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Quantitative Analysis of Oxytetracycline Residues in Honey by High Performance Liquid Chromatography

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Abstract

In this study, a simple, sensitive, selective and precise HPLC method is developed for the determination of oxytetracycline in bulk and honey samples. Separation of oxytetracycline was achieved on a Kromosil C18 analytical column (250 mm × 4.6 mm I.D., 5 µm particle size). The mobile phase was a mixture of acetonitrile and water (85:15 v/v) at a flow rate of 1.5 ml/min. The method was linear in the range of 1–6 µg/ml. The LOD and LOQ values are found to be 0.05 and 0.10 µg/ml, respectively. The developed method was successfully applied for the determination of the oxytetracycline in honey sample following extraction of the oxytetracycline with McIlvaine buffer and Solid Phase Extraction system. All the honey samples that were analyzed for oxytetracyline residues had higher residue levels than the recommended maximum residue level for honey. The method described in this study would be useful for routine monitoring of oxytetracycline residues in honey.

Keywords: Oxytetracycline, HPLC, honey, european union regulations.

Introduction

Antibiotics are among the most frequently prescribed medications in modern medicine. Antibiotics cure disease by killing or injuring bacteria. Today, over 100 different antibiotics are available to cure minor as well as life-threatening infections[1]. Broad spectrum antibiotics are effective against a broad range of microorganisms in comparison to narrow spectrum antibiotics.

In the livestock industry, antibiotics are used abundantly. There are three main uses of antibiotics in livestock production. Antibiotics can be used as a therapeutic, as a growth promoter and as a prophylactic. Therefore antibiotics are necessary to society-used properly or not. The commercial importance of antibiotics is not only for treating infections in humans but also for food production and keeping animals and plants disease free[2].

Antibiotics are screened for any negative effects on humans or other mammals before approval for clinical use. However, some antibiotics have been associated with a range of adverse effects[3]. Side effects range from mild to very serious depending on the antibiotics used, the microbial organisms targeted and the individual patient. Adverse effects range from fever and nausea to major allergic reactions including photodermatitis and anaphylaxis[4].

Honey is a mixture of sugars and other compounds. With respect to carbohydrates, honey consists of mainly fructose (about 38.5%) and glucose (about 31%), making it similar to the synthetically produced inverted sugar syrup, which is approximately 48% fructose, 47% glucose, and 5% sucrose[5]. It contains only trace amounts of vitamins or minerals. Honey also contains minute amounts of compounds thought to function as antioxidants like chrysin, pinobanksin, vitamin C, catalase, and pinocembrin. The specific composition of any batch of honey depends on the flowers available to the bees that produced the honey[6].

Although the mechanism of its action is not fully understood, since ancient times honey has been widely used throughout the world as a healing medicine[7,8]. It is mainly used for the treatment of eye diseases, cough, thirst, phlegm, hiccups, and blood vomit, leprosy, diabetes, obesity, worm infestation, vomiting, asthma, diarrhoea and healing wounds and also used as a natural preservative and sweetener in many ayurvedic preparations.

Due to less production of honey, northern countries importing honey from eastern countries. Antibiotics have been added to honey as preservatives by the honey exporters[9,10]. Oxytetracycline (figure-1) is a broad-spectrum antibiotic commonly used in human and veterinary medicine for therapeutic and prophylactic purposes. In apiculture beekeepers use oxytetracycline against the bacterial diseases that affects honey bees[11]. As a result, oxytetracycline residues can be detected at trace levels in honey of treated bees. Oxytetracycline residues show a relatively long half-life and may show direct toxic effects on consumers[11]. Therefore, the presence and maximum residue values of oxytetracycline residues in honey should be regulated.

