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Original article

Comparative stability of oxytetracycline and tylosin in sugar syrup[1]

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Abstract – Measurements of OTC stability in apicultural dosage forms have relied on methods (fluorescence, bactericidal effects) that are not specific to OTC. We have measured the stability of OTC and tylosin in sucrose syrup using high-performance liquid chromatography. At 34 °C in the dark, OTC in syrup made from highly purified sucrose had a half life of 7.6 days. Tylosin was considerably more stable, with a half life of 186 days; the half life of the total tylosin complex was 287 days. In syrup prepared from commercial-grade sugar, both materials were less stable, with OTC having a half life of 6.3 days and tylosin about 75 days. Microbiological data paralleled these findings, with a rapid decrease of inhibition zone against *Paenibacillus larvae* with OTC, and a persistence of inhibition with tylosin. © Inra/DIB/AGIB/Elsevier, Paris

oxytetracycline / tylosin / syrup / foulbrood / stability

1. INTRODUCTION

Oxytetracycline hydrochloride (OTC, Terramycin) has been used since the early 1950s [4, 7] for the prevention and control of American and European foulbrood, which are caused by two species of bacteria, *Paenibacillus (Bacillus) larvae* and *Melissococcus pluton*, respectively. One of its advantages is that it breaks down rapidly in solution and is therefore unlikely to contaminate honey. It is currently the only approved treatment for these diseases in the United States, and has been for many years. Recently, however, strains of *P. larvae* showing some tolerance to OTC have been

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[1] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture

discovered in a limited number of beekeeping operations (Shimanuki and Knox, unpublished data). This was not unexpected, as any time there is only one treatment for a disease or other pest, there is an increased chance of development of resistance.

In preparation for the inevitable development of resistance, the USDA has been screening alternate materials for many years. Tylosin was first reported as a candidate treatment for foulbrood in 1970 [5, 12]. Its usefulness has been confirmed in a recent paper [18]. None of these papers refers to analytical methods or stability of the antibiotic in syrup or other media.

Early assays for OTC relied on the inhibition of growth of a test organism, such as *Erwinia* sp. [3] or *Bacillus cereus* var. *mycoides* [9, 20]. Tylosin was also analyzed by such methods ([13] and references therein). While sensitive, bacteriological methods are incapable of distinguishing between antibiotics, nor are they able to assay individual compounds in mixtures. In addition, determinations of tetracyclines by fluorescence measurements of calcium complexes have been used [1]. While more selective, this method was still not able to distinguish between OTC and other tetracyclines, all or most of which fluoresce similarly.

High-performance liquid chromatography (HPLC) assays are now the preferred method for analysis of many substances of biological interest. Such methods have allowed both good sensitivity and simultaneous assay of various tetracyclines in honey [2, 10, 16, 19] as well as other samples, such as milk and meat (for example, see [14]). Tylosin can also be analyzed similarly, but using different elution systems [11, 13], for a recent review see [6]. For analysis in syrup formulations, a simplified form of this assay sufficed for determination of both antibiotics, since the extensive purification required for complex matrices was not required in this case. This analytical system was not optimized for tylosin, but could be used after making allowances for the differences in light absorption and the differences in retention time on the HPLC column.

For summaries of analytical methods for antibiotics, see Oka et al. [17] and Volume 812 of the Journal of Chromatography A (1998).

2. MATERIALS AND METHODS

Oxytetracycline hydrochloride, tylosin tartrate, and SigmaUltra grade sucrose were obtained from Sigma. A commercial sucrose, 'Sno-White Granulated Sugar' (anonymous), was obtained from our bee-feeding supply. Oxalic acid dihydrate (ACS reagent grade), acetonitrile (HPLC grade), and methanol (HPLC grade) were obtained from Aldrich.

Syrup was prepared by weight in volume, either 50 or 70 % as indicated, using reagent grade water (Nanopure, Barnstead/Thermolyne, Dubuque, IA). A weighed amount of either OTC or tylosin was dissolved in a small amount of syrup in a volumetric flask, then diluted with additional syrup to volume. Final antibiotic concentration was 200 $mg \cdot L^{-1}$. Unless otherwise noted, solutions were stored in the dark except for a few hours on the days of analysis, when they were in normal fluorescent room light.

