Detection of Tetracycline, Doxycycline, Chlortetracycline, and Oxytetracycline Antibiotics in Nineveha Drug Wastewater

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Abstract

This paper describes a simultaneous method for the determination of tetracycline (TC), and tetracycline's derivatives; doxycycline (doxysam), chlortetracycline (chlor-TC), and oxytetracycline (oxy-TC), in Nineveh Drug Factory (NDF) antibiotic industrial water course effluent, using solid-phase extraction followed by liquid chromatography separation. The identification of the target antibiotics in environmental samples was based on comparison of their HPLC peaks with those of the corresponding reference standard solutions. It was found that compromisingly satisfactory separation of the investigated antibiotics and stable base line could be achieved by using acetonitrile–formic acid solution as a binary mobile phase system. Still the compounds were separated on a Sep-Pak C18 cartridge with a resolve CN guard column using gradient elution and UV wavelength of 280 nm. The reported data indicated that tetracycline is the most frequently detected antibiotic in the samples. It was detected in five out of eight effluent water samples, with the maximum concentration of 0.412 µg/l. Doxycycline were detected in four with a maximum of 0.349 µg/l, chlortetracycline was found in only one sample (0.358 µg/l), while oxytetracycline was not detected.

Keywords: Nineveh Drugs Factory, High-Performance Liquid Chromatography (HPLC), antibiotics, tetracycline, NDF, Cep-pak18, oxytetracycline, chlortetracycline, doxycycline

Acronyms And Abreviations

Chlor-TC: Chlortetracycline
D.D: Distilled, deionized water
HPLC: High performance liquid chromatography
LOD: Limit of detection
NCDCR: National Center for Drug Control and Research
NDF: Nineveh Drug Factory
Oxy-TC: Oxytetracycline
S.D.I: Samara Drug Industry
TC: Tetracycline
t_R :Retention time in minutes
UV: Ultra violet wave

Introduction

Antibiotics are widely used to treat bacterial diseases and infections for both humans and animals. Some antibiotics also are used as growth promoters in animal feed additives. The human- and veterinary-use antibiotics are excreted unchanged or in metabolites and might gain entry to the natural waters through the incomplete removal during sewage treatment processes, agricultural runoff, and/or direct municipal wastewater discharge [1, 2]. The presence of antibiotic residues in aquatic environment has caused increasingly environmental and ecological concerns [3, 5] because antimicrobial compounds have the potential to promote antibiotic resistance in pathogens, changing the community structure/diversity of native bacteria. Some antibiotics are suspected to have direct toxicity to certain aquatic microorganisms [4]. Occurrence information of antibiotics is limited and most previous studies that were conducted in Europe and in U.S.A could be different from that of Iraq . However, the previous studies provide guidance for identifying the classes of antibiotics that are more persistent in the environment. Among the antibiotics prescribed in Iraq drug industries, tetracycline antibiotics were identified to be the most important water contaminants because of their high quantities of use, low susceptibility toward biotic and abiotic transformation and weak to moderate adsorption to sediments [7]. Yet despite the long history of antibiotic usage in Iraq, information regarding their production and use patterns is severely limited due to the lack of coordinated and comprehensive monitoring and documenting efforts, knowing that large amounts of antibiotic usage may result in their presence in environmental waters because up to 90 percent of the administered antibiotics can be excreted without undergoing metabolism [5].

Robust and sensitive analytical techniques have been developed to extract, concentrate, and detect these compounds in complex water matrices. The official methods of analysis for the tetracycline in the Code of Federal Regulations [4] are based on time-consuming microbiological, column chromatographic and UV spectrometric determinations. Several studies aimed at simplifying these analyses by high-performance liquid chromatography (HPLC) and have been published in the literature [1, 3, 6, 8].

This study focuses on the separation of the most commonly encountered commercial tetracycline antibiotics on the basis of their retention times (t_R) in effluent samples collected from Nineveh Drug Factory (NDF) industrial waste water treatment plant that utilizes a range of simple conventional treatment processes.

Sensitive analytical methods using solid phase extraction, followed by high performance liquid chromatography (HPLC) with fluorescence detection analysis, have been developed to improve the current knowledge on the occurrence level of these antibiotics in the water system as contaminants.

Site Description And History

The state company for Nineveh Drug Industries and Medical Appliances is in Mosul city in the north of Iraq. It is located at a distance of 10 Km to the north of Mosul on the highway between Mosul and Dohuk, in the left side of the city near AL-Karama Quarter.

