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Short communication

# HPLC determination of lincomycin in premixes and feedstuffs with solid-phase extraction on HLB OASIS and LC–MS/MS confirmation

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**Abstract**

A rapid clean-up procedure based on solid-phase extraction (SPE) and HPLC determination of lincomycin in premixes with UV detection is described. After extraction of lincomycin from premix with extraction solvent the extract is applied to OASIS HLB column treated with methanol and water. Lincomycin is eluted with methanol and effluent is analysed on analytical column (phenyl) using mobile phase consists 0.2% phosphoric acid in water and acetonitrile (875:125, v/v). Detection is performed at 208 nm. Quantitation is carried out using external standard. The mean recovery of lincomycin was $105.0 \pm 7.3\%$, in concentration range of 250–750 mg kg$^{-1}$, and $99.8 \pm 3.7\%$, in concentration range of 10,000–150,000 mg kg$^{-1}$. The limit of determination, based on a signal-to-noise ratio of 10:1, was 5.2 mg kg$^{-1}$. LC–MS/MS confirmation of lincomycin is also presented. Identification was performed by monitoring two pairs of multiple reaction monitoring ions from the parent ions (*m*/*z* 407.2 → 126.1 and 407.2 → 359.2) at the defined retention time window and by matching of the specific tolerance of relative abundance of major ions as stated in the European Union Commission Decision 2002/657/EC.
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## 1. Introduction

Lincomycin [methyl 6,8-dideoxy-6{[(1-methyl-4-propyl-2-pyrrolidyl)carbonyl]amino}-1-thio-D-*erythro*-α-D-*galacto*-octapyranoside] is a sulfur-containing pyranoside broad-spectrum antibiotic synthesized by *Streptomycin lincolnensis* [1] which shows in vitro and in vivo activity comparable to that of erythromycin against *Staphylococci*, *Streptococci*, and *Diplococci* [2,3]. Its chemical structure was shown by Hoeksema et al. [4]. It is used in both human and veterinary medicine.

Traditionally, lincomycin in complete feeds, supplements, premixes and veterinary preparations is determined by microbiological assay [5–7] or thin-layer chromatography [8]. However, it is very difficult to differentiate lincomycin from other substances using microbiological methods, which moreover require considerable expenditure of time and specialized skills. Microbiological and TLC methods showed poor sensitivity, accuracy and selectivity, and therefore nowadays are used mainly column separation techniques. Gas chromatographic procedures require elaborate extraction and derivatization steps (pre-column derivatization into volatile esters) [9–11].

In the literature, there are many HPLC methods with ultraviolet, electrochemical [12,13] and MS detection [14–16] for the determination of lincomycin in food of animal origin and pharmaceutical dosage forms [17]. Lincomycin has only a weak UV absorbance in the low wavelength range (210 nm), and with a few exceptions [17–19], HPLC with photometric detection does not allow the sensitive determination of lincomycin in complicated matrix. Determination of lincomycin in fermentation beers using ion-pair reversed-phase LC on octylsilica gel with UV detection at 214 nm was reported too [20]. Sulfur-containing antibiotics that do not contain fully oxidized sulfur can be detected electrochemically. The electrochemical detection process for sulfur compounds on noble metal electrode surfaces has been described by LaCourse and co-workers [21–23]. Method for quantitation of lincomycin residues in tissues by ion-pair reversed-phase LC

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with electrochemical detection [24] is highly selective for lincomycin.

To date, no report has been published using such method for animal premixes. The purpose of this study was to develop a rapid, simple and sensitive quantitative HPLC method for determination of lincomycin in premixes using a phenyl column for chromatographic separation followed by UV detection at 208 nm. Since at this region many UV-absorbing components presented in analyzed samples could interfere, the selectivity of separation had to be optimized

## 2. Experimental

### 2.1. Chemicals and materials

Solvents, acetonitrile and methanol, were of HPLC grade (Merck, Germany). Water purified on Milli-Q system (Millipore, USA) was used. Other chemicals were of analytical grade. Extraction solvent was made by combining 950 ml water and 50 ml methanol. Carrez solution I was prepared by dissolution of 21.9 g dehydrated zinc acetate in water, then 3 ml glacial acetic acid was added and solution was diluted to 100 ml with water. Carrez solution II was prepared by dissolution of 10.6 g potassium ferrocyanide in 100 ml water.

