

OXYTETRACYCLINE RESIDUES IN HONEY ANALYZED BY LIQUID CHROMATOGRAPHY WITH UV DETECTION

Anna Gajda^{1*}, Andrzej Posyniak¹,
Andrzej Bober², Tomasz Błądek¹, Jan Żmudzki¹

¹Department of Pharmacology and Toxicology, National Veterinary Research Institute,
Partyzantów 57, 24-100 Pulawy, Poland

²Department of Honey Bee Diseases, National Veterinary Research
Institute, Partyzantów 57, 24-100 Pulawy, Poland

*corresponding author: anna.gajda@piwet.pulawy.pl

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S u m m a r y

A liquid chromatography method with UV detection for determination of oxytetracycline (OTC) in honey has been developed. The samples were extracted with the solution of oxalic acid. The clean-up procedure was performed by solid phase extraction (SPE) using polymeric Strata X and carboxylic acid cartridges. Chromatographic separation was carried out on the Luna C8 analytical column with mobile phase consisting of acetonitrile-0.02 M oxalic acid. The method has been successfully validated according to the requirements of the European Decision 2002/657/EC and this method is used in routine control of oxytetracycline in honey samples. The limit of detection (LOD) and limit of quantification (LOQ) of the presented method were 10 and 12.5 µg/kg, respectively. The developed method has also been verified in quantitative determination of oxytetracycline residues in honey after experimental treatment with this product in bee colonies.

Keywords: oxytetracycline, honey, residues, validation, depletion, HPLC-UV method.

INTRODUCTION

American foulbrood (AFB) and European foulbrood (EFB) are destructive, infectious bacterial diseases in honeybees (*Apis mellifera*), which can severely decrease the honey bee population and honey production (Gojmerac, 1980). American foulbrood spores are highly-resistant and can remain vital in combs and honey for many years. To control outbreaks of these infections in honeybee colonies, tetracyclines are shown to be effective. These compounds are wide spectrum antibiotics with properties to prevent the growth of bacteria by inhibition of protein synthesis. Oxytetracycline belongs to the tetracyclines group and is commonly applied by beekeepers.

There are many regulatory problems and inconsistencies associated with the use of the oxytetracycline antibiotic in apiculture. According to The European Medicine

Agency (EMA) summary opinion, regarding the establishment of maximum residue limit (MRL) for oxytetracycline in honey, a provisional MRL of 25 µg/kg has been set, which will expire on 01.01.2014, for the parent drug of OTC and its 4-epimer (EMA Summary Report, 2008). However, in accordance with the Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010, no MRL exists for this compound in honey and it is banned in Europe. So, the European Union (EU) does not allow the use of oxytetracycline, or anything else from the tetracyclines group, for treatment of honeybees. The presence of any detectable residues of these antibiotics could mean that the honey cannot be commercialized in the EU. Unlike the EU countries, other countries like the United States and Brazil, allow the use of OTC for treatment of AFB and the maximum residue limit for

this compound has been set as 200 µg/kg (USFDA, 2008; Peres et al., 2010).

Oxytetracycline can persist in honey and may lead to drug resistance, biological adverse effects, and allergic reactions in humans. For consumer protection, determination of this antibiotic in honey samples is of considerable importance.

Many analytical studies for the determination of tetracyclines residues in honey have been described. However, due to the complexity of this matrix, it is difficult to obtain a sufficiently sensitive method for honey. To remove the matrix effect, a more rigorous clean-up procedure is required. The analysis of OTC in honey is carried out using HPLC with different detection methods, such as ultraviolet (Sporns et al., 1986; Oka et al., 1987; Vinas et al., 2004; Victorita et al., 2007), fluorescence (Argauer and Moats, 1991; Pena et al., 2005; Fujita et al., 2008; Peres et al., 2010), and mass spectrometry (Khong et al., 2005). Only a little information is available concerning the effect of storage on OTC residues in honey after hive treatment (Thompson et al., 2005). Sensitive analytical method for the analysis of OTC in honey. Additionally, the

The aim of this study was to improve on the previously described (Zhou et al., 2009) selective and depletion time after experimental administration of OTC to honeybee colonies by liquid-sucrose-solution dosing was studied. The stability of OTC in honey under laboratory conditions was also examined.

MATERIAL AND METHODS

Reagents

All reagents used were of analytical grade. The oxytetracycline (OTC) standard with a 99% degree of purity, was obtained from Sigma-Aldrich Chemical Company (USA). Acetonitrile, methanol, and Bakerbond SPE cartridges (carboxylic acid 500 mg/3 ml) were from J.T. Baker. Oxalic acid dihydrate (ACS) was from POCh Gliwice (Poland). Strata X (100 µg, 3ml) polymeric cartridges were

from Phenomenex. Additionally, ethyl acetate was obtained from POCh (Poland). Water was purified using the Milli-Q system.

