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Short Communication

Biotic transformation of anticoccidials in soil using a lab-scale bio-reactor as a precursor-tool

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ABSTRACT

Two anticoccidial agents, salinomycin and robenidine, heavily used in the worldwide veterinary meat production, were investigated for their potential biotic degradation by cultured soil bacteria. The degradation-study was performed in lab-scale bio-reactors under aerobic and anaerobic conditions incubated for 200 h with a mixed culture of soil bacteria. Samples were analyzed by LC-MS/MS and potential transformation products were tentatively identified. Salinomycin was degraded under aerobic conditions and traces could be found after 200 h, however, seems more persistent under anaerobic conditions. Four transformation products of salinomycin were discovered. Robenidine was degraded under aerobic and anaerobic conditions, however, traces of robenidine were observed after 200 h. Five biotic transformation products of robenidine were discovered.

1. Introduction

Anticoccidial pharmaceuticals or coccidiostats are intensively and legally used in meat production industry as prophylactic veterinary feed additives and as growth promoters (European Food Safety Authority, 2009; US Food and Drug Administration, 2009). These drugs possess antimicrobial and antiparasitic potency of which the primarily aim is to reduce outbreaks of the parasitic disease coccidiosis. As stated in recent reviews anticoccidials are now reported in several environmental compartments (Hansen et al., 2009a) and have also been identified as potential environmental contaminants that will pose an eco-toxicological risk (Hansen et al., 2009b). Despite their heavy application and possible eco-toxicological risk, the knowledge on environmental fate and effects of these emerging contaminants is limited. In many countries it is not required to monitor the usage of feed additives, thus no complete picture of the consumption data is available. However, the usage is monitored in Denmark, where total consumption of anticoccidials reflects that ionophores are used in the highest amounts, salinomycin being the most heavily applied (Statens Serum Institut et al., 2004). The more potent synthetic anticoccidials are consumed in smaller quantities, where robenidine is the most used (Statens Serum Institut et al., 2004). Previous research investigated the abiotic stability and antibacterial potency of salinomycin and robenidine (Hansen et al., 2009c), and the environmental fate of anticoccidials are emerging as a focus area, nevertheless, only scarce biodegradation data is available for salinomycin (Vertesy et al., 1987; Schlusener and Bester, 2006; Schlusener et al., 2006; Ramaswamy et al., 2010), while no data is available for robenidine. Likewise another ionophore, monensin, has initially been investigated and the soil dissipation was found to strongly depend on soil organic matter and water content with half-lives up to 23 d (Yoshida et al., 2010).

The aim of this initiative was to describe a fast methodology to discover environmental realistic transformation products using lab-scale batch bio-reactors combined with LC-MS^2, which later can be applied to investigate the 23 approved anticoccidials worldwide (Hansen et al., 2009a). Salinomycin and robenidine were selected among the coccidiostatics to simulate biotransformation under aerobic and anaerobic conditions, providing initial soil degradation profiles and preliminary information on transformation products. The obtained data will improve environmental risk assessment and observed transformation products can be eco-toxicologically evaluated (Hansen et al., 2009b).

2. Materials and methods

2.1. Chemicals and materials

Salinomycin SV sodium (purity 94%), was purchased from Sigma–Aldrich, Germany and robenidine hydrochloride (purity 96%)

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was obtained from Qmx Laboratories, Thaxted, Essex, United Kingdom. Stock solutions of 1000 ppm were prepared in dimethylsulfoxide. Nutrition media was prepared from 5 g sodium chloride, 3 g yeast extract, 1 g glucose and 5 g peptone in 1 L MilliQ water. All chemicals were of analytical grade. Bio-reactors (1 L in volume) were obtained from Skandinavisk Glasblæseri, Copenhagen, Denmark. Nitrogen was supplied by a generator and air was obtained from a compressed air system.

2.2. Extraction of bacteria from soil

A well-described agricultural Danish soil (Jyndevad) was used (pH 6.9, content of calcium, magnesium, sodium and potassium ions were 5.19, 0.71, 0.09 and 0.19 cmol(+) kg^{-1}, respectively, orthophosphate was 56.5 mg kg^{-1} soil, nitrogen and carbon content was 0.12% and 2.43%, respectively, the distribution of clay, silt and sand was 5%, 1% and 94%, respectively). The soil has not been exposed to antibiotics for the last 8 years. Separation of bacteria from soil was done using a Nycodenz density gradient as described elsewhere (Lindahl and Bakken, 1995).

