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Original article

Analysis of oxytetracycline in extender patties

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Abstract – Antibiotic extender patties, consisting of sugar, vegetable shortening, and oxytetracycline (OTC, usually as the hydrochloride), are used for treatment/prevention of foulbrood in honey bees. An analytical method was developed to determine the concentration in the patties and to allow a study of deterioration over time. Trituration of a sample with EDTA-treated C_{18} silica gel, removal of the shortening with isooctane, and elution of the OTC with methanol/acetonitrile gave a solution that could be analyzed by HPLC. Different concentrations and total amounts of OTC have been recommended by different authors. Not all newly-procured samples contained the same level of OTC, and the concentration decreased about 4% per year with storage at room temperature.

oxytetracycline / solid-phase extraction / HPLC / American foulbrood / European foulbrood

1. INTRODUCTION

Oxytetracycline hydrochloride (OTC, Terramycin[1]) has been used since the early 1950s [7, 10] for the prevention and control American and European foulbrood in honey bees (*Apis mellifera* L.), which are caused by two species of bacteria, *Paenibacillus* (*Bacillus) larvae* and *Melissococcus pluton*, respectively. 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showing some tolerance to OTC have been discovered in a limited number of beekeeping operations (Shimanuki and Knox, unpublished). 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.

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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 U.S. Department of Agriculture.

Three methods of OTC application have been commonly used: a dusting with OTC in powdered sugar repeated several times at weekly intervals [6, 8]; a solution of OTC in syrup fed to the bees [4]; and now most common, an 'extender patty' consisting of OTC, sugar, and vegetable shortening [23, 24]. The syrup formulations are undesirable due to the instability of OTC in syrup [5, 11], and the dust formulations are unpopular with beekeepers since the requirement for repeated treatments renders them too labor-intensive for convenient use.

Early assays for OTC relied on the inhibition of growth of a test organism, such as *Erwinia* sp. [6] or *Bacillus cereus* var. *mycoides* [13, 22]. 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 than bacterial assays, this method was still not able to distinguish between OTC and other tetracyclines, 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, 14, 15, 17, 18, 20, 21] as well as in other samples, such as milk and meat (for example, see [19]).

A wide variety of OTC dosages in various size extender patties have been reported in the literature and as unpublished recommendations (see below), but not always with data supporting the efficacy of the dosage given. Furthermore, the amount of the patty actually consumed by the bees, as opposed to being discarded from the hive as trash, is not known. OTC doses have ranged from 200 mg [12] to 1240 mg [23, 24]. Recommendations during the last decade have tended toward a total of 700–1000 mg of OTC/patty, regardless of patty size [3, 16, other unpublished recommendations]. Patty sizes have ranged from 170 g (Mann-Lake product label) to 590 g [3, 16] to 'a wax paper covered patty was kept on the top bars throughout the experimental period' [9]. OTC concentrations in patties range from 1 mg/g to 5.9 mg/g (calculated from references above). This wide variation in both concentration and total dosage makes an analytical method desirable for active ingredient in these products.

A study was conducted to determine the OTC content of commercially-available patties used for foulbrood prevention and control, and to determine the stability of active ingredient in storage. In the case of extender patties, the large amount of grease mixed with the antibiotic and sugar caused difficulties in analyses developed for normal biological matrices. Liquid/liquid partition failed, since thick emulsions resulted on shaking samples with organic solvents and water. A satisfactory separation was achieved by a modification of the matrix solid-phase dispersion technique of Long et al. [14, 15].

2. MATERIALS AND METHODS

2.1. Reagents and samples

New extender patty mixes were purchased from bee supply companies (Brushy Mountain, Glorybee, Lapp's Bee Supply) or had been in laboratory stock for varying lengths of time. Short storage times were obtained by keeping patties at room temperature (nominally 20–25 °C) for varying lengths of time, then placing them in the refrigerator. Unless otherwise indicated, all were stored at 5 °C, and time at 5 °C was not counted toward 'age'. Oxytetracycline hydrochloride and ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate (molecular biology grade) were obtained from Sigma. Oxalic acid dihydrate (ACS reagent grade), acetonitrile (HPLC

grade), and methanol (HPLC grade) were obtained from Aldrich. EDTA acid was a technical grade material ('Cyquest Acid') from American Cyanamid. The C_{18} silica gel was salvaged from unused Waters Prep 500 cartridges in laboratory stock. Since this form of C_{18} silica is no longer available, Supelclean LC-18 silica (Supelco) was tried and gave similar results.