Antibiotic determinations were carried out by direct injection of syrup solutions onto a 3 mm × 250 mm column packed with C_{18} silica gel (Supelcosil LC-18-DB, 5 μm particle size, Supelco, Inc., Bellefonte, PA), using a 20 μL injector loop. Flow rate was 0.7 $mL \cdot min^{-1}$ of methanol–acetonitrile–0.01 M oxalic acid (1:1.5:2.5 by volume) [15]. Antibiotic concentrations were determined with a SpectraSYSTEM UV2000 detector attached to an SP4400 integrator (Thermo Separation Products, San Jose, CA). OTC was determined at a wavelength of 350 nm, while tylosin concentrations were determined at a wavelength of 280 nm. Range was 1.0 absorbance units full scale for all analyses. Each point is the average of three injections. Injections of a series of standards in the range 2 ng–2 μg gave a linear plot with an average detector response of 4 348 detector units/ng for OTC and 3 050 detector units/ng for tylosin. The retention time of OTC was 3.26 min, and the main peak of tylosin (tylosin A) had a retention time of 9.2–10 min, depending on the age of the

oxalic acid solution. Other members of the tylosin complex eluted from 7–13 min (*figure 1*). Since the concentrations in these solutions were quite large, we did not investigate the detection limits for these antibiotics, which would be required for residue assays in honey, for example.

Data reduction and plotting were performed with GraphPad Prism ver. 2.01 (GraphPad Software, Inc. San Diego, CA).

Bacterial sensitivity was determined by our standard method, using *P. larvae*. A stock spore suspension (2×10^8 spores·mL^{-1}) was prepared by mixing the scale (dried remains of diseased honey bee larvae) with sterile water in a screw-capped tube. Before each use, the suspension was heat-shocked at 80 °C for 10 min of effective time to kill any non-spore-forming bacteria. For the bioassay, 0.2 mL of the stock suspension was spread over the surface of freshly prepared brain–heart infusion agar (BHI) plates (brain–heart infusion fortified with thiamine hydrochloride (0.1 mg·L^{-1}), 2 % agar, and adjusted to pH 6.6 with hydrochloric acid). For each test, aliquots (25 µL) from the antibiotic/syrup solutions were applied to paper assay discs (6.35 mm, no. 740-E, Schleicher and Schuell). The discs were positioned in the center of the BHI plates and the plates were incubated at 34 °C. The antibiotics diffuse into the medium, and the diameters of the zones of inhibition were measured after 72 h. We did not make any correction for disc diameter.

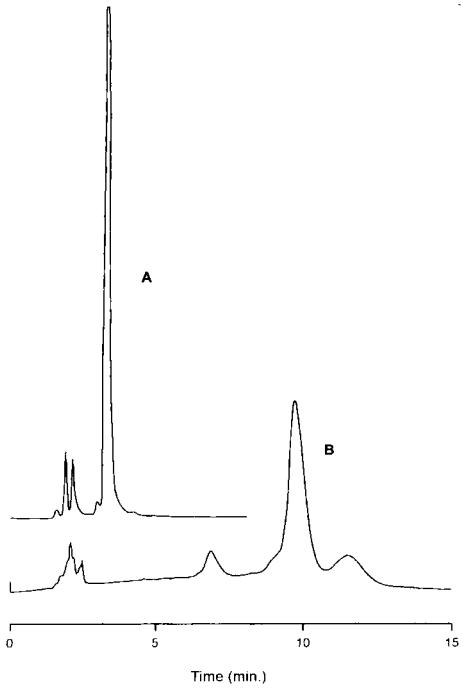

Figure 1. HPLC traces of OTC (curve A) and partially degraded tylosin (curve B). The small peaks from 1.5–2.5 min are impurities eluting near the solvent front.

3. RESULTS AND DISCUSSION

HPLC determinations showed that the OTC concentration decreased much more rapidly than did that of tylosin. Data fit well with a single-phase exponential decay model. In a preliminary run, in 50 % syrup at room temperature in room light, a half life for OTC of about 21 days was determined, compared to a half life for tylosin in excess of 35 days; however, the solutions became moldy so quickly that these data are not very accurate, and are not shown. The first long-term run, in 70 % syrup prepared from highly purified sucrose, and kept at 34 °C in the dark, the half life for OTC was approximately 7.6 days and a decrease to 10 % of initial concentration took 31 days (*figure 2a*). The second run, under the same conditions, but in 70 % syrup prepared from commercial sucrose produced a half life of approximately 6.3 days and a 10 % point in 17.5 days (*figure 3a*). These half lives are a little shorter than the values of 12–14 days observed in honey [2, 19]. The commercial sugar syrup was noticeably darker in color and became moldy faster. Both solutions darkened considerably after a few days as the OTC decomposed.