Standard solutions

Stock standard solution (1 mg/mL) which had been prepared by weighing 10.0 ± 0.1 mg of standard substances and dissolving them in 10 ml of methanol, was stable for six months when stored at a temperature below -18 °C in amber glass. Working standard solutions (100 µg/mL, 10 µg/mL) prepared in acetonitrile by diluting suitable aliquot of stock standard were stable for one month, stored at $2 - 8$ °C in amber glass. Working standard solutions in the mobile phase were prepared on the day of analysis.

Extraction and two-step clean-up

Three grams of honey were mixed with 20 ml of 0.02 M oxalic acid buffer, pH 4.0. This mixture was then vortexed for 5 min. and put in an ultrasonic bath to mix well for 15 min. After the extraction procedure, the supernatant was poured into Strata X polymeric columns which were earlier preconditioned with 5 ml methanol and a 10 ml extraction buffer solution. After percolation of the whole solution, the columns was washed with 10 ml of 5% methanol in water and dried (under vacuum) for 10 min. Finally, the analytes were eluted from the polymeric cartridges with 15 ml ethyl acetate. The ethyl acetate eluate was transferred into a carboxylic acid cartridge, previously conditioned with 5 ml ethyl acetate. The cartridge was washed with 10 ml methanol and dried completely. The oxytetracycline was eluted with 4 ml mixture of 0.02 M oxalic acid (pH 2.0) and acetonitrile (6:4, v/v). The eluate was evaporated to a 2 ml volume under a stream of nitrogen at 40 °C. After vortex mixing, the solution was ready to analyse.

LC-UV analysis

The instrumental analysis was performed using the Varian Prostar HPLC system, equipped with a quaternary pump, autosampler, column oven, and UV/Vis detector ($\lambda=355$ nm), controlled by Galaxie Workstation software. Chromatographic

analyses were performed on Luna (Phenomenex) C8 column (5 μm , 250 mm x 4.6 mm) with mobile phase consisting of (A) acetonitrile : (B) 0.02 M oxalic acid (pH 4.0) in gradient mode at 1.0 ml/min flow rate. A gradient elution program was started from 16% of A. After 1 min., an increase was done to 25% of A up to 5 min., and at 5 min. to 10 min. a decrease to 16 % was done and kept for 3 min. The column oven temperature was controlled at 30 °C. The injection volume of 100 μl was used.

Validation

Samples of honey were spiked with the OTC working solution to levels corresponding to 12.5, 25, and 50 $\mu\text{g}/\text{kg}$, respectively. The recovery was evaluated by comparing the concentrations in the spiked samples having known amounts of analyte, to the concentrations in standard solution. The six spiked samples with OTC were analysed within three different days. Based on these spiked sample replicates, the precision (repeatability and reproducibility) of the method was determined. Linearity was tested by preparing amatrix-matched calibration curve on five levels corresponding to 12.5, 25, 50, 75, and 100 $\mu\text{g}/\text{kg}$. The correlation coefficient was evaluated. The detection limits (LOD) and limit of quantification (LOQ) of the method were calculated. Additionally, during the validation process, the decision limit ($CC\alpha$) and detection limit ($CC\beta$) were evaluated.

Bee colonies and treatment

Colonies of honeybees (*Apis mellifera*) were maintained and owned by the Department of Honey Bee Diseases of the National Veterinary Institute in Puławy, Poland. The colonies were housed in wooden hives and were allowed free flight during the study. The hives had frames which were: 360 x 260 mm (length x height). None of the colonies showed clinical signs of disease. Before the start of experiment, OTC analyses were realized on honey collected in every comb of every hive. Bees from three colonies (hive 1, hive 2, hive 3) were fed with 1 litre

saccharose syrup containing OTC. The syrup was prepared by mixing 0.5 g of OTC in a 1 litre sugar (saccharose/water 50:50 v/v) solution. Dosing of OTC was initiated at the end of June and repeated two times - every 7 days. Bees from hive 4 received saccharose syrup without OTC. Honey samples were taken from the frames in the hives prior to the beginning of the dosing, and at 1 and 12 months after the end of the dosing. The honey samples were collected from each hive separately and filtered through cloth into a clean glass jar labelled with the colony number, then stored at an ambient temperature before being analysed. To determine the stability of OTC in honey under laboratory conditions, samples from the first collection were kept at 20 °C and analysed for 17 months.