Aqueous-ethanolic EDTA dipotassium salt was prepared from EDTA acid (29.2 g, 100 mmol), KOH (13.2 g, 200 mmol assuming 85% assay) in water, filtration, and refiltration after several days. Dilution to 200 mL gave a 0.5M stock solution. This stock solution (10 mL) was diluted with water (50 mL), ethanol (190 mL, solution cloudy), and additional water (10 mL, solution clear). The final concentration was thus 19.2 mM EDTA dipotassium salt in 73% (v/v) ethanol. C_{18} silica gel was slurried in this solution and the silica was filtered off with suction and dried in air over the weekend.

2.2. HPLC conditions

Antibiotic determinations were carried out by injection of antibiotic eluate 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. Eluents were mixtures of methanol-acetonitrile-0.01 M oxalic acid. Initial ratio was 20:30:50 [19], and some later runs were carried out with a ratio of 30:45:25 (see below). Flow rate was 0.7 mL/min. 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. 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 of 2 ng–2 μg gave a linear plot with an average detector response near 4 350 detector units/ng for OTC; standards were run for each set of extractions and the factor obtained was used for that data set. All concentrations in this paper are expressed as OTC hydrochloride; the concentrations of OTC base would be 92.7% of these values. The retention time of OTC was 3.26 min (eluent ratio of 30:45:25 gave a retention time of 4.4 min, see below), but this became longer as the oxalic acid component of the eluent aged, to the point that the solution needed to be replaced.

2.3. Extraction and sample preparation

Initial separations of OTC from patty mixes entailed trituration of a patty sample (0.5 g) with C_{18} silica gel (2 g) containing EDTA disodium salt and oxalic acid (0.05 g each) in a glass mortar. This mixture was transferred to a 10-mL plastic syringe barrel containing a 1.5 cm filter paper (Whatman #1). Additional C_{18} silica (0.5 g) was used as a dry wash of the mortar, and was then transferred to the syringe barrel. After placement of another sheet of filter paper over the silica, a syringe plunger (without the rubber gasket) was used to compress the packing bed. Isooctane (10 mL) was passed through the column to elute the shortening. Other hydrocarbons such as hexane or heptane would presumably also be suitable. After adding ethyl acetate (2 mL, otherwise the methanol/acetonitrile eluent would not mix with the isooctane on the column, preventing flow), the OTC was eluted into a 50 mL volumetric flask with methanol/acetonitrile (1:1) until the eluate reached the graduation mark. Aliquots of this solution were injected onto the HPLC column.

Since the concentrations of OTC in extender patties are in the range of 1–6 mg/g, the concentration in the eluate solution is also quite large. We did not investigate the detection limits, which would be required for residue assays in honey, for example.

Data reduction and plotting were performed with GraphPad Prism ver. 3.0 (GraphPad Software, Inc. San Diego, CA).

3. RESULTS AND DISCUSSION

Initial experiments indicated that the shortening component of the patty could be eluted effectively with 10 mL of isooctane. The OTC was initially eluted with 10 mL of methanol/acetonitrile (1:1), but additional solvent removed additional analyte. An experiment was performed with duplicate 0.5 g extender patty samples using ten successive 10-mL portions of methanol/acetonitrile, and analyzing each sample twice. The total OTC eluted was 2.922 ± 0.069 mg (mean ± SEM), with 2.794 ± 0.065 mg in the first fraction, 0.100 ± 0.013 mg in the second, and 0.012 ± 0.001, 0.006, and 0.003 in fractions 3–5. The first 5 fractions represented 99.9% of the total removed by all ten fractions, so 50 mL of eluate was chosen for all succeeding analyses.