Tylosin was considerably more stable than OTC under these conditions, having decreased in purified sucrose syrup only to 76.5 % of the initial concentration after 83 days (*figure 2*). Extrapolation of the regression line suggested the half life for tylosin under these conditions would be approximately 186 days. These half lives represent the decrease of tylosin A, the

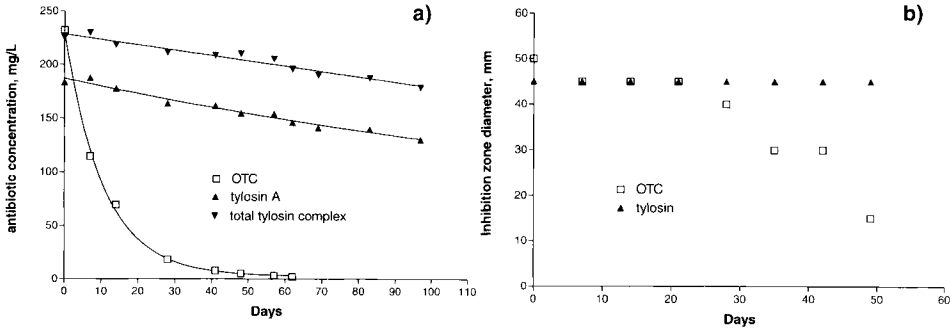

Figure 2. a) Stability of OTC and tylosin in 70 % sugar syrup prepared from purified sucrose. r^2 values: OTC 0.9979, tylosin A 0.9712, total tylosin complex 0.9428. b) Inhibition of *P. larvae* by OTC and tylosin.

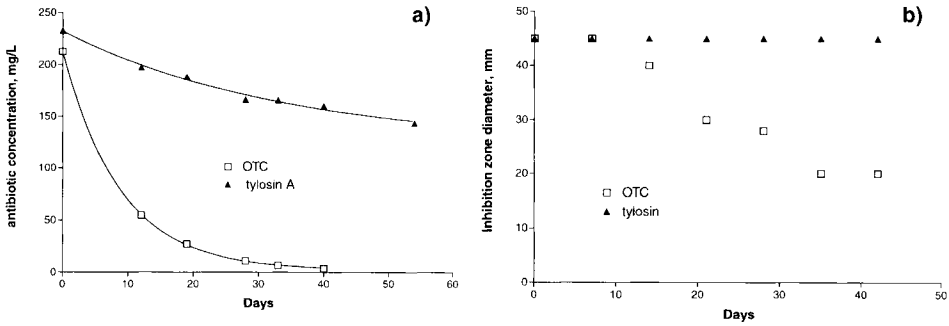

Figure 3. a) Stability of OTC and tylosin in syrup prepared from commercial sugar. r^2 values OTC 0.9997, tylosin A 0.9625. b) Inhibition of *P. larvae* by OTC and tylosin.

major component of the antibiotic complex. Other components are usually present, and hydrolysis of tylosin A can give other members of the complex, most of which are also active antibiotics [8]. The decrease rate of the total tylosin complex was even slower (note the upper line in *figure 2*), since the relative percentage of other members of the tylosin complex increased over time as the tylosin A was converted into related compounds. The half life for the entire tylosin complex is approximately 287 days. A similar decrease in stability was observed for tylosin in commercial sugar syrup, except that tylosin A had a half life of only about 75 days. The stability of the tylosin complex was not plotted separately for this run.

The differences in stability for the antibiotics in purified sucrose syrup versus commercial sucrose syrup are presumably due to traces of metal ion impurities, since metal ions, such as iron or copper, are known to catalyze oxidative deterioration of biological materials. The SigmaUltra grade sucrose is especially purified to minimize metallic impurities.

The high levels of antibiotics in the syrup samples and the lack of interfering substances enabled us to use a simplified analytical method for this work. The presence of interferences in environmental samples means that this analysis would require modification to be used for residue levels of these antibiotics, particularly in honey.

Microbiological assays for run 2 (*figure 3b*) paralleled the HPLC results. Inhibition zones for tylosin remained constant at 45 mm over time, while inhibition by OTC began to decrease after 14 days. Results for run 1 (*figure 2b*) were similar, but OTC retained activity slightly longer than for run 2, paralleling the increased stability observed by HPLC. Tylosin apparently has some mold-inhibiting properties. The OTC solutions became too moldy for the microbiological assay after 6–8 weeks (no inhibition zone was visible under the mold growth on the plates), while the tylosin solutions showed almost no mold growth, either visually or on the BHI plates.

