer (ammonium formate:formic acid:acetonitrile:water, 1:5:50:950, v/v/v/v) through a 250 mm × 2.1 mm, 5 μm Hypersil C18 column (Alltech, Deerfield, USA) and a corresponding C18 guard column (7.5 mm × 4.6 mm), at a flow rate of 0.2 ml min^{-1}. Identification and detection of analytes were carried out by an API 4000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, USA) equipped with an ESI turbo ion source operated at positive mode. A built-in software (Analyst, ver-

sion 1.3, Applied Biosystems) was used for system control and data processing.

2.3. Calibration standards

A stock standard solution of 1000 μg ml^{-1} of lincomycin in methanol was prepared and stored in a freezer at −20 °C before use. Two lincomycin working standards of 10 μg ml^{-1} (WS1) and 50 μg ml^{-1} (WS2) were freshly prepared by dilution of the stock standard solution prior to analysis. A stock internal standard (clindamycin) solution of 1200 μg ml^{-1} and a working standard of 60 μg ml^{-1} were prepared in the same manner as the lincomycin standard. The stock standard solutions of lincomycin and clindamycin were found to be stable for about 2 months under the above storage conditions. Because of different MRLs in different matrices, two independent six-point standard calibration curves covering the ranges of 25, 50, 100, 250, 500 and 1000 μg kg^{-1} for milk and muscles samples and 50, 100, 200, 500, 1000 and 3000 μg kg^{-1} for organ samples, respectively, were prepared. Each of the matrix spiked calibration standards was prepared independently by adding a quantitative volume (2.5–30 μl) of either one of the two working lincomycin standards and 60 μl of the working clindamycin standard into a blank (drug-free) sample (1 g of sample was used for milk and muscle matrix; and 0.5 g of sample was used for organ matrix) in a 15 ml-plastic tube. After spiking of the standards, each of the mixtures was left at room temperature for 10 min before addition of 2 ml of acetonitrile and then sonication for 5 min. Afterwards, each of the mixture was further mixed thoroughly by a vortex mixer (Maxi Mix II, Thermolyne) for 1 min to complete the deproteinization of samples. The clear supernatants were transferred to new centrifuge tubes after centrifugation (5810R, Eppendorf, Hamburg, Germany) at 4000 rpm for 10 min at 5 °C. The above extraction procedure was repeated by adding another 1 ml of acetonitrile to each of the remnants. Corresponding supernatants were combined and finally made up to 10 ml with distilled water. The extracts were then defatted by liquid-liquid partition with 2 ml of *n*-hexane. After centrifugation, the aqueous layers were separated, filtered through 0.45 μm PTFE filter discs and ready for use as a freshly prepared calibration standard.

2.4. Preparation of spiked milk samples

Spiked samples at two concentrations were prepared by separately spiking 15 μl (equiv. to 150 μg kg^{-1}) and 45 μl (equiv. to 450 μg kg^{-1}) of WS1 into 1 g of a blank milk sample in a 15 ml-plastic centrifuge tube (Sarstedt, Newton, USA). Ten microlitres of clindamycin working standard (equiv. to 600 μg kg^{-1}) was then added into each of the samples. The spiked samples were then subjected to the same extraction and cleanup procedures as described for preparation of calibration standards prior to LC/MS analysis. All sample extracts were confirmed stable by storing at −70 °C for 2 weeks before analysis.

2.5. Preparation of spiked muscle and organ samples

Blank muscle and organ tissues were thoroughly blended in a homogenizer (Ultra-Turax T25, IKA, Staufen, Germany) for 10 min. Spiked muscle samples at two concentrations were prepared by separately spiking 10 μl (equiv. to 100 μg kg^{-1}) and 30 μl (equiv. to 300 μg kg^{-1}) of WS1 into 1 g of a blended muscle sample. Similarly, for kidney and liver samples, 25 μl (equiv. to 500 μg kg^{-1}) of WS1 and 15 μl (equiv. to 1500 μg kg^{-1}) of WS2 into 0.5 g of drug-free kidney and liver samples, respectively. Ten microlitres of clindamycin working standard was added in all these samples (equiv. to 600 μg kg^{-1} in muscle and 1200 μg kg^{-1} in organ samples). Treatment and cleanup for the spiked samples was the same as that described for the spiked milk samples. All sample extracts were stable by storing at −70 °C for 2 weeks before analysis.