Recovery of OTC from C_{18} silica gel that had not been treated with EDTA/oxalic acid was low and variable (only 1.15–1.55 mg recovered of a 2 mg spike). Difficulties with reproducibility necessitated changes in the method of application of the chelating agents to the silica gel. In the initial method, EDTA and oxalic acid were added as crystalline solids [14, 15]. This was satisfactory in the published cases of catfish and milk, since these matrices are largely aqueous and distributed the chelators evenly over the silica during trituration. In our case, this did not happen, and the recovery of OTC in spiked blank extender patty samples was low and uneven (1.25–2.08 mg recovered of 2.0 mg spike, mean recovery 1.80 ± 0.36 SD for 22 samples). Attempted dissolution of EDTA disodium salt and oxalic acid in water/ethanol failed, as disodium EDTA had insufficient solubility. EDTA acid (1.96 g), oxalic acid (2.5 g) and KOH (2.16 g) were dissolved by heating in water (45 mL) and ethanol (50 mL). C_{18} silica gel (100 g) was added and the slurry was dried on a rotary evaporator. Use of this silica mixture gave recoveries of 'OTC' that were higher than expected by about 10%, but the peak was asymmetrical, with the major part occurring about 0.15 min before the retention time for OTC, with a shoulder representing OTC. It seemed at the time that the treated silica was causing decomposition of OTC. The 'decomposition product' was not identified, but comparison with standards indicated that it was not β-apo-OTC or 4-epi-OTC. An alternative explanation was later found, see below.

C_{18} silica filter-coated with the dipotassium salt of EDTA gave better results. A 'standard patty' was prepared from sucrose (67 g), Crisco (33 g), BHT (100 mg), and OTC hydrochloride (500 mg). Four analyses of this 'standard' patty (4.97 mg/g OTC hydrochloride) gave recoveries ranging from 4.76 mg/g to 4.91 mg/g (mean ± SEM 4.82 ± 0.03, mean ± SD 4.82 ± 0.06) for four samples for a mean recovery of 97.0%.

For early runs the solvent mixture used was methanol-acetonitrile-0.01 M oxalic acid (20:30:50 by volume) [19] at 0.7 mL/min. Later experiments used 0.7 mL/min of the same solvents at a ratio of 30:45:25, which gave longer retention time and baseline separation from a small peak preceding the OTC peak. With this eluent mix, however, the separation of OTC from other tetracyclines was inferior to that given by the 20:30:50 mix. The objectives of the analysis and the possible presence of other tetracyclines would determine the eluent mixture chosen.

One phenomenon was observed with this system, however; the retention time of the standard OTC solutions was different from the retention times of the material eluted from the columns. It is likely that this is related to the 'decomposition' discussed above. Four injections of patty analyte gave a retention time of 4.220 ± 0.014 min (mean ± SD) and an OTC standard gave retention time 4.408 ± 0.022 min. The width at half height for both was 1.5 mm, corresponding to 9 seconds. An approximately 1:1 mixture of the two samples, however, gave a single peak with a retention time of 4.310 ± 0.014 min, with the same half width.

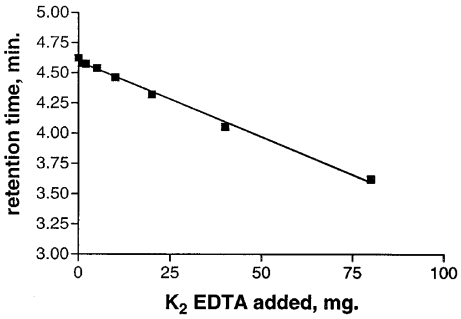

Figure 1. Change in retention time of OTC in presence of potassium EDTA. OTC concentration 2.5 mg in 50 mL, concentrations not corrected for changes in volume caused by EDTA additions or withdrawals for injection.