The company of Nineveh Drug Industry (N.D.I) was founded in 22/4/2002, as it was a small factory subsidiary to the state company for Drug Industries and medical Appliances in samara (S.D.I).

The company occupies an area of about 7500 m^2 of which 5000 m^2 is a factory, consists of three factories and eight departments. The anti-biotic department is one of the effective departments, where tetracycline was of the first pharmaceuticals produced in this department.

The NDI had a simple combined wastewater treatment plant that consists of primary sedimentation tank, followed by aeration tank using air bubbles diffusion method. There after effluent was to be discharged directly to lands of a nearby valley.

Experimental Works

Materials

Pure standards of tetracycline (Samacycline), doxycycline (Doxysam), chlorotetracycline, and oxytetracycline, were obtained from National Center for Drug Control and Research (NCDCR) laboratories in the Ministry of Health, in the purest available grades, and were used without further purification.

Stock solutions of 0.5µg/ml of each were prepared by weighing the pure substances and dissolving them in distilled, deionized, resin filtered water (DD. water) and stored at 4^0C in the dark. These solutions were used to make up all working solutions and standards, by diluting portions of the stokes with (DD. water).

Sampling and samples enrichment

Water samples were collected in January, February, April and May (2010). Effluent samples were obtained by immersing 5l-high-density polyethylene jugs in raw watercourse at the antibiotic plant site. During sampling, the bottles were pre rinsed with sample for three times before a final sample was collected. Appropriate amount of sodium azide (0.5 g/l) was added to each sample immediately after sampling to inhibit potential biodegradation [1]. Samples were placed on ice during transport and were stored at 4°C. All samples were processed within 48 h from the time of collection. Upon reaching the HPLC laboratory in the Science and Technology Ministry in Baghdad, wastewater samples were placed into 1000 ml glass bottles, and extracted using a solid-phase extraction apparatus Sep-Pak C18 cartridge to enrich samples. Prior to extraction, the cartridges were conditioned with methanol and deionized water.

Prior to each trace enrichment, ten-milliliter rinses, of methanol followed in sequence by D.D water, then acetonitrile, then a second D.D water, and finally methanol rinse, served to both desorbs any organic impurities and also to wet the packing [6 , 8]. The sample was separated by means of a cartridge at a flow rate of 5 ml/min. The cartridge was connected directly to the HPLC port, so that all antibiotics in the water sample could be passed without any loss. Such connection enables the analysis of the antibiotics at very low concentration despite the extracted sample volume being small.

Stock Standard

Stock standard solutions were prepared by weighing the pure substances and dissolving them in distilled, deionized, resin filtered water (D.D. water), stored at 20°C in the dark. These solutions were used to make up all working solutions and standards, by diluting portions of the stokes with (D.D water).

Quantification

The method limit of detection (LOD) was determined using calibration curves prepared from spiked pure water samples. Five hundred milliliter volumes of pure water were spiked to

contain 0.1, 0.25, 1.0 and 2.5 mg of the four target analytes, with duplicate samples extracted at the higher concentrations (0.25, 1.0 and 2.5 mg) and four replicates extracted for the lowest concentration (0.1 mg).

The detection limits of the modified acquisition method were calculated to be between 0.030 and 0.075 µg/l (Table 1). Quantification of target analytes was based on external calibration curves, which were constructed from a plot of the peak area ratio of the analyte signal for the product ion of highest intensity versus concentration.

HPLC Equipment

The experiments were carried out in a Shimadzu high performance liquid chromatography (HPLC) system, equipped with a variable wave length detector (water model), and a 20 µl volume injector (u6k model). 10µl of the samples were injected into the column and a fixed-wavelength detector (Model 440, Waters Associates) operated at 280nm were used for all analysis. After investigating several different columns and mobile phases, it appeared that separation of tetracycline and tetracycline derivatives were best accomplished on a stationary phase of stainless-steel column (250mm x 4.6 i.d.) packed with 5-µm particles of C18 reversed-phase packing material. The column was washed with acetonitrile after use each day, using a program of 30 minutes linear gradient of acetonitrile: 0.02M KH_2PO_4, (56:44 to 80:20), pH 4.0 as mobile phase, flow rate 2.0 ml/min (600 psi), sensitivity of 2×10^{-3}.

All solutions were continuously degassed ultrasonically for one hour before use [6 , 8].

The characteristic chromatographic profiles obtained during combined standard analysis are shown in Figs.[1, 2, 3 and 4].