The extracts were cleaned up using separation unit Baker SPE 12G System (J.T. Baker, USA) on OASIS HLB Cartridge columns (Waters, USA).

### 2.2. Instrumentation

Sample extraction was performed on laboratory horizontal shaker. All chromatographic experiments were carried out using a liquid chromatograph system consisting of Alliance 2695 and PDA detector W2996 (all Waters, USA). The system was controlled by data station PC Compaq using Millennium software (Waters, USA).

The HPLC/MS equipment consisted of a Waters Alliance 2690 system (Waters, UK), connected to a Micromass Quattro Premier Mass Spectrometer with Z Spray$^{TM}$ API source operating in positive ion electrospray (ESI) mode (Micromass UK, UK). The MS system was controlled by the Masslynx software Version 4.0.

### 2.3. Chromatographic conditions

HPLC separations were performed on a 150 mm × 4.6 mm, 4 μm Phenomenex Synergi Polar-RP Column (Phenomenex, USA) and on a 150 mm × 3.0 mm, 4 μm RPAmide C16 (Supelco, USA) as alternative column. The mobile phase was 875:125 (v/v) 0.2% phosphoric acid in water–acetonitrile and 950:50 (v/v) 0.2% phosphoric acid in water–acetonitrile as alternative mobile phase for RPAmide column. Mobile phases were prepared by mixing volume to volume of the components. The flow rate was 0.8 ml min$^{-1}$, the detection wavelength 208 nm, the injection volume was 50 μl, the column was thermostated at 35 °C and the run time was 8 min.

The mobile phase for MS–MS experiments had the following composition acetonitrile–water–formic acid (125:875:1, v/v) and separation was performed on a 150 mm × 4.6 mm, 4 μm Phenomenex Synergi Polar-RP Column (Phenomenex, USA). The flow rate was 0.5 ml min$^{-1}$, injection volume was 5 μl.

### 2.4. Standard preparation

The standard of lincomycin (Fluka, Germany; purity 102.7%) was dissolved in acetonitrile at a concentration of 1000 mg l$^{-1}$ to obtain the standard stock solution.

### 2.5. Sample preparation

The real samples of premixes and compounded feeds were homogenized and grinded to particles of 0.5 mm and less. A portion (from 1.0 to 2.5 g of premix sample and 10.0 g of compounded feed sample) was weighed into a 100-ml volumetric flask, 80 ml extraction solvent was added, and this mixture was shortly shaken by hand. The sample was extracted for 10 min on a horizontal shaker and then for 5 min in ultrasonic bath. Dissolved proteinanceous substances were precipitated with Carrez solution I (1 ml) and Carrez solution II (1 ml). This mixture was shortly shaken by hand and volumetric flask was filled to volume 100 ml with extraction solvent.

The preconcentration was performed on an OASIS HLB Cartridge column. After filtration, 1–5 ml of filtrate was applied on an OASIS HLB SPE column (previously activated with 5 ml methanol and 5 ml water) and the cartridge was washed with 2 ml of extraction solvent and with 2 ml of water. The SPE column was dried under vacuum for 30 s, and then lincomycin was eluted with 5 ml of methanol, collected in a 25-ml volumetric flask. The volumetric flask was filled to the mark with 0.2% phosphoric acid. The solution was injected into the liquid chromatograph. If necessary the extract solution was filtered through a 0.45 μm membrane filter before injection.

## 3. Results and discussion

### 3.1. Development and optimization of the HPLC method

Early method development highlighted limitations placed on the chromatography due to the physico-chemical properties of lincomycin. Lincomycin UV absorbance is too weak for quantitation above 208 nm, so the possible mobile phase composition was limited. Hence, HPLC method development was limited to an acetonitrile/phosphoric acid mobile phase using Polar-RP Phenyl and RPAmide C16 columns at low UV wavelengths and to variation of pH, temperature and volume fraction ($\varphi$) of organic solvent in mobile phase mixture.