2.6. Quality assurance and validation

The purity of the lincomycin reference standard was confirmed to be not less than 95% by cross-checking of two different brands of commercial standard. Clindamycin is an analogue lincosamide and produces similar antibacterial effects as lincomycin, and has been shown to be effective in the treatment of infections caused by susceptible anaerobic bacteria or susceptible strains of gram positive bacteria such as streptococci, staphylococci and pneumococci. Clindamycin is not co-administered with lincomycin or administered independently to the same animal. Therefore,

clindamycin is a suitable internal standard for quantification of lincomycin or vice-versa. Qualitative identification was based on the presence of two product ions at *m*/*z* 126.1 and 359.2 within a defined retention time window, whilst quantification was based on the relative ratio of the sum of the peak areas at *m*/*z* 126.1 and 359.2 for lincomycin to that of the internal standard (peaks at *m*/*z* 126.1 and 377.2) with reference to the respective ratios of the standards from the calibration curve. Based on a statistical approach for calibration [14], the acceptance criteria for the correlation coefficient (*r*) of the six-point calibration curve was adopted to be equal to or better than 0.995; and goodness-of-fit of coefficients of the calibration curves, calculated as the relative standard deviation (RSD) of the relative response factors of all calibration points, was set to be within 15%. Limit of quantification (LOQ) was defined as the lowest calibration concentrations and limit of detection (LOD) was estimated using the criterion of a signal-to-noise (S/N) ratio of 10 with reference to that of the lowest calibration standard. Accuracy, in terms of the absolute difference between the experimental mean and the respective nominal values; and precision, in terms of the RSD were assessed by intra-day and inter-day variation of spiked samples of replicate analysis of spiked samples at two different concentrations within the calibration range. Three independent quality check standards within the calibration range were used to check the validity of the calibration curve (trueness within $100 \pm 10\%$). Analytical bias was estimated by evaluating the accuracy and precision of measuring lincomycin in blind samples, each of which was administered with a known concentration of lincomycin; as well as the relative percentage difference (RPD) of replicate analysis of blind samples of two selected matrices between two operators.