RESULTS

The specificity of the method was checked by analyzing different types of honey samples. No interfering peaks were detected in the OTC retention time. The typical HPLC chromatograms of blank sample and sample spiked with OTC are shown in Fig. 1. In this method, the separation was made using C8 analytical column, with a mobile phase containing acetonitrile and 0.02 M oxalic acid. A sharp, symmetrical peak was obtained. The two step clean-up system with polymeric

Table 1.

Validation results of analytical procedure for the determination of oxytetracycline in honey

Parameters	Results		
Compound	Oxytetracycline		
Correlation coefficient (r)	0.9994		
Linearity (working range), $\mu\text{g}/\text{kg}$	12.5 - 100		
Detection limit (LOD), $\mu\text{g}/\text{kg}$	10		
Limit of quantification (LOQ), $\mu\text{g}/\text{kg}$	12.5		
Decision limit ($CC\alpha$), $\mu\text{g}/\text{kg}$	29.8		
Detection capability ($CC\beta$), $\mu\text{g}/\text{kg}$	35.9		
Level of spiked samples ($\mu\text{g}/\text{kg}$)	12.5	25	50
Repeatability, CV% (n=6)	3.8	4.2	5.7
Reproducibility, CV% (n=18)	4.9	6.5	7.9
Recovery, %	75.4	74	71

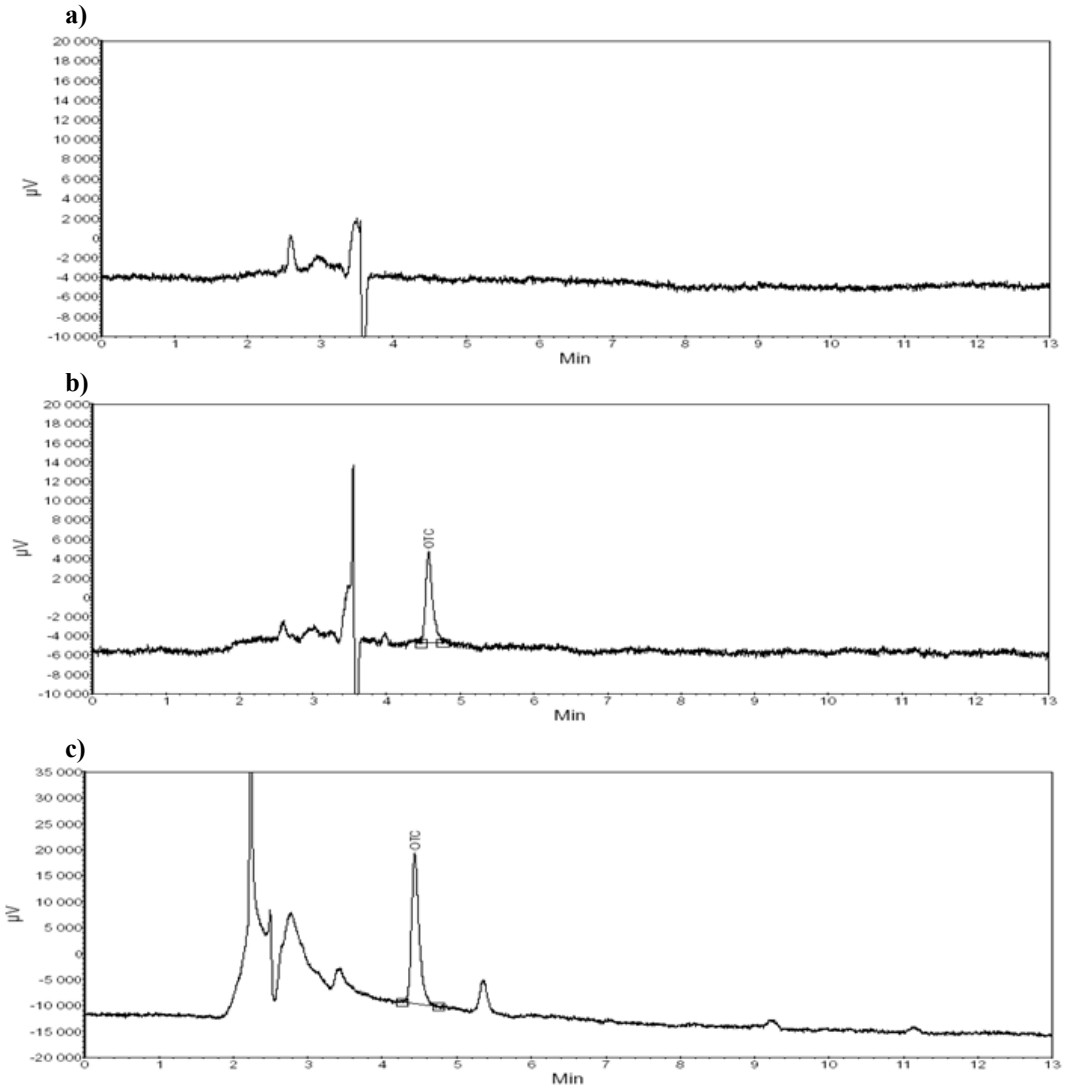


Fig. 1. Typical chromatograms of: a) blank honey sample; b) honey sample spiked with OTC at LOQ level; c) honey sample after OTC treatment.