If there were two compounds present, there should be two peaks, or at least a broadening of the peak, since the separation is significantly greater than the peak width. Changing the solvent composition of the standard had no effect on the retention time, but addition of EDTA to the standard reduced retention time proportionally to amount added. To 50 mL of OTC standard solution containing 2.5 mg of OTC hydrochloride were added amounts of dipotassium EDTA (as a 0.5 M solution in water) from zero to 80 mg (Fig. 1). The retention times decreased approximately linearly from 4.62 min with no addition to 4.32 min with 20.0 mg added. Further additions at levels of 40 and 80 mg gave further reductions to 4.05 and 3.62 min, but a second broad peak at longer retention time appeared. Elution of 2.5 g of EDTA-treated silica gave 15.6 mg of residue, which from the observed curve would be expected to reduce the retention time by 0.2 min. The observed reduction was 0.19 for the run cited at the beginning of the paragraph, and 0.21 min for standards run on the same day as the EDTA curve.

OTC contents of purchased extender patty mixes from Manufacturer A were consistent with the labeled dose of 1000 mg/patty (nominally 170 g) when purchased from several distributors. A second manufacturer did not list a concentration on the label, and the concentration found was less than in Manufacturer A's product. These data are shown in Table I. These data are subject to several assumptions: when we received them the patties were new or had been stored under conditions where no decomposition occurred and that no decomposition occurred during our storage at 5 °C.

OTC content of extender patties decreased slowly with length of storage at room temperature. Data are shown in Table I as well as in Figure 2. The time for 25% decrease is about 75 months (about 4%/year).

The OTC content of both commercial mixes was within the recommended ranges for foulbrood control, and the slow decomposition at room temperature suggests that correctly-mixed or commercially-prepared

Table I. OTC contents of commercial terra-patty mixes.

Sample (mg)	Manufacturer	mg OTC/g	Suggested dose	OTC/dose
1	A	6.15 ± 0.24	patty (170 g)	1 045
2	A	5.97 ± 0.21	patty	1 015
3 (R.T.[1] ~ 9 months)	A	5.79 ± 0.15	patty	984
4 (R.T. ~ 2 years)	A	5.72 ± 0.06	170 g	972
5 (R.T. ~ 34 months)	A	5.25 ± 0.24	170 g	893
6	B	3.60 ± 0.14	170–226 g	612–816[2]

[1] R.T. = Room temperature (~25 °C).
[2] The directions say to use a second patty if necessary.

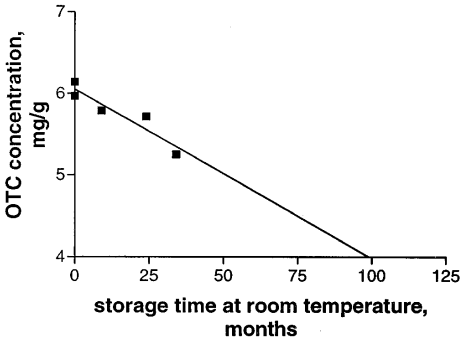

Figure 2. OTC extender patty storage stability at room temperature. Extrapolation of the regression line suggests that a 25% decrease in concentration would take ca. 75 months.

patties, stored at room temperature or below, should retain adequate antibiotic activity for several years. Storage at elevated temperatures (for example, in a closed shed out in the sun during the summer) would be expected to lead to more rapid inactivation. Low dosages of OTC are more likely to result from patties improperly mixed by beekeepers, improper storage conditions, or from incorrect placement within the hive. Since only part of the patty is consumed, the rest being discarded by the bees, the position in the hive is important. Wilson et al. [23] report that 'Adult bees apparently removed the patty, not because they enjoyed eating it, but rather because it was in their way and annoyed them'. Whenever low concentrations of active ingredient are present over a long period of time, whether from patty composition (for whatever reason) or from placement in the hive in a location where the bees take a long time to remove the patty, the chance of resistance is increased.