Résumé – Étude comparative de la stabilité de l'oxytétracycline et de la tylosine dans le sirop de sucre. L'hydrochlorure d'oxytétracycline (OTC) est utilisé depuis le début des années 50 pour prévenir et traiter la loque américaine et la loque européenne des abeilles mellifères (*Apis mellifera* L.). C'est actuellement le seul produit de traitement autorisé pour ces maladies aux États-Unis. La tylosine est un autre antibiotique qui a été mentionné comme candidat pour traiter la loque en 1970, mais il n'est pas encore autorisé pour cet usage. Les premières mesures de stabilité dans les produits de nourrissement, sirop, poudre ou pâte, reposaient sur des méthodes non spécifiques, qui comprenaient les tests d'inhibition bactériennes pour les deux produits et la fluorescence pour l'OTC. Nous avons mesuré la stabilité de l'OTC et de la tylosine dans le sirop à l'aide de la chromatographie liquide haute performance (HPLC). À 34 °C et à l'obscurité, l'OTC dans du sirop de saccharose à 70 % très purifié a une demi-vie de 7,6 j (*figure 1a*) et aucun autre pic n'apparait dans la zone d'analyse. Dans les mêmes conditions la tylosine est beaucoup plus stable, ne diminuant que de 25 % en trois mois. (*figure 2a*). Sa demi-vie est estimée à 186 j environ, une partie de la diminution correspondant à la décomposition en d'autres substances du complexe de la tylosine présents dans le produits de départ. Dans ces conditions la demi-vie du complexe global de la tylosine est d'environ 287 j. Dans du sirop préparé à partir de saccharose du commerce, les deux produits sont moins stables : l'OTC a une demi-vie de 6,3 j et la tylosine d'environ 75 j (*figure 3a*). Les données microbiologiques confirment les résultats de l'HPLC : un test sur plaques contre *Paenibacillus larvae* montre une décroissance rapide de la zone d'inhibition avec l'OTC et une persistance de l'inhibition avec la tylosine (*figure 2b, 3b*).

loque américaine / oxytétracycline / tylosine / antibiotique / stabilité / sirop

Zusammenfassung – Vergleich der Stabilität von Oxytetracyclin und Tylosin in Zuckersyrup. Seit den frühen 50er Jahren wurde Oxytetracyclin-hydrochlorid zur Vorbeugung und Behandlung von Amerikanischer und Europäischer Faulbrut bei Bienen angewendet. In den Vereinigten Staaten stellt es zur Zeit das einzige von der FDA genehmigte Behandlungsmittel gegen diese Krankheiten dar. Ein weiteres für die Behandlung der Faulbrut in Frage kommendes Antibiotikum ist Tylosin, über dessen Wirksamkeit 1970 erstmalig berichtet wurde. Dieses ist aber zur Zeit nicht für diese Verwendung zugelassen. Frühe Messungen der Stabilität dieser Substanzen in Syrup, Staub und Futterteig (extender patties) waren auf unspezifische Methoden wie bakterielle Hemmtests für beide der Antibiotica oder Fluoreszenztests für OTC angewiesen. Wir haben die Stabilität von OTC und Tylosin in Syrup mittels Hochleistungsflüssigkeits-chromatographie (HPLC) bestimmt. Bei 34 °C in Dunkelheit hatte OTC in hochreinem 70 % igen Zuckersyrup eine Halbwertszeit von 7,6 Tagen (*Abb. 1a*), und es traten innerhalb des Analysebereiches keine weiteren Peaks in Erscheinung. Tylosin war erheblich stabiler und nahm

unter den gleichen Bedingungen über 3 Monate nur um 25 % ab (*Abb. 2a*). Es hatte eine hochgerechnete Halbwertszeit von etwa 186 Tagen, wobei ein Teil dieser Abnahme auf den Zerfall von anderen in dem Ausgangsmaterial enthaltenen Anteilen des Tylosinkomplexes zurückzuführen war. Für den gesamten Tylosinkomplex ergab sich damit eine Halbwertszeit von ungefähr 287 Tagen. In aus kommerziellem Zucker hergestelltem Syrup waren beide Substanzen weniger stabil . Hier betrug die Halbwertszeit von OTC 6,3 Tage, die von Tylosin 75 Tage (*Abb. 3a*). Die Ergebnisse mikrobiologischer Untersuchungen entsprachen diesen HPLC Befunden, wobei die Hemmzone in einem Plattentest gegen *Paenibacillus larvae* bei OTC rasch abnahm, während sie mit Tylosin über längere Zeit erhalten blieb (*Abb. 2b* und *3b*).

Oxytetracyclin / Tylosin / Syrup / Faulbrut / Stabilität

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