2.3. Batch bio-reactors

Six sterile bio-reactors were added 400 mL autoclaved (121 °C for 20 min) nutrition media. Three reactors were kept at aerobic conditions by constant bubbling of compressed air and three reactors were kept at anaerobic conditions by constant bubbling of nitrogen. Room temperature was at all times kept at 19 ± 1 °C. Furthermore the reactors were shielded from light and incubated for 3 d before spiking with 200 μL of the Nycodenz bacteria extract to all reactors and incubated another 24 h before spiking to 10.0 ppm of salinomycin and robenidine in separate reactors and one matrix-matched blank (i.e., all reactors were spiked to 1% dimethylsulfoxide by volume) at both conditions. At the end of the study (200 h) the 400 mL media remaining in each of the six bio-reactors was liquid–liquid extracted with dichloromethane (3 × 10 mL), and further evaporated (nitrogen) and reconstituted in 500 μL mobile phase A.

2.4. Chemical analysis

Bio-reactor samples of 1000 μL were centrifuged at 15,000 g for 15 min and 200 μL supernatant was transferred to a HPLC vial and analyzed within 1 h. Sampling was done at 0, 22, 68 and 200 h. Agilent 1100 series HPLC coupled to a Sciex API3000 ESI-MS/MS system was used with 5 μL injections (autosampler temperature controlled to 4 °C) onto a Phenomenex MAX-RP C_{12} 2.0 × 150 mm, 4 μm column with a 4.0 mm guard column kept at 40 °C. An isocratic 300 μL min^{-1} flow of 10/90 or 40/60 mobile phase A/B was used for salinomycin and robenidine analysis, respectively. Mobile phase A consisted of 95/5 water/acetonitrile (v/v) and 5 mM ammonia acetate, while mobile phase B consisted of 5/95 water/acetonitrile (v/v) also with 5 mM ammonia acetate. The MS was operated in positive MRM mode monitoring 334.1 > 137.9 (40 V) and 336.1 > 139.1 (40 V) ion transitions for robenidine, and 773.5 > 431.5 (75 V) and 773.5 > 531.5 (50 V) for salinomycin. Other MS parameters; declustering, focusing, entrance and collision cell exit potential was set to 20, 200, 10 and 15 V, respectively. Additionally, full scan spectrums of each sample were acquired. Nitrogen was used as curtain, nebuliser, auxiliary and collision cell gases with flow rates of 8, 6, 6 and 4 L min^{-1}, respectively. Source temperature and spray voltage was 400 °C and 4500 V. A Valco valve was used as a diverter between the HPLC and the MS, and was set to infuse into the MS from 5.0 to 9.0 min. Retention times were 6.2 and 7.2 min for salinomycin and robenidine, respectively.

3. Results and discussion

3.1. Salinomycin

Degradation of salinomycin is substantial under aerobic conditions compared to anaerobic as seen in Fig. 1A. Already after 68 h at aerobic conditions no traces of salinomycin could be found. The aerobic data complies with previous findings by Vértesy et al., where salinomycin degraded in 20 h, even though the experiments were done with enzymes extracted from a pure bacteria culture and not by direct exposure to living bacteria (Vertesy et al.,

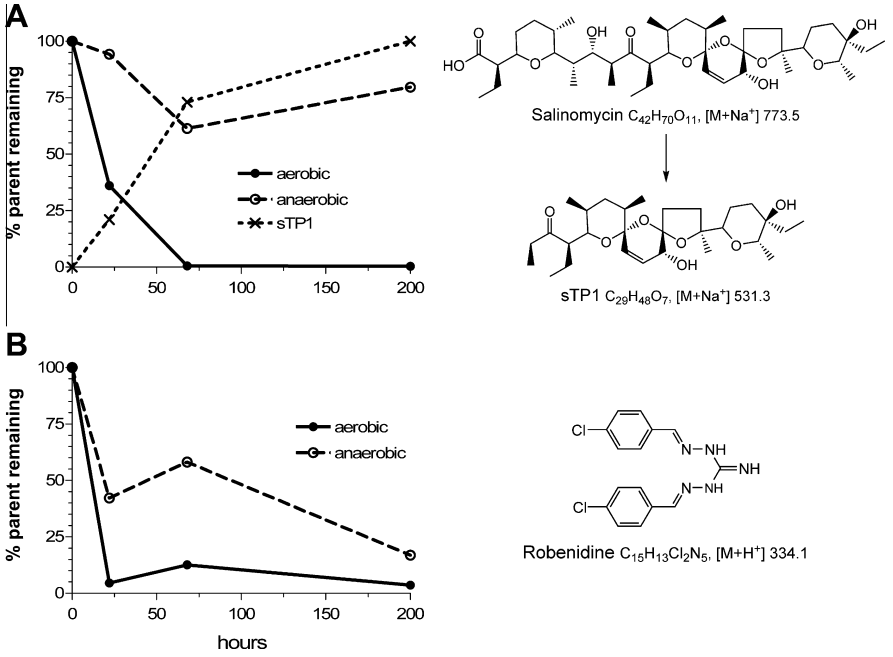

Fig. 1. Degradation profiles of salinomycin (A) and robenidine (B) under aerobic and anaerobic conditions. Dashed line with crosses displays the generation of transformation product 1 (sTP1) from salinomycin. The chemical structures of salinomycin, sTP1 and robenidine are shown to the right.