Résumé – Analyse de l'oxytétracycline dans les pâtés à longue durée d'action. L'hydrochlorure d'oxytétracycline (OTC, Terramycine) est un antibiotique utilisé depuis de nombreuses années pour prévenir et traiter la loque américaine et la loque européenne (NDLR : pas d'AMM en France). Le pâté à longue durée d'action (PLDA) est constitué de sucre, de graisse végétale et d'OTC, et posé sur la tête des cadres dans la ruche. C'est une méthode d'application largement répandue. Les méthodes d'analyse mises au point pour détecter l'OTC dans le miel ne peuvent être appliquées aux PLDA, car l'extraction est rendue difficile par la forte teneur en lipides. Dans ce cas la méthode de dispersion de la phase solide a été modifiée afin de détecter l'OTC. La trituration d'un échantillon de gel de silice C_{18} traité à l'EDTA, l'élimination des lipides par l'isooctane et l'élution de l'OTC par le méthanol/acétonitrile (1:1) ont permis d'obtenir une solution qui a pu être analysée par chromatographie liquide haute pression (HPLC). L'analyse d'un mélange de PLDA préparé au laboratoire a donné un taux de recouvrement de l'OTC de 97 %. Un échantillon du commerce, libellé comme contenant 1 000 mg OTC/ pâte, en contenait légèrement plus lorsqu'il était frais (Tab. I), alors qu'un mélange provenant d'un autre fournisseur, sans précision de la teneur en OTC, en contenait énormément moins. La teneur en OTC décroît d'environ 4 % par an en cas de stockage à température ambiante (Fig. 2).

oxytétracycline / antibiotique / loque / extraction phase solide / HPLC

Zusammenfassung – Analyse von Oxytetracyclin in Futterteig für Langzeitbehandlung. Oxytetracyclin-Hydrochlorid (OTC, Terramycin) ist seit vielen Jahren zur Prävention und zur Kontrolle von Europäischer und Amerikanischer Faulbrut der Bienen in Benutzung. (redaktionelle Anmerkung: gilt nicht für Deutschland). Eine weit verbreitete Methode ist die Applikation durch „Langzeit Futterteig", der aus Zucker, pflanzlichem Fett und OTC besteht und der oben auf die Rähmchen gelegt wird. Die

Analyse von OTC in Honig kann nicht direkt für eine Analyse im Futterteig angewendet werden, weil die Extraktion durch den hohen Fettgehalt schwierig ist. In diesem Fall wurde eine Modifikation der Methode der Festphasen Dispersion gewählt, um OTC zu bestimmen. Der Futterteigprobe wurde mit einem mit EDTA behandelten C18 Silica Gel pulverisiert, danach konnte das Fett mit Isooctan entfernt werden. Die folgende Elution von OTC aus dem Gel mit Methanol/Acetonitril (1:1) ergab eine Lösung, die mit dem HPLC analysiert werden konnte. Die Analyse eines frischen Futterteigs, der mit dem Gehalt von 1000 mg OTC/Teig gekennzeichnet war, enthielt etwas mehr OTC. Dagegen enthielt die Mischung eines anderen Anbieters, der keine Gehaltsangabe gemacht hatten, wesentlich weniger. Bei einer Speicherung bei Raumtemperatur nahm der OTC Gehalt etwa 4 % pro Jahr ab.

Oxytetracyclin / Festphasenextraktion / HPLC / Faulbrut

REFERENCES

[1] Argauer R.J., Gilliam M., A fluorometric method for determining oxytetracycline in treated colonies of the honey bee, *Apis mellifera* J. Invertebr. Pathol. 23 (1974) 51–54.

[2] Argauer R.J., Moats W.A., Degradation of oxytetracycline in honey as measured by fluorescence and liquid chromatographic assays, Apidologie 22 (1991) 109–115.

[3] Delaplane K.S., Lozano L.F., Using Terramycin in honey bee colonies, Am. Bee J. 134 (1994) 259–261.

[4] Farrar C.L., Treating bee diseases, Gleanings Bee Cult. 84 (1956) 207–211, 218.

[5] Gilliam M., Argauer R.J., Stability of oxytetracycline in diets fed to honeybee colonies for disease control, J. Invertebr. Pathol. 26 (1975) 383–386.