The mobile phase was optimized to reach the capacity factor $k \geq 1.5$, theoretical plate number $N \geq 3000$ and asymmetry factor $t_a \leq 1.4$. The experimental parameters of optimized chromatographic method were determined using calibration solution of standard (at concentration of 10 mg l$^{-1}$).

The pH and ratio of acetonitrile to phosphoric acid were optimized with the set conditions at 30 °C, 208 nm wavelength, 0.2%

phosphoric acid and flow rate 0.8 ml $min^{-1}$ on a Phenomenex Synergi Polar-RP Phenyl column and on a RP Amide C16 column. To the test robustness of developed method the pH of mobile phase was always adjusted with potassium hydroxide (5 M) to pH 2.0, 2.25, 2.50, 2.75 and 3.00. The pH of mobile phase had no influence on retention of lincomycin and response of UV detector in the studied range.

To evaluate the influence of organic solvent fraction in mobile phase, Eq. (1) can be used:

$$\log k = \log k_a - m\varphi \tag{1}$$

where $k_a$ is the (extrapolated) value of $k$ for $\varphi = 0$ (in this case it corresponds to retention in 0.2% phosphoric acid) and $m$ is a constant for each solute [25,26]. The calculated Eq. (1) for volume fraction $\varphi = 0.05$–$0.20$ is consecutive: $\log k = 1.2313 - 5.9922\varphi$ ($r = -0.9894$) for Phenomenex Synergi Polar-RP Phenyl column. The calculated correlation coefficient $r$ was poor, so Eq. (1) have been re-calculated for narrower volume fraction $\varphi = 0.075$–$0.15$: $\log k = 1.2364 - 6.4460\varphi$ ($r = -0.9983$). Eq. (1) for volume fraction $\varphi = 0.025$–$0.10$ using RPAmide C16 column is consecutive: $\log k = 0.9827 - 6.7324\varphi$ ($r = -0.9936$). The above equations allow prediction of retention of lincomycin in studied chromatographic systems (for mentioned ranges of volume fractions).

The effect of temperature on the retention in RP-HPLC has been previously examined, e.g., by Melander et al. [27]. The expected temperature dependence of retention can be expressed using van't Hoff's equation [28]:

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln\frac{V_S}{V_M} = A + \frac{B}{T} \tag{2}$$

where $\Delta H^\circ$ and $\Delta S^\circ$ are the standard enthalpy and standard entropy in chromatography system, $R$ the gas constant, $V_S$ the stationary phase volume, $V_M$ the mobile phase volume, and $A$ and $B$ are the constants dependent on chromatographic system. In presented study linear van't Hoff plots have been obtained over narrow temperature range (30–50 °C). The calculated Eq. (2) for temperature range 30–50 °C is consecutive: $\ln k = -0.061 + 280.2/T$ ($r = -0.9957$) for Phenomenex Synergi Polar-RP Phenyl column. Eq. (2) for the same temperature range using RP Amide C16 column is consecutive: $\log k = -1.149 + 782.1/T$ ($r = -0.9916$). The above equations allow prediction of retention of lincomycin in studied chromatographic systems (for mentioned ranges of temperature). The suitable temperature for separation of lincomycin is 35 °C.

Using obtained information concerning to behavior of lincomycin in studied chromatographic systems the useful experimental conditions were selected and separation of lincomycin from matrix components in a short analysis time (below 8 min) was achieved. The optimal mobile phase contains 125 volumes of acetonitrile and 875 volumes of 0.2% phosphoric acid. Typical chromatogram of an extract of premix analyzed under proposed chromatographic conditions is shown in Fig. 1.

