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Determination of tylosins A, B, C and D in bee larvae by liquid chromatography coupled to ion trap-tandem mass spectrometry

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ABSTRACT

A LC–MS/MS method has been developed to simultaneously quantify tylosins A, B, C and D in bee larvae, compounds currently used to treat one of the most lethal diseases affecting honey bees around the world, American Foulbrood (AFB). The influence of different aqueous media, temperature and light exposure on the stability of these four compounds was studied. The analytes were extracted from bee larvae with methanol and chromatographic separation was achieved on a Luna C_{18} (150 × 4.6 mm i.d.) using a ternary gradient composed of a diluted formic acid, methanol and acetonitrile mobile phase. To facilitate sampling, bee larvae were initially dried at 60 °C for 4 h and afterwards, they were diluted to avoid problems of pressure. MSD-Ion Trap detection was employed with electrospray ionization (ESI). The calibration curves were linear over a wide range of concentrations and the method was validated as sensitive, precise and accurate within the limits of quantification (LOQ, 1.4–4.0 ng/g). The validated method was successfully employed to study bee larvae in field tests of bee hives treated with two formulations containing tylosin. In both cases it was evident that the minimal inhibitory concentration (MIC) had been reached.

1. Introduction

American Foulbrood (AFB) is an infectious disease of *Apis mellifera* honey bee larvae [1] that affects apiculture worldwide. This disease is caused by the Gram-positive spore-forming *Paenibacillus larvae* subsp. *Larvae* [2], which can produce over one billion spores in each infected larva. The spores are extremely heat stable and resistant to chemical agents, and only the spores are capable of inducing the disease. Infection can be transmitted to larvae by nurse bees or by spores remaining at the base of a brood cell. Although the larvae stages of worker bees, drones and queens are susceptible to infection, infected queens and drone larvae are rarely observed under natural conditions. Moreover, the susceptibility of larvae to AFB diminishes as the larva age [3]. AFB is a highly contagious disease and exchanging combs containing the remains of diseased brood is the most common means by which the disease spreads from colony to colony [4]. In addition, the spread of the disease may also be facilitated by feeding or robbing spore laden honey or bee bread, packaged bees and the introduction of queens from infected colonies. Similarly, wax used to produce the comb foundations and that is contaminated with *P. larvae* spores can also spread the disease.

In geographical areas where AFB is more prevalent, antibiotic treatment appears to offer an alternative to the burning of diseased colonies. The antibiotic oxytetracycline hydrochloride (OTC) is one of the most commonly employed to treat AFB and it has been used worldwide for decades to control the disease. Indeed, the accumulation of this antibiotic in honey has been determined in several studies [5–7]. However, tetracycline resistant strains have been identified in the USA, Canada and Argentina [8–13], and so other antibiotics, such as tylosin and lincomycin have also been proposed to control AFB [14–19].

When the incidence of AFB is high or during epidemics (such as the epidemic affecting some Spanish regions in 2008), therapeutic approaches like the use of antibiotics must be combined with prophylactic actions to help professionals control the appearance of the disease and avoid having to assume the high cost associated with the burning of affected beehives. For this reason, it is interesting to examine the potential to use alternative antibiotics for disease treatment. To be effective, such antibiotics must reach the larvae at a dose sufficiently high as to prevent the growth of the bacteria for long enough periods of time. Subsequently, the treatment must be reapplied to maintain the inhibitory concentration in the targets (larvae). In previous laboratory assays, we found that the inhibitory

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dose of tylosin for Spanish strains of *P. larvae* is 0.12 μg/ml and no resistance to tylosin was detected with any isolate of the Foulbrood pathogen [20]. These values indicated that very low concentrations of tylosin were required to inhibit the growth of *P. larvae* in field conditions, as suggested elsewhere [14].

The tylosin series of macrolides (Fig. 1) comprises several related substances of which tylosin A (TA) is the major component. Other minor constituents include tylosin B or desmycosin (TB), tylosin C or macrocin (TC) and tylosin D or relomycin (TD). These four compounds each contribute to the potency of tylosin [21], although tylosin A has the strongest activity, and all of them can be found in the technical product. These natural products exhibit distinct antibacterial activity against numerous gram-positive bacteria and mycoplasma, binding to their ribosomes and inhibiting protein synthesis [22].

In order to optimize the dose, it is necessary to develop an analytical method to determine the true concentration of tylosin in bee larvae after application of this antibiotic in field conditions. Several studies and even reviews have been published on the use of LC–MS/MS or LC–MS to analyze tylosin in several matrices, like water [23–26], honey [27–31] and food matrices [32–35], but not in bee larvae. In most of these studies only tylosin A was analyzed, although tylosin B was also studied in one report [28], and tylosins A, B, C and D have been determined in honey [27] and water [23]. As there are no prior references to studies in bee larvae, we set out to define a procedure to prepare the samples and to optimize a sensitive LC–MS/MS (MSD-Trap) method [27] in order to detect the lowest amounts of tylosins in this matrix. We also performed some studies to assess the influence of stressful (alkaline, oxidative and acidic) media and laboratory conditions (temperature, light) on tylosin stability. Although there have been some attempts to define the pH, light and temperature stability of these compounds [36–39], these have only been performed on tylosin A.