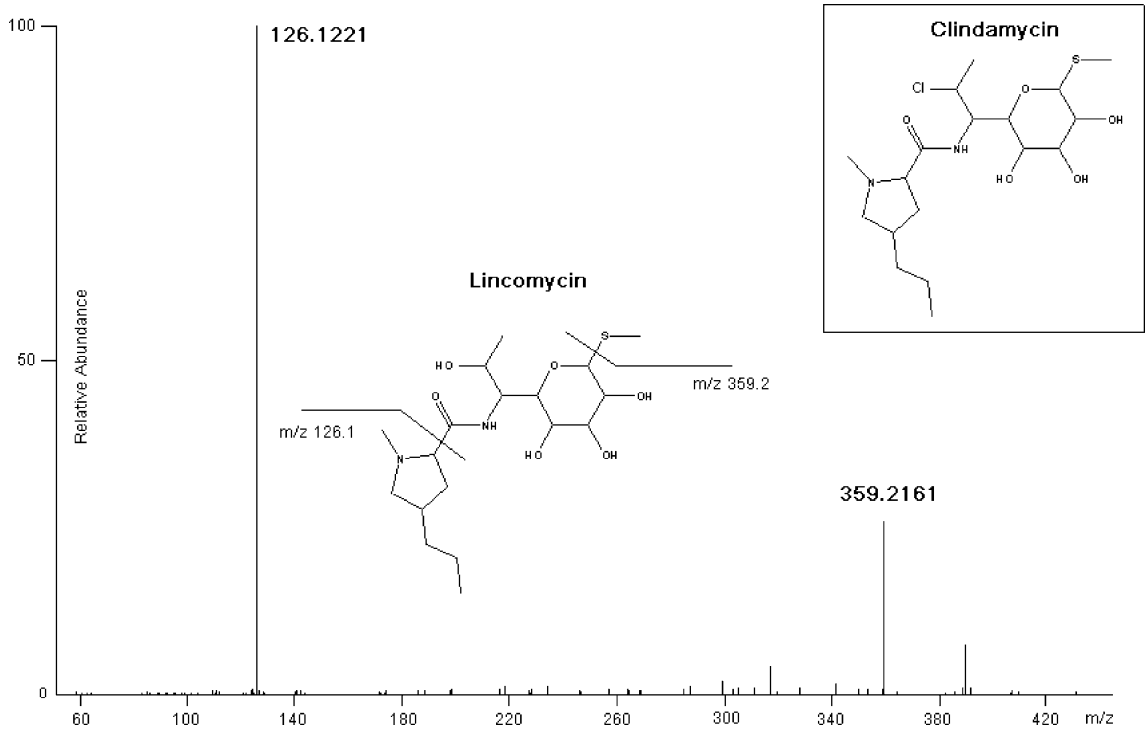

Fig. 1. Tandem mass (MS/MS) spectrum of lincomycin was obtained by infusion of 200 ng ml^{-1} standard solution in 0.1% formic acid at 10 μl min^{-1} into the LC/MS/MS (operating conditions refers to the text). Formation of product ions at *m*/*z* 126.1 and 359.2 from parent ions (*m*/*z* 407.2) was represented by the bond cleavages shown in the lincomycin structure. Chemical structure of the internal standard, clindamycin was illustrated at the top right corner box.

3. Results and discussion

3.1. Optimization of the MS operating conditions

The molecular-weight related peak of lincomycin was identified by infusing a 200 ng ml^{-1} standard solution in 0.1% formic acid at 10 μl min^{-1} into the triple quadruple mass spectrometer. The dominant protonated ion $[M+H]^+$ that was obtained in the positive ionization mode at *m*/*z* 407.2 was used as the precursor ion in optimizing the required MS/MS parameters. Two major product ion peaks at *m*/*z* 126.1 and 359.2 were found to give the highest intensity in the following settings: flow rate of nebulizing gas at 40 (arbitrary unit); sheath gas at 50; curtain gas at 10 and collision gas at 6; needle spraying potential at 5.0 kV; turbospray temperature at 500 °C for desolvation; declustering potential at 75 V; collision potential at 39 V (for *m*/*z* 126.1) and 27 V (for *m*/*z* 359.2); exit potential at 8 V (for *m*/*z* 126.1) and 20 V (for *m*/*z* 359.2). As illustrated in Fig. 1, the mass fragments at *m*/*z* 126.1 and 359.2 correspond to the 3-propyl-*N*-methylpyrrolidine ion and the loss of thiomethanol molecule from the precursor, respectively.

For clindamymin, the product ions are *m*/*z* 126.1 and 377.2 from the parent $[M+H]^+$ ion at *m*/*z* 425.2. The MS/MS settings were almost the same as that of lincomycin, with only the alteration of the exit potential at 26 V (for *m*/*z* 377.2).

3.2. LC–MS/MS analysis

The LC effluents of sample extracts were sprayed onto the cone and focused by the lens and skimmer into the mass analyzer. The dwell time for each monitoring channel was 200 ms with a mass window of 1.0 *m*/*z*. A matrix blank was included in every batch of analysis for checking of interference. All the blank samples were found not to contain significant amount of endogenous substances that could mask the peaks from the analyte and internal standard. It is evident that the present work has made advances compared with already published work [7,8,11]. No sign of interference from in-source fragmentation of the analyte and internal standard was observed in all samples. Lincomycin was eluted at 4.0–4.1 min and clindamycin at 6.9–7.0 min with satisfactory peak symmetry and resolution. Typical chromatograms of milk, muscle and organ samples were shown in Fig. 2. The retention of lincomycin on C18 columns was affected by the composition difference between the mobile phase and the solutions containing the analytes. For instance, the retention time of standard in 0.1% formic acid is approximately 0.8 min longer than that in the mobile phase. Subsequently, on making up the combined acetonitrile extracts of different samples to 10 ml with ammonium formate buffer, the retention time shifts were rectified. However, lincomycin was found to be unstable upon storing in a mixture of ammonium formate buffer and acetonitrile; with the concentration of lincomycin decreased significantly (for more than 50%) after storage for 1 week. Eventually, the extracts were finally made up with distilled water in which lincomycin is stable and could be preserved intact for at least 2 weeks in freezer at −70 °C before analysis. A number of C18 reversed-phase columns have been tested and all gave similar chromatographic results. Hypersil column was chosen because it gives a better separation between lincomycin and clindamycin than others.

The absolute responses of lincomycin and the internal standard were initially found to have gradually declined after each successive injection. For example, a sequence of 50 injections could lead to an overall signal drop by approximately 60%. Although the diminished responses do not adversely affect the precision of analysis upon the application of internal standard correction, it indicates that co-eluted endogenous substances might have accumulated in the LC–MS interface region suppressing the ionization process. Thus, to minimize the suspected accumulation of endogenous materials in the MS system, the LC effluents from 0 to 2 and 8.5 to 10 min were diverted to the drain without entering the MS system to avoid contamination of ion source and reduce ion suppression of analytes [15]. In addition, a 3 min-acetonitrile wash was inserted after injection of every five samples. Under such an arrangement, the absolute responses of lincomycin and the internal standard were observed to be constant throughout the course of the analysis.