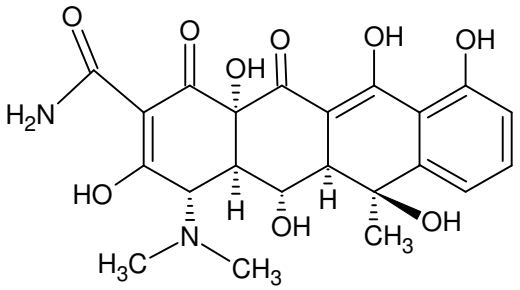

Figure-1
Chemical structure of oxytetracycline

Different analytical methods are reported for the determination oxytetracycline residues in honey. These include HPLC with UV detection[12-17], HPLC with fluorescence detection[18-22], Liquid chromatography with mass spectrometry[11,23-26], capillary high performance liquid chromatography[27], HPTLC[28], high performance capillary electrophoresis[29], Immunological assay[30], Capillary zone electrophoresis[31] and spectrophotometric methods[32]. The above reported methods suffers from one or more drawbacks such as use of internal standard, expensive detectors, cumbersome procedure, long analysis time, less sensitive, lack of precision and accuracy.

Hence, an attempt has been made to develop a simple, sensitive, cost effective and reliable HPLC with UV detection method for estimation of oxytetracycline residues in honey without the use of internal standard.

Material and Methods

Mobile phase: The chemicals and solvents used for the preparation of mobile phase are of HPLC grade. Milli-Q-water was used throughout. The mobile phase used for the analysis of oxytetracycline consists of a mixture of acetonitrile and water in the ratio of 85:15 *v/v*. The mobile phase is also used as diluent for the preparation of oxytetracycline standard solutions. The mobile phase was filtered through a 0.45 µm Millipore membrane filter and sonicated for 15 minutes for degassing prior to use.

Instrumentation and chromatographic conditions: Chromatographic separation was performed on an isocratic High Pressure Liquid Chromatography system (Shimadzu HPLC class VP series, Shimadzu Corporation, Kyoto, Japan) with two LC-10 AT, VP pumps, variable wavelength programmable UV/Visible detector SPD-10A, VP, CTO-10AS VP column oven, SCL-10A, VP system controller. A 20 µl Hamilton syringe was used for injecting the samples. Data were analyzed by using PEAK software. Double beam UV-VIS spectrophotometer Model UV-VIS 2301 (Tech-comp limited, Hong Kong, Japan) was used for spectral studies. Degassing of the mobile phase was done by using Ultrasonic Bath Sonicator (Loba Chemie Pvt. Ltd. Mumbai, India). Samples were weighed by using Denver electronic Weighing Balance (Denver instruments, Colorado, USA.).

Separation was achieved isocratically with a Kromosil C18 analytical column (250 mm × 4.6 mm I.D., 5 µm particle size), under reversed phase chromatographic conditions, eluted with a mixture of acetonitrile and water (85:15 *v/v*) as the mobile phase at flow rate of 1.5 ml/min. Detection was carried out by absorbance at 360 nm. The analysis was carried out at an ambient temperature and injection volume was 20 µl.

Standard solutions: The bulk form of oxytetracycline was obtained from Matrix Laboratories Limited, Hyderabad, India and was used as received. The stock standard solution of oxytetracycline (1 mg/ml) was prepared in mobile phase. Six series of oxytetracycline working standard solutions at the concentration values of 1, 2, 3, 4, 5 and 6 µg/ml were prepared from the stock standard solution by apt dilution with the mobile phase.

Sample collection: Seven different brands of honey samples were collected from local markets of Tenali, Guntur Dt. Andhra Pradesh, India. For each brand 6 honey samples were collected. Raw honey sample was collected from Repalle, Guntur, Dt,Andhra Pradesh, India. The samples were stored at −20°C until analysis.

Extraction of oxytetracycline residues from honey samples: The oxytetracycline residues from honey samples were extracted with optimized extraction method as described by Pagliuca G. et. al.[33]. Five gram honey sample was taken and dissolved in 20 ml of 0.1 M Na_2EDTA-Mcllvaine buffer at pH 4. The solution was vortexed for 5 minutes, filtered and made ready for Solid Phase Extraction (SPE) clean-up procedure. After extraction, 60 mg of sample was loaded on a Sampli Q OPT 3 ml cartridge previously conditioned with 1 ml methanol and 1 ml water. The SPE cartridge was then washed with 10 ml water. Finally, the sample was eluted with 1 ml ethyl acetate (Sdfine-Chem limited, Mumbai, India) directly in sample tube. After evaporating the solvent at 40ºC under nitrogen stream, the residues were reconstituted with 1 ml of mobile phases.