and carboxylic SPE columns was found suitable for purification purposes. The use of the 355 nm wavelength (λ) was the most appropriate for oxytetracycline.

During the validation process, the limit of detection (LOD), and limit of quantification (LOQ) were established. Linearity and detection parameters, and precision and recovery of the method are shown in Table 1. The variation coefficients and mean extraction recoveries are satisfactory. The method was linear and showed good precision.

After experimental medication of OTC to honeybee colonies, all honeys showed positive results by HPLC with concentration ranges from 17 300 $\mu\text{g}/\text{kg}$ to 34 000 $\mu\text{g}/\text{kg}$ at the first month after treatment. In honey harvested from colonies after 12 months (one overwintering period), the concentration of OTC was below LOD of the method. In the control colonies no OTC was detected. The stability data of OTC in honey under laboratory conditions are presented in Table 2. In honey stored at an ambient temperature, the concentration

Table 2.

Stability of oxytetracycline in honey
stored under laboratory conditions

Date of analysis	Concentrations of oxytetracycline in honey [$\mu\text{g}/\text{kg}$]		
	Hive 1	Hive 2	Hive 3
July 2009	17 300	26 100	34 000
September 2009	4 600	5 900	8 900
January 2010	1 450	1 240	1 990
June 2010	780	680	840
December 2010	348	268	425

of OTC slightly decreased and the honey was analysed for a period of 17 months after treatment.

DISCUSSION

Honey is a very complex matrix to analyse. It contains several organic and inorganic constituents. Among them are: glucose and fructose (75%), oligo saccharides, vitamins, aliphatic acids, amino acids, and proteins. Due to the composition of the honey matrix, it is difficult to obtain sufficient sensitivity in a method, especially when classic detectors are used.

A crucial point in drug residue analysis is the sample extraction step, which requires the isolation of the residues from a biological matrix. The effective isolation of OTC residues from honey is more difficult, because the OTC is high polar molecule and binds with sample proteins. Additionally this compound connects with metal ions. In many studies for the determination of tetracyclines in honey, an extraction using sodium acetate buffer (Huq et al., 2006), McIlvaine - EDTA buffer (Oka et al., 1987; Vinas et al., 2004; Pena et al., 2005; Zhou et al., 2009), sodium succinate buffer (Victorita et al., 2007), and oxalate buffer (Khong et al., 2005) was used to overcome these undesirable properties. The value of pH extraction buffer has a big influence on the chemical behaviour of tetracyclines. Three pH values of oxalic acid buffer (2.0, 4.0, and 6.0) were tested in the present work.

The best results were obtained with oxalic acid pH 4.0. Additionally, the use of hexane for the elimination of the honey endogenous compounds adsorbed on the column was checked, but the results were not satisfactory.

To success of analytical method is to achieve clean sample after sample preparation step. The use of combination neutral polymeric sorbent and weak cation exchange (carboxylic) sorbent was described for determination of tetracycline in propolis (Zhou et al., 2009). A more detailed study on clean-up efficiencies with a weak cation exchange polymeric sorbent (strata X-CW) and a combination of neutral and strong cation exchange sorbent (strata X plus Strata-Screen-C or strata X-C) was reported (Huq et al., 2006). It was also proposed and described metal chelate affinity column (MCAC) in clean-up procedure for determination tetracyclines residues in honey (Thompson et al., 2005; Fujita et al., 2008) or application DSC-phenyl SPE cartridges (Vinas et al., 2004). When only neutral polar polymeric sorbent was used, the interference peaks due to matrix effect by LC-MS/MS were observed (Khong et al., 2005).

In our study, during clean-up optimization, different kind of sorbents and cartridges for clean up efficacy were compared. After the use of C18 sorbent or nexus cartridges, the extract was not clean enough with many interferences and too low recovery. Two polymeric packings: Oasis HLB and Strata X was tested, separately. However, these cartridges were not efficient in eliminating the honey

matrix constituents. As it was found, the use of two step clean-up with Strata X and carboxylic acid columns was most efficient because of the cleanest extract.