Finally, we successfully validated the method developed by analyzing samples of bee larvae obtained from field tests on honey bee colonies infected with AFB and treated with three formulations, two of them containing different amounts of tylosin, with the aim of finding a useful dose.

2. Materials and methods

2.1. Materials and chemicals

Tylosins A, B and C were obtained from Professor Hoogmartens (Katholieke Universiteit Leuven, Leuven, Belgium), Tylosin D was obtained from the European Council Pharmacopeia (Strasbourg, France) and the Tylosin technical product (tylosin tartrate$_{TP}$) was generously donated by Laboratorios Calier S.A. (Barcelona, Spain). Formic acid and roxithromycin (internal standard, IS) were purchased from Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). HPLC grade methanol and acetonitrile were both supplied by Labscan Ltd. (Dublin, Ireland), and analytical grade glacial acetic acid, sodium hydroxide and *hydrochloric acid* (37%, m/v) were obtained from Merck (Darmstadt, Germany). Syringe filters (17 mm Nylon 0.45 μm) were purchased from Nalgene (Rochester, NY, USA) and deionised water was obtained from a Milipore Mili-RO plus system together with a Mili-Q system (Bedford, MA, USA).

A 5810R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany) was also used. Finally, a drying oven from Selecta (Barcelona, Spain) was used in the stability tests.

2.2. Preparation of standard solutions

Individual standard stock solutions (drugs and roxithromycin) were prepared in methanol at a concentration of 200 mg/L. An intermediate standard solution was prepared by combining portions of each standard stock drug solution, and this intermediate standard solution was diluted daily with water to produce a set of working standards.

Matrix-based standards were made by extracting 2 g of the larvae obtained from beehives not treated with tylosin tartrate$_{TP}$ but that were spiked with the antibiotics studied. This material was reconstituted from the dried residue with 1 mL of a 1:1 (v/v) methanol:water mixture. All the standards and stock solutions were kept in the dark at +4 °C until analysis and they were stable for over one month.

2.3. Stability of the compounds

Several tests were performed to check the stability of the four compounds in an aqueous media under different conditions. Firstly, the stability of the tylosins was checked in acidic, oxidative and alkaline media. The influence of light exposure under the precise experimental conditions of the laboratory and temperature on the stability of the compounds was also studied by exposing solutions of the four compounds to different conditions.

2.4. LC–MS/MS system

An Agilent Technologies 1100 series LC/MSD Trap XCT (Palo Alto, CA, USA) instrument was used with electrospray ionization in positive ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump and an autosampler with a column oven. The system was controlled by an Agilent ChemStation for LC Rev. A. 10.02 and MSD Trap Control version 5.2. Data analysis was performed by Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 3.2, all of them form Agilent Technologies (Palo Alto, CA, USA).

A Luna 5 μm C_{18} (2) 100 Å (150 × 4.60 mm i.d.) analytical column was used for LC separation, and it was protected by a C_{18} guard column (4 × 2.0 mm i.d., both from Phenomenex, Torrance, CA, USA). After the optimization study, the mobile phase components used were 1% formic acid in water (solvent A), methanol (solvent B) and acetonitrile (solvent C), applied at a flow rate of 0.5 mL/min in a gradient mode as follows: (i) 0 min (A–B–C, 63:37:0, v/v/v); (ii) 10 min (A–B–C, 80:0:20, v/v/v); (iii) 18 min (A–B–C, 74:0:26, v/v/v); (iv) 20 min (A–B–C, 64:0:36, v/v/v); (v) 22 min (A–B–C, 63:37:0, v/v/v); (vi) 32 min (63:37:0, v/v/v). The injection volume and column temperature were 50 μL and 25 °C respectively. All ESI-MS analyses were performed using multiple reaction monitoring (MRM) in ultra scan mass range mode, scanning from m/z 100 to 1000. The ESI-MS/MS detection conditions were optimized for each compound, and with the exception of the capillary voltage and some fragmentation parameters (Table 1), the other parameters studied have the same effect on the sensitivity of each tylosin. The optimal MS/MS conditions were set as follows: Drying gas (N_2) temperature 350 °C; Drying gas (N_2) flow of 9 L/min; Nebulizer pressure at 40 psi; Trap drive 35; Skimmer 40 v: Octopole RF amplitude 130 V; Capillary exit 105.0; Max. Accumulation time 200 ms; ion charge control (ICC) 200,000; and Delay 5 ms.

Among the other MS/MS parameters the transitions monitored for each compound are summarized in Table 1. Matrix matched calibration curves with an internal standard were used for quantification, where relative response factors for the most intense MS/MS transition of each antibiotic were compared to the intensity of the transition monitored for roxithromycin. These calibration curves were constructed by plotting the ratios between the signal (areas) of each tylosin and the internal standard in the y-axis, against the concentration values in the x-axis. A weighting factor of $1/x^2$ was applied to the linear regression analysis and a second MRM transition was monitored for confirmatory purposes.