General analytical procedure: Working standard solutions equivalent to 1 to 6 µg/ml of oxytetracycline were prepared by appropriate dilution of the stock standard solution with the mobile phase. Twenty µl aliquot of each solution was injected into the column in triplicate. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1.5 ml/min. The peaks were recorded at 360 nm. The linearity curve was constructed by plotting peak area versus concentration of the oxytetracycline. The concentration of the unknown was read from the calibration graph or computed from the regression equation derived using the mean peak area-concentration data

Quantification of oxytetracycline in honey samples: The extract prepared from honey samples, as described in section "Extraction of oxytetracycline residues from honey samples",

was further diluted appropriately with the mobile phase for the analyses of oxytetracycline residues by the proposed method. The honey sample solution was injected into HPLC system in duplicate. The chromatograms were recorded. The area under the peak was calculated. The concentration of oxytetracycline in the honey samples were calculated using the corresponding calibration curve or corresponding regression equation.

Results and Discussion

Method Development: A series of trials was conducted, with different analytical columns and with varied proportions of the organic solvent and water, to develop an appropriate reverse phase liquid chromatographic method for quantification of oxytetracycline in bulk and honey samples. Finally a typical chromatogram with better peak shape and low retention time was obtained with acetonitrile and water in the ratio of 85:15 *v/v* at a flow rate of 1.5 ml/min. The chromatographic separation was performed on Kromosil C18 analytical column (250 mm × 4.6 mm I.D., 5 µm particle size) by injecting 20 µl and analyte was detected with ultraviolet detector set at 360 nm. The retention time of oxytetracycline was found to be 6.81 minutes. The optimized conditions were given in table-1.

Table -1
Optimized chromatographic conditions

Parameter	Optimized condition
Column	Kromosil C18 (250 mm × 4.6 mm I.D., 5 µm particle size)
Mobile phase	Acetonitrile: Water (85:15 *v/v*)
Flow rate	1.5 ml/min
Detection wavelength	360 nm
Injection volume	20 µl
Column temperature	25 ± 1^0C
Retention time	6.81 minutes

Method validation: The validation was performed with above developed RP-HPLC method for estimation of oxytetracycline in bulk and honey samples according to ICH guidelines. Various parameters were evaluated such as system suitability, linearity, sensitivity, selectivity, precision and accuracy.

System Suitability: System suitability was performed to prove the adequacy of the resolution and repeatability of the system. System suitability was assessed by injecting six replicate injections of the oxytetracycline working standard solution (6 µg/ml) and parameters such as peak area, theoretical plates, retention time, and peak asymmetry were evaluated. The percentage relative standard deviation for the parameters was determined. The results were shown in table-2. The results reported are within the limits.

Table-2
System suitability parameters

Parameter	Mean value*	% RSD	Acceptable limits
Retention time	6.81	1.158	% RSD < 2.0
Peak area	6701	0.269	% RSD < 2.0
Theoretical plates (N)	4513	0.429	N > 2000
Peak asymmetry (P)	1.45	0.592	P < 1.5

* Average of five determinations

Linearity: To assess the linearity of the proposed method, calibration curve were constructed by plotting the peak area of the working standard solutions in the range of 1-6 µg/ml *vs* the oxytetracycline concentration. The regression data of six series of calibration curve are indicated in table- 3. From the results obtained the proposed method was found to be linear.