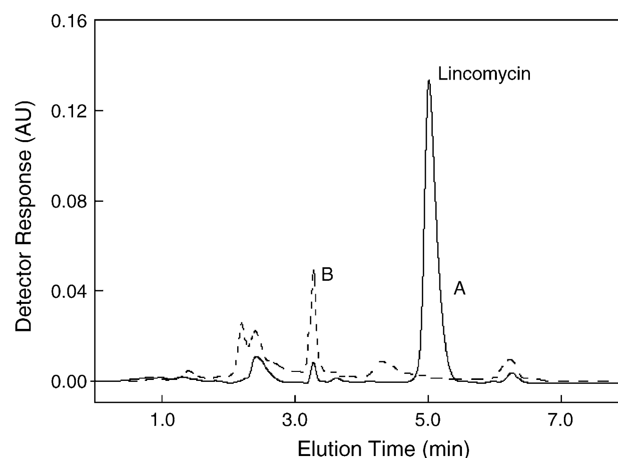


Fig. 1. Chromatograms of lincomycin in real premix sample (content 100,000 mg $kg^{-1}$); A, extract of real premix sample; B, blank extract. Capacity factor $k = 2.35$, plate number $N = 3500$, asymmetry factor $t_a = 1.4$.

### 3.2. Linearity, limit of detection and limit of quantitation

A set of six standard solutions at the following concentrations was prepared: 0.2, 2.0, 4.0, 8.0, 20 and 60.0 mg $l^{-1}$. Each of them was analyzed in duplicate. The calibration curve was constructed by plotting the peak area against the concentration and the calibration equation was calculated using linear regression analysis. It showed slope 28,163, $y$-intercept 4772 and correlation coefficient of 0.9999 what indicates an excellent linearity. The calibration curve was prepared in range from 0.2 to 60 mg $l^{-1}$, which is satisfactory with regard to actual content of lincomycin in premixes.

The average limit of detection of lincomycin (based on a detector signal-to-noise ratio 3:1) was 0.075 mg $l^{-1}$; the average limit of quantitation of lincomycin (based on a detector signal-to-noise ratio of 10:1) was 0.26 mg $l^{-1}$. The found limit of detection and limit of quantitation correspond to 1.5 and 5.2 mg $kg^{-1}$, respectively, in a real feed sample using the treatment described in the experimental section. The baseline noise was measured using four different chromatograms of the blank feed extracts in the region of retention time of lincomycin using chromatographic software. All of the above-presented limits were verified experimentally by measuring blank feed samples fortified with lincomycin (for above calculated amounts). All calculated limits are sufficiently low with regard to expected amounts of lincomycin in real samples.

### 3.3. System suitability

The system suitability test is performed to assure that the analytical method can be executed with the existing HPLC system. A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of a system suitability/calibration standard (at concentration of 10 mg $l^{-1}$) were made. Area and retention time relative standard deviation, asymmetry factor $t_a$ and efficiency (as plate number $N$) for the five injections were determined. For all samples anal-

yses, the asymmetry factor $t_a$ was $\leq 1.4$, efficiency $\geq 3000$ and area %R.S.D. $\leq 1.0\%$.

### 3.4. Optimization of sample preparation

Solid-phase extraction was used as an important step of the sample preparation. The extraction solvent (5% methanol in water) was tested as rinsing solvent to eliminate sample matrix components, which might interfere in HPLC determination. The extraction solvent did not cause any loss of analyte during cartridge rinsing up to 5 ml of solvent volume. Quantitative elution of lincomycin from SPE cartridge is apparent after 5.0 ml of methanol. The reproducibility and recovery of solid-phase extraction was determined from five repetitions. The reproducibility expressed as R.S.D. was 0.6% and recovery was 98.8% for concentration of 8 mg l$^{-1}$ of lincomycin.

### 3.5. Accuracy and precision

#### 3.5.1. Premixes

Model samples of premix were prepared to test the accuracy of the developed method. Different amounts of lincomycin were added to the mixture of subsequent components 60% wheat and 40% calcite to prepare samples with different concentration levels. For each level, six analyses were performed. The results and statistical parameters are summarized in Table 1. The average overall recovery at the 10,000, 50,000, 100,000 and 150,000 mg kg$^{-1}$ levels was 99.8% with a standard deviation of 3.7%. Determined contents ($c_d$) were compared with expected ones ($c_e$) using linear regression. The regression equation (significance level $P = 0.95$) was $c_d = (-47.49 \pm 1129.8) + (1.004 \pm 0.012)c_e$ and $R^2 = 0.9999$. The first and second constants were not statistically different from zero and one, respectively. It can be concluded that analytical method gives accurate results for premixes.