The entire LC–MS/MS analysis, including the acetonitrile wash, requires approximately 15 min per sample and a total of more than 90 samples could be analysed within 24 h. It is evident that the simple extraction and cleanup protocol allows rapid sample preparation and high throughput instrumental analysis.

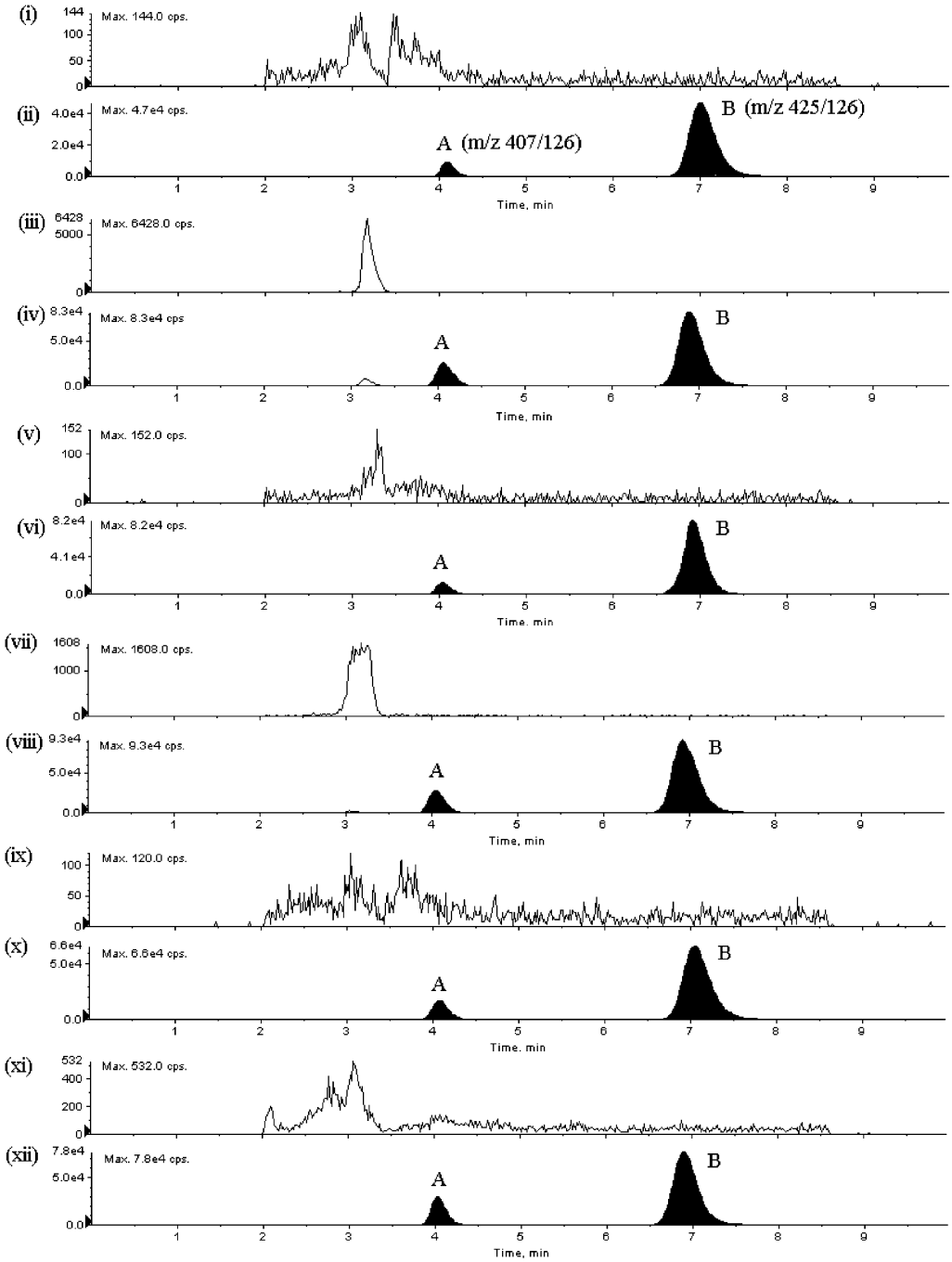

Fig. 2. Total ion chromatogram of (i) blank bovine muscle, (iii) kidney, (v) liver, (vii) milk, (ix) chicken muscle and (xi) liver; and selective ion chromatograms (ii, iv, vi, viii, x and xii) of the corresponding samples spiked with lincomycin (A) at 100–200 $\mu g\,kg^{-1}$ and clindamycin (B).