Table-3
Linearity and regression data

Parameter	Value
Linearity range (µg/ml)	1-6
Regression equation (Y = a + bc)*:	
Slope (b)	1112.85
Intercept (a)	65.857
Correlation Coefficient (r)	0.9996

Sensitivity: The sensitivity of the method was assessed by determining the parameters like limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ for the oxytetracycline were based on the lowest detectable peak that had signal/noise = 3 and 10, respectively. The obtained values (LOD = 0.05 µg/ml and LOQ = 0.10 µg/ml) were satisfactory and indicate that the method is sensitive.

Selectivity: The selectivity of the method was established by checking the potential interference with the components from the mobile phase and honey sample. The chromatograms of pure oxytetracycline, blank mobile phase and honey sample are recorded and compared (figure-2, 3 and 4). No interference was observed for any of the components from the mobile phase and honey sample. The results indicated the selectivity of the method for the assay of oxytetracycline in honey sample.

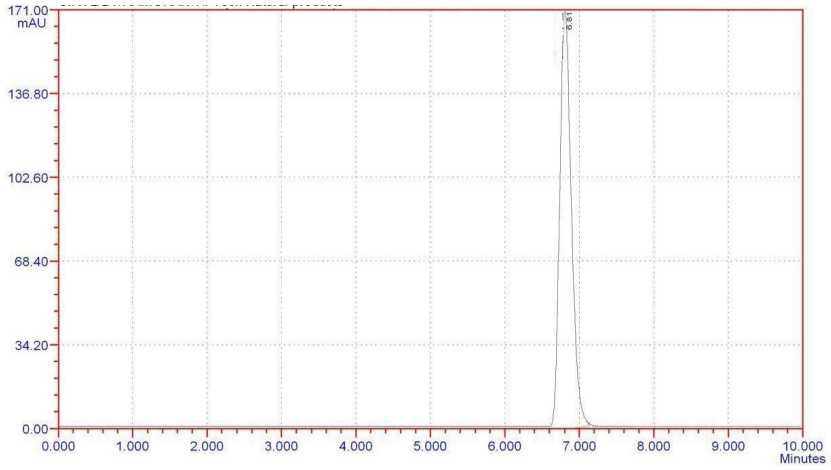

Figure-2
Chromatogram of pure oxytetracycline

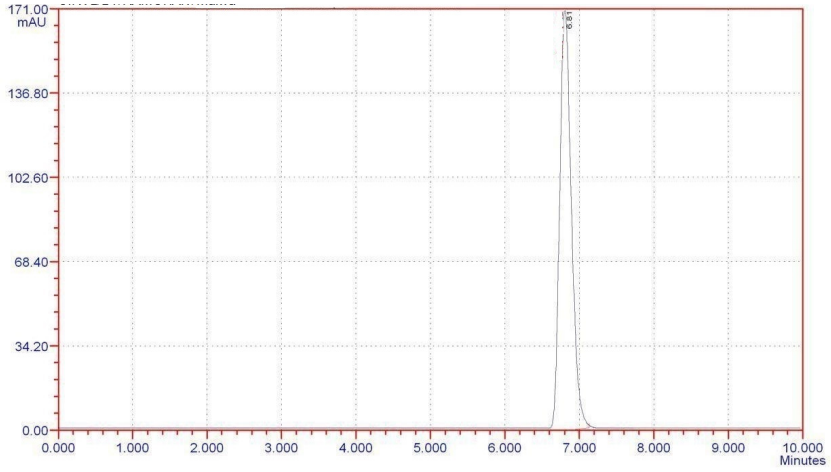

Figure -3
Chromatogram of oxytetracycline in honey

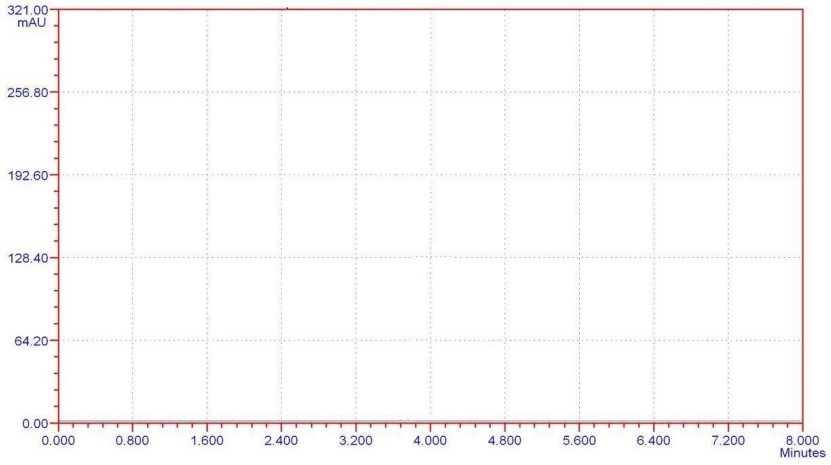

Figure-4
Chromatogram of mobile phase blank

Precision: The method precision studies were carried out by injecting six replicates of oxytetracycline working standard

solutions with the same concentration (6 µg/ml). The percentage relative deviation of the peak areas was calculated from the chromatograms and results (RSD = 0.596%) obtained were within the limits of 2%. Therefore, the proposed method was found to be precise.

Accuracy: The accuracy of the method was assessed by calculating the recovery studies of the oxytetracycline at three different concentration levels (50%, 100%, and 150%) by standard addition method. A known amount of oxytetracycline (within linearity range) was added to prequantified sample solution. Three replicates of each concentration were injected into the HPLC system. The mean percentage recovery of oxytetracycline was varied between 99.00 and 99.80%. The recovery results indicating that the developed method was found to be accurate. The results were shown in table- 4.

Table- 4
Percentage recovery results of oxytetracycline