#### 3.5.2. Compounded feeds

Model samples of feeds were prepared to test the accuracy of the developed method. Different amounts of lincomycin were added to the compounded feeds for pig to prepare samples with different concentration levels. For each level, six analyses were performed. The results and statistical parameters are summarized in Table 2. The average overall recovery at 250, 500 and 750 mg kg$^{-1}$ levels was 100.4% with a standard deviation of 4.2%. Determined contents ($c_d$) were compared with expected ones ($c_e$) using linear regression. The regression equation (significance level $P = 0.95$) was $c_d = (25.01 \pm 123.74) + (0.9393 \pm 0.2227)c_e$ and $R^2 = 0.9998$. The first and second constants were not statistically different from zero and one, respectively. It can be concluded that analytical method gives accurate results for feed.

Table 1
Results and statistical parameters for analyses of model premix samples ($n = 6$)

| Statistical parameters | | | | |
|---|---|---|---|---|
| Expected value (mg kg$^{-1}$) | 10000 | 50000 | 100000 | 150000 |
| HPLC assay, average (mg kg$^{-1}$) | 9756 | 50396 | 100507 | 150362 |
| Relative standard deviation (R.S.D.) (%) | 7.4 | 4.3 | 3.0 | 2.3 |
| Recovery (%) | 97.6 | 100.8 | 100.5 | 100.2 |

Table 2
Results and statistical parameters for analyses of model compounded feed samples ($n = 6$)

| Statistical parameters | | | |
|---|---|---|---|
| Expected value (mg kg$^{-1}$) | 241.6 | 483.2 | 724.8 |
| HPLC assay, average (mg kg$^{-1}$) | 254.4 | 474.0 | 708.2 |
| Relative standard deviation (R.S.D.) (%) | 2.1 | 1.4 | 3.1 |
| Recovery (%) | 105.3 | 98.1 | 97.7 |

### 3.6. Intermediate precision

The intermediate precision of the method was assessed during 2 days. On each day the same premix sample (110,000 mg kg$^{-1}$) was six times analyzed by different analysts at the same equipment. The approximate lincomycin concentration in the analyzed solutions was about 45 mg l$^{-1}$. Results are shown in Table 3. One-way ANOVA was carried out to determine statistical difference between two sets of data. According to calculated results, the difference between the sets was not statistically significant at 95% confidence level ($F_{value}$ (1.247) < $F_{crit}$ (5.050)).

### 3.7. Analysis of real samples

The developed method was verified on real samples of different commercial premixes. Table 4 shows a comparison of assay

Table 3
Intermediate precision of the method

| | Analyst 1 ($n = 6$) | Analyst 2 ($n = 6$) | Intermediate precision ($n = 12$) |
|---|---|---|---|
| Mean (mg kg$^{-1}$) | 103358 | 98043 | 100700 |
| Standard deviation (S.D.) (mg kg$^{-1}$) | 6130 | 5490 | 6203 |
| Relative standard deviation (R.S.D.) (%) | 5.9 | 5.6 | 6.2 |
| Confidence (at 95% level) (mg kg$^{-1}$) | 6433 | 5761 | 3941 |

Table 4
Results of assay lincomycin in four different commercial brands

| | Concentration declaration (mg kg$^{-1}$) | Concentration found (mg kg$^{-1}$) |
|---|---|---|
| Lincofarm 110 px (Chemifarma, Italy) | 110000 | 104400 |
| Lincomycina 11% (Chemifarma, Italy) | 110000 | 100700 |
| Linkomicin 110 N premix (Tekro, Czech Republic) | 110000 | 106900 |
| Premix of lincomycin and spectinomycin (Biofaktory, Czech Republic) | 11000 | 10100 |

values with declared contents in samples obtained from three different producers.