[6] Girardeau J.H. Jr., The occurrence of dry-mix-fed drugs in larval food of the honey bee, J. Econ. Entomol. 58 (1965) 878–880.

[7] Gochnauer T.A., Drugs fight foulbrood diseases in bees, Minn. Home Fm. Sci. 9 (1951) 15.

[8] Gochnauer T.A., The response of normal package colonies of bees to antibiotic feeding, Am. Bee J. 97 (1957) 104–106.

[9] Hoopingarner R., Nelson K., American foulbrood cleanup rate using three Terramycin treatments, Am. Bee J. 128 (1988) 120–121.

[10] Katznelson H., Arnott J., Bland S.E., Preliminary report on the treatment of European foulbrood of honey bees with antibiotics, Sci. Agric. 32 (1952) 180–184.

[11] Kochansky J., Knox D., Shimanuki H., Comparative stability of oxytetracycline and tylosin in sugar syrup, Apidologie 30 (1999) 321–326.

[12] Lehnert T., Shimanuki H., European foulbrood disease control in honey bee colonies used for blueberry and cranberry pollination, Am. Bee J. 120 (1980) 429–30.

[13] Lehnert T., Shimanuki H., Oxytetracycline residues in honey following three different methods of administering the antibiotic, Apidologie 12 (1981) 133–136.

[14] Long A.R., Hsieh L.C., Malbrough M.S., Short C.R., Barker S.A., Matrix solid-phase dispersion isolation and liquid chromatographic determination of oxytetracycline, tetracycline, and chlortetracycline in milk, J. Assoc. Off. Anal. Chem. 73 (1990) 379–384.

[15] Long A.R., Hsieh L.C., Malbrough M.S., Short C.R., Barker S.A., Matrix solid-phase dispersion isolation and liquid chromatographic determination of oxytetracycline in catfish (*Ictalurus punctatus*) muscle tissue, J. Assoc. Off. Anal. Chem. 73 (1990) 864–867.

[16] Lozano L.F., Protect your bees now! Use of Terramycin in honey bee colonies, Am. Bee J. 136 (1996) 572–574.

[17] Matsuka M., Nakamura J., Oxytetracycline residues in honey and royal jelly, J. Apic. Res. 29 (1990) 112–117.

[18] Oka H., Ikai Y., Kawamura N., Uno K., Yamada M., Harada K.-I., Suzuki M., Improvement of chemical analysis of antibiotics XII. Simultaneous analysis of seven tetracyclines in honey, J. Chromatogr. 400 (1987) 253–261.

[19] Oka H., Ikai Y., Hayakawa J., Masuda K., Harada K.-I., Suzuki M., Improvement of chemical analysis of antibiotics. Part XIX: Determination of tetracycline antibiotics in milk by liquid chromatography and thin-layer chromatography/fast atom bombardment mass spectrometry, J. Assoc. Off. Anal. Chem. Int. 77 (1994) 891–895.

[20] Oka H., Ikai Y., Hayakawa J., Harada K.-I., Asukabe H., Suzuki M., Himei R., Horie M., Nakazawa H., MacNeil J.D., Improvement of chemical analysis of antibiotics. 22. Identification of residual tetracyclines in honey by frit FAB/LC/MS using a volatile mobile phase, J. Agric. Food Chem. 42 (1994) 2215–2219.

[21] Sporns P., Kwan S., Roth L.A., HPLC analysis of oxytetracycline residues in honey, J. Food Prot. 49 (1986) 383–388.

[22] Wilson W.T., Residues of oxytetracycline in honey stored by *Apis mellifera*, Environ. Entomol. 3 (1974) 674–676.

[23] Wilson W.T., Elliott J.R., Lackett J.J., Antibiotic treatments that last longer, Am. Bee J. 110 (1970) 348–351.

[24] Wilson W.T., Elliott J.R., Hitchcock J.D., Antibiotic extender patties for the control of American foul brood, J. Apic. Res. 10 (1971) 143–147.