Level (%)	Concentration of oxytetracycline (µg/ml)			Recovery (%)	Average recovery (%)
	Fixed	Spiked	Recovered		
50	2	1	2.97	99.00	99.43
100	2	2	3.98	99.50	
150	2	3	4.99	99.80	

Application of the method for the determination of oxytetracycline in honey samples: The developed and validated method was successfully applied for the quantification of oxytetracycline in honey samples. The results were summarized in table-5. For the present study, 7 branded honey samples and one raw honey sample was collected from the local markets at Tenali and Repalli, respectively.

Table -5
Assay of oxytetracycline in honey samples

S.No	Sample	Branded honey*	Concentration of oxytetracycline (µg/kg)
1	Sample -1	Brand - 1	0.12 ± 0.25
2	Sample -2	Brand - 2	0.09 ± 0.43
3	Sample -3	Brand - 3	0.17 ± 0.57
4	Sample -4	Brand - 4	0.08 ± 0.96
5	Sample -5	Brand - 5	0.17 ± 0.57
6	Sample -6	Brand - 6	0.11 ± 0.39
7	Sample -7	Brand - 7	0.05 ± 0.48
8	Sample -8	Raw honey**	0.54 ± 0.49

* All the branded honey samples were collected at local markets of Tenali ** Raw honey was collected from Repalli

Oxytetracycline was detected in all the honey samples. The concentration of oxytetracycline was found to be high in raw honey sample (0.54 µg/kg) followed by brand 5 and 3 (0.17 µg/kg), brand 1 (0.12 µg/kg), brand 6 (0.11 µg/kg), brand 2 (0.09 µg/kg), brand 4 (0.08 µg/kg) and brand 7 (0.05 µg/kg) samples. The European Union Regulations 396/2005 (2013) has recommended zero level tolerance for oxytetracycline residues in honey. However, the oxytetracycline residues were higher than maximum residue limit established by European Union Regulations[34].

Conclusion

A rapid, simple, accurate, precise, and selective HPLC with UV detection method has been developed for the quantification of oxytetracycline and honey samples. The developed method was successfully employed for the simple and rapid determination of oxytetracycline in honey samples with good precision and accuracy. The results of assay of the oxytetracycline in the selected honey samples indicated that in most of the collected samples the antibiotic residues were higher than the maximum residue limits set by European Union Regulations. Thus the proposed method is useful in the monitoring and quantification of the oxytetracycline in honey samples.

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