Table 1
Calibration ranges, coefficient coefficients and detection limits of lincomycin in different matrices

Matrix	Range ($\mu g\,kg^{-1}$)	Correlation coefficient (r)	Goodness-of-fit coefficient (%)	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)
Milk	25–1000	0.9991–0.9999	8.2–12.8	1.5	25
Bovine muscle	25–1000	0.9996–0.9999	5.4–9.5	8.8	25
Chicken muscle	25–1000	0.9990–0.9999	6.6–8.7	4.3	25
Bovine liver	50–3000	0.9990–0.9999	5.3–8.8	7.3	50
Chicken liver	50–3000	0.9989–0.9999	3.9–7.6	7.8	50
Bovine kidney	50–3000	0.9988–0.9999	5.2–10.7	4.7	50

3.3. Method validation

Standard calibration curves of lincomycin in different matrices were found to be linear ($r > 0.999$, goodness-of-fit coefficients <13%) in the range of 25–3000 $\mu g\,kg^{-1}$ (Table 1) over a period of 2-month trial. The mass signals tend to deviate from the norm beyond 3000 $\mu g\,kg^{-1}$ and a new calibration or sample dilution is required for quantification of higher concentrations. These LOQ values (25 $\mu g\,kg^{-1}$ in milk and muscles, 50 $\mu g\,kg^{-1}$ for liver and kidney, respectively) show that the present method is sufficiently sensitive to measure lincomycin at the MRLs in various matrices. The signal-to-noise ratios at the lowest concentration ranged from 28.8 to 220; and the LOD were estimated to be 1.5–8.8 $\mu g\,kg^{-1}$. As summarized in Table 2, accuracy and precision for intra-day and inter-day assay of all sample matrices were found to be 94.4–108% and 1.3–5.5%; and 96.9–108% and 3.4–7.8%, respectively. The bias, in terms of trueness, of the QC samples at three different concentrations (Table 3) in milk, muscle, liver and kidney were in the range of −14.0 to +7.2%, −10.5 to +8.4%, −4.5 to +10.0% and −5.1 to +11.0%, respectively. When applying the method to measure lincomycin in blind samples for all tested sample matrices, the overall results were found to be acceptable with accuracy in the range of 93.9–107% and precision within 8.5%. Two operators were selected to test the validity of the method by analyzing independently blind samples of two selected matrices (chicken muscle and bovine kidney). The means of the results ($n = 6$) from the two operators were consistent: the accuracy and precision of the first person were 96.8 and 6.2%; and 95.2 and 0.5%, respectively, in the analysis of chicken muscle and bovine kidney; whilst the values

Table 2
Intra-day ($n = 7$) and inter-day ($n = 4$–6) accuracy and precision of spiked samples during a 2-month trial

Matrix	Concentration ($\mu g\,kg^{-1}$)	Intra-day		Inter-day	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Milk	150	108	5.1	107	7.8
	450	94.4	1.3	98.6	4.5
Bovine muscle	100	99.5	1.5	99.2	4.1
	300	102	4.2	102	4.9
Chicken muscle	100	102	1.4	101	5.5
	300	95.9	3.5	98.8	4.9
Bovine liver	500	107	3.5	108	5.4
	1500	101	2.0	102	4.5
Chicken liver	500	98.3	5.5	96.9	6.0
	1500	101	2.3	100	3.4
Bovine kidney	500	101	2.4	102	5.9
	1500	106	5.3	102	6.1

Table 3
Evaluation of method bias by analysis of QC samples ($n = 4$), blind samples ($n = 8$) and inter-person variation ($n = 6$)

