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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 ± 7.3%, in concentration range of 250–750 mg kg^{-1}, and 99.8 ± 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.

Keywords: HPLC determination; LC–MS/MS confirmation; Lincomycin; Premix; Validation

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 (φ) 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%