In several studies it have been presented the usage of C18 analytical columns (Pena et al., 2005; Huq et al., 2006; Zhou et al., 2009), PLRP-S (Argauer and Moats, 1991; Thompson et al., 2005) or Amide C16 column to analyse tetracyclines in honey (Vinas et al., 2004). In our method, the C8-bonded silica column was used based on the results of Neils and De Leenheer (1980), who preferred this packing to the more common C18 material (Sporns et al., 1986; Peres et al., 2010). Tetracycline can form chelate complexes on the reversed - phase column and they can appear as tailing peaks. In order to avoid these properties and obtain symmetrical, non - tailed peaks, oxalic acid as mobile phase is recommended (Oka et al., 1987; Vinas et al., 2004; Zhou et al., 2009).

The experimental administration of OTC to honeybee colony showed, that OTC is transferred to honey in high concentration (up to 34,000 µg/kg). OTC is not very stable in honeybee combs and the level of this compound quite fast decline. Based on the Dinkov et al. results study, the estimated elimination half-life for OTC in honey after bees treatment was 38.37 days (Dinkov et al., 2005). Some experiments were done to determine the stability of OTC in honey and aqueous systems (OTC half-life) depending on buffer pH and temperature. It was found that OTC in honey is almost five times as stable as OTC in diluted honey (Sporns et al., 1986). According to Martel et al. (2006) study, at 35 °C, which is the internal hive temperature, the degradation of tetracycline (TC) is twice faster than at ambient temperature. A half-life for this compound (at 35 °C in the dark) was estimated as 121 days. The stability of TCs after 60 days of storage in honey samples fortified at 500 µg/kg was evaluated by Peres et al. (2010). Thompson et al. (2005) reported, that the time for OTC residues to decline

to the LOQ of 0.05 mg/kg is approx 14- 16 weeks. It was also suggest, that OTC method of application has big influence on OTC residues in honey. Application as a powdered icing sugar results in lower concentration level than dosing in liquid sucrose.

CONCLUSION

The HPLC-UV method, described in this study, was developed to analyse the residues of OTC in honey samples. The two step clean-up procedure was found suitable to obtain extract clear enough without any interferences. The satisfactory validation results and verification of presented method in experiment proved that proposed method is sensitive enough and suitable for routine identification of OTC in honey. It was also found that OTC duration in honey stored in laboratory was longer than in hives.

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ANALIZA POZOSTAŁOŚCI OKSYTETRACYKLINY W MIODZIE METODĄ CHROMATOGRAFII CIECZOWEJ Z DETEKTOREM UV

Gajda A., Posyniak A., Bober A.,
Błądek T., Żmudzki J.

S t r e s z c z e n i e

Opracowano metodę chromatografii cieczowej z detektorem UV do oznaczania oksytetracykliny (OTC) w miodzie. Do ekstrakcji próbek wykorzystano roztwór kwasu szczawiowego. Do oczyszczania zastosowano zestaw ekstrakcji do fazy stałej (SPE) z użyciem kolumnienek polimerycznych Strata X oraz kolumnienek karboksylowych. Rozdziału chromatograficznego dokonano przy użyciu kolumny analitycznej Luna C8 z fazą ruchomą składającą się z acetonitrylu i 0.02 M roztworu kwasu szczawiowego. Metoda została zwalidowana zgodnie z wymogami Decyzji Komisji Europejskiej 2002/657/EC i jest stosowana w rutynowych badaniach kontrolnych oksytetracykliny w próbkach miodu. Opracowana metoda została zweryfikowana w ilościowym oznaczaniu pozostałości oksytetracykliny w miodzie po eksperymentalnym podaniu pszczołom tego antybiotyku. Celem przeprowadzonego doświadczenia było określenie, jak długo oksytetracyklina może pozostawać w miodzie. Badano także stabilność oksytetracykliny w warunkach laboratoryjnych. Wyniki badania stabilności wskazują, że oksytetracyklina w miodzie przechowywanym w warunkach laboratoryjnych ulega powolnemu zmniejszaniu. W pobranych do badań próbkach miodu stwierdzono zawartość OTC w stężeniach od 17 300 µg/kg do 34 000 µg/kg. Przeprowadzone badania wskazują, że OTC w znacznych ilościach przedostaje się do miodu po jej zastosowaniu u pszczoł. Po 12 miesiącach od podania antybiotyku pszczołom, stężenie w miodzie było poniżej wyznaczonej granicy wykrywalności metody (10 µg/kg).

Słowa kluczowe: oksytetracyklina, miód, pozostałości, walidacja, zanikanie, metoda HPLC-UV.