Matrix	Trueness of QC samples (%)			Blind samples		RPD (%)
	L	*M*	*H*	Accuracy (%)	Precision (%)	
Milk	−14 to 5.7	−5.0 to 11.9	−9.4 to 7.2	98.4	3.3	–
Bovine muscle	−6.2 to 7.3	2.1 to 8.4	−0.1 to 6.1	102	3.2	–
Chicken muscle	−3.6 to 3.5	−10.5 to 4.2	−10.1 to 2.4	93.9	4.0	0.02–5.7
Bovine liver	−1.9 to 6.7	2.6 to 8.9	−2.6 to 6.1	98.9	8.5	–
Chicken liver	0 to 10.0	−3.3 to 5.1	−4.5 to 6.3	107	5.1	–
Bovine kidney	−2.7 to 11.0	−2.4 to 4.3	−5.1 to 3.4	97.5	3.8	1.0–1.7

Trueness was determined by comparing the difference between the experimental values and the nominal concentrations of QC samples ($L = 50$, $M = 100$ and $H = 500\,\mu g\,kg^{-1}$ for milk and muscle samples; and $L = 200$, $M = 500$ and $H = 2000\,\mu g\,kg^{-1}$ for organ samples, respectively). RPD is the absolute percent difference between the mean values obtained by two independent operators.

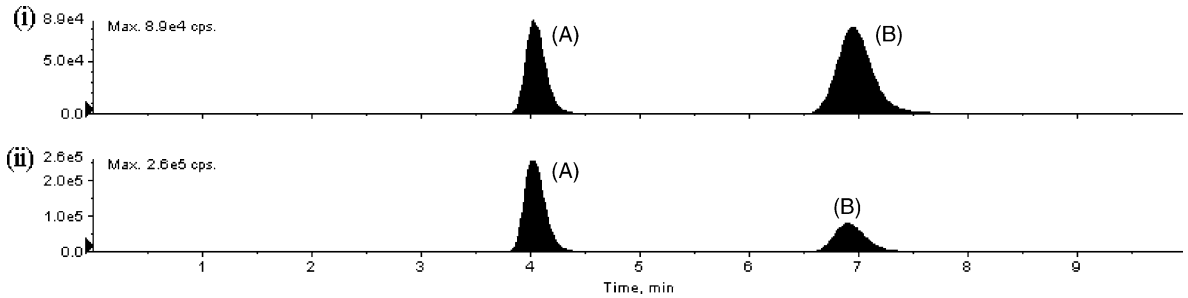

Fig. 3. Chromatograms showing the presence of lincomycin (Peak A) in the muscle and (ii) in the liver of a chicken after oral dosing of the subject at 25 mg per day for 7 days.

were 92.9 and 2.6%; and 94.0 and 2.5%, respectively, for the second person. RPD between persons were within 5.7% (Table 3). The findings reflect the robustness of the method that is shown to be suitable for the determination of lincomycin at trace levels in milk and animal tissues.

3.4. Application to real chicken samples

Two chickens weighed approximately 1.4 kg each were independently given an oral single dose of 50 and 25 mg of lincomycin hydrochloride for seven consecutive days. The chickens were slaughtered 1 h after administration of the last dose. Muscle and liver of the two chickens were taken, homogenised separately and have their concentrations of lincomycin determined by the method under discussion. As shown in Fig. 3, lincomycin was detected with no significant endogenous interference from the muscle and liver matrices. Mean concentrations (±S.D.) of lincomycin in muscle and liver ($n = 4$) were found to be 540 ± 24 and $4250 \pm 121\,\mu g\,kg^{-1}$, respectively, for the chicken dosed at 50 mg per day, and 235 ± 8.7 and $2370 \pm 94\,\mu g\,kg^{-1}$ for the one dosed at 25 mg per day, respectively. As the lincomycin concentration in the liver at higher dose was observed to be beyond the calibration range in the initial runs, it was necessary to dilute the sample by mixing with appropriate portion of blank liver sample during sample treatment. The experiments indicate that the developed method is applicable to the determination of lincomycin in real samples.

4. Conclusion

The presented LC–MS/MS method for determination of lincomycin in bovine milk and animal tissues is accurate, precise and sensitive. The major advantages over previous analytical methods for determination of lincomycin are (i) simple sample treatment, (ii) improved reproducibility with the employment of

a suitable internal standard (iii) improved sensitivity and selectivity and (iv) less time-consuming analytical cycles. It is the first published LC–MS/MS method for trace analysis of $\mu g\,kg^{-1}$ level of lincomycin in a variety of food and biological matrices. In addition, the simple sample preparation cleanup procedure and efficient instrumental analysis allows the method to be of fast sample turnover and of high throughput. The present study demonstrates the sensitivity and selectivity of LC–MS/MS technique in determination of trace veterinary drug residues in complex matrices.

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