### 3.8. LC–MS/MS confirmation

UV detection at 208 nm has to be considered as non-selective and sometimes it can be necessary to carry out confirmation of presence of analyte in sample by mass spectrometry. Effectiveness of ionization of the analyte was investigated by analyzing an appropriate amount of the standard (50–100 ng $ml^{-1}$) under different modes of ionization (electrospray positive and negative, respectively). As the negative ionization mode did not give significant signals for analyte, it was not selected for further experiments. The parent ion was used as the precursor for formation of MRM fragments in tandem mass spectrometry. Further MS–MS experiments were performed to generate the major product ion fragments. The final MS conditions were achieved by optimizing of the capillary voltage, desolvation temperature, gas flow and ion-focussing potentials whilst continuously infusing 0.4 μg $ml^{-1}$ standard solution at a flow rate of 0.5 ml $min^{-1}$.

The following MS–MS parameters were used: capillary voltage: 3.1 kV; cone voltage: 25 V; source temperature: 120 °C; desolvation temperature: 350 °C; collision energy: 24 eV; collision gas pressure: $2.3 \times 10^{-3}$ mbar ($N_2$).

In HPLC parameters we used same chromatographic column and composition of mobile phase as HPLC-UV method, we changed the flow rate to 0.5 ml $min^{-1}$ because it is more optimal for ESI ionization and we decreased injection volume to 5 μl due the higher sensitivity of MS instrument.

Identity of lincomycin was confirmed by the presence of two fragments (at *m/z* 126.1 and 359.2) from the precursor ion at the defined retention time window and matching of the specific tolerance of relative abundance of the major ions as stated in the Commission Decision 2002/657/EC [29]. As illustrated in Fig. 2, *m/z* 126.1 corresponds to the 3-propyl-*N*-methylpyrrolidine ion (A) and *m/z* 359.2 is due to the loss of thiomethanol molecule (B) from the respective parent ion of lincomycin. MS–MS method could by used for quantitation especially for low concentration, but for the feed sample in which the concentration of lincomycin is sufficient for HPLC-UV method, mainly we used MS–MS for confirmation. Quantitation was based on the relative ratios of the summation of peak areas of major ions of the analytes with reference to the respective ratios of the calibration standards. The average limit of quantitation of lincomycin (based on lowest positive signal) is 0.1 mg $kg^{-1}$. Fig. 3 show the reconstructed MRM chromatogram that was obtained for lincomycin in spiked control compounded feeds sample. The concentration of lincomycin in the spiked feed control was 250 mg $kg^{-1}$.

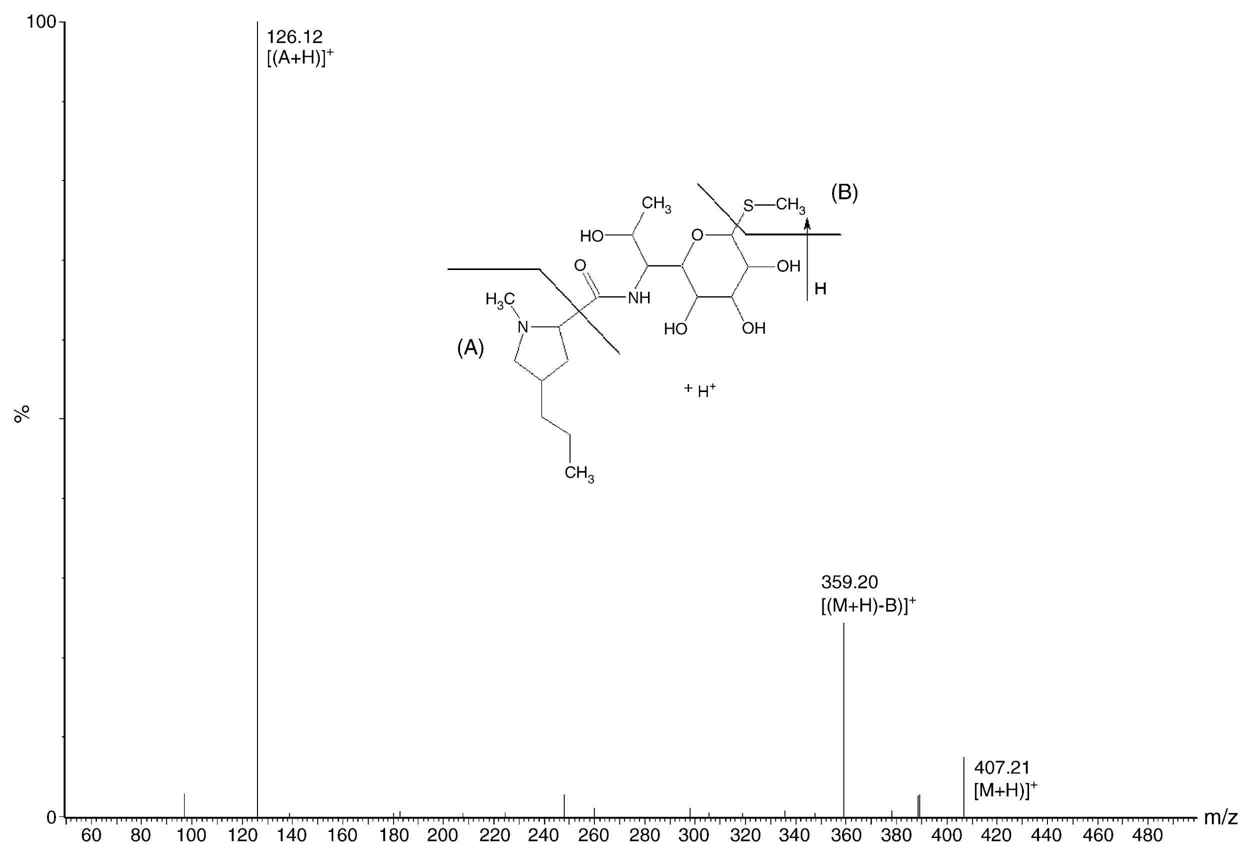


Fig. 2. A tandem mass spectrum of lincomycin (100 ng $ml^{-1}$ in 0.1% formic acid in water) with collision-induced dissociation of quasimolecular ion ($[M+H]^+ = 407$) leading to daughter ions at *m/z* 126.1 and 359.2.

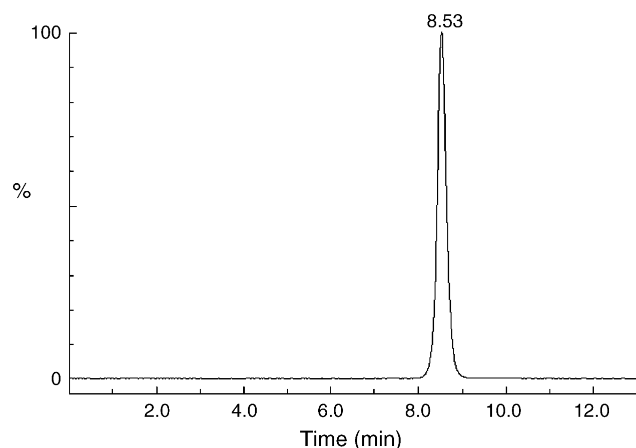


Fig. 3. Reconstructed MRM chromatogram of ions (at *m/z* 359 and 126) for control compounded feed fortified with 250 mg $g^{-1}$ of lincomycin.

## 4. Conclusion

The developed HPLC procedure allows short analysis (below 8 min) with satisfactory UV detection and it is convenient for determination of lincomycin in premixes and feeds in content ranging from 250 to 150,000 mg $kg^{-1}$. In comparison with described methods for determination of lincomycin, developed HPLC method is very simple, rapid and enough sensitive for determination in premixes and feeds without derivatization step. Elimination of interfering compounds, without loss of target analyte, is achieved. Evaluation of method demonstrates satisfactory statistical parameters for its application to lincomycin determination in studied matrices. Liquid chromatography coupled with mass spectrometry is rapidly becoming the method of choice for the determination of lincomycin in feeds. The use of confirmation ions (*m/z* 126 and 359) provides additional confidence in the identification of drug.

Preparation of samples in series and short chromatographic run also offers the application of developed method in routine laboratory assays.

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