Quality assurance and quality control

A field blank and a procedural blank consisting of high purity water were included in each batch of sampling event and laboratory treatment, respectively, as controls of sampling and laboratory contamination. A solvent blank (acetonitrile) and an instrumental blank (redistilled water) were injected into the instrument every time before running a sample sequence to check potential analytical interferences.

Acetonitrile was injected into the column after running a sample of potentially high-level concentration to avoid possible cross-contamination. For routine determination, samples were performed in duplicate and the mean values were adopted.

Hplc Analysis

Day-to-day variation in detector response and column performance was significant enough, that standard curves had to be prepared prior to each set of chromatographic analysis.

The components of standard mixture were run individually, to determine the order of elution of each component with respect to the mixture components as in Fig.1. No difference was noted in the retention time of the component when they were injected; either individually or in the blend.

The retention time and response of each compound was determined by using a 50µl Hamilton syringe, injecting 2-4µl in the injection loop of 20 µl separately of the stock or dilute stock solution of each derivative. Before sampling, the column was cleaned with 50-50 percentage acetonitrile-water until base line stability was obtained. Stable base line and single corresponding peak for each chromatographed component are criteria for governing the purity of the components; no further purification was needed. None of the field blanks showed detection of any of the analytes, indicating that there was no cross-contamination during sample collection and also suggesting that the method is not susceptible to false positives due to matrix effects.

A phosphate buffer was used to maintain a constant pH throughout the test, in an attempt to improve elution, the pH of the mobile phase was reduced by the addition of small amount of acetic acid [1, 6 , 8].

Compound Identification and Quantification

The identification of the target antibiotics in environmental samples was based on comparison of their HPLC peaks with those of the corresponding reference standard solutions, table 1, and Figs. [1, 2, 3, 4].

The concentrations were calculated relative to the internal standards and retention times, by using the following equation [1, 6, 8, and 9]:

$$(Wu)\ \mu g/ml = (Au \times Ws) / (A_S) \quad \ldots\ldots..(1)$$

Where Wu =concentration of the sample in µg/ml,

Au = area of the sample peak;

Ws = concentration of the standard in µg/ml;

As = area of the standard peak;

Results And Discussion

The results show that except for the oxy-TC all other target analytes were detected in the antibiotic plant effluent. TC is the most frequently detected antibiotic in the samples. It

was detected in five (of eight) waste water samples, with the maximum concentration of 0.412 µg/l. Doxycycline was detected in two (with a maximum of 0.439 µg/l), and chlor-TC was found in only one waste water sample (0.459 µg/l). TC has been detected widely in European and North American rivers. Median and maximum levels of TC of 0.16 and 1.9 µg/l, respectively, were reported in American streams [10 , 11], which was comparable to the level in the NDF wastewater effluent. TC also was detected at 0.03 to 0.085 µg/l and a maximum concentration of 0.48 µg/l in German rivers [12 , 13].

Chlor-TC were reported 20 ng/l in river waters in Switzerland [18], which was about 1: 2.5 in magnitude of the NDF industrial effluent (0.358 µg/l) in the present study. The same can be said about receiving waters, with the difference that several mechanisms tend to decrease these concentrations (e.g. biodegradation, sorption, photo degradation). Although these levels may not pose direct risks to human health via contaminated drinking water nevertheless, the continuous introduction of these chemicals in the environment makes them "persistent, "regardless of their environmental half -life. Some TAs may reach drinking water supplies at trace levels, and some of these chemicals may survive drinking water treatment to be introduced in potable water distribution systems. In addition, the effects of this exposure through drinking water consumption are far from being understood, and the fact that these chemicals are not present alone makes it even harder to understand their possible combined effects.

Conclusion

HPLC has proven to be a fast and selective separation method for isolation of pure compounds from crude mixtures.

The effluent of Nineveh wastewater treatment plants revealed concentrations of three of the four test compounds, indicating that a chronic exposure to low levels of antibiotics exists as the result of incomplete elimination by current wastewater treatment processes.

Results from this study show that the wastewater treatment plant processes are not effective in the complete removal of the target antibiotics. However, since only the plant effluent was sampled, and the effluent was to be discharged to nearby valley, it would be likely that some of these compounds would also take place in nearby surface and ground water, yet it is not possible at this time to quantify the presence of antibiotics in these waters.

The emission of antibiotics into the environment should be reduced as an important part of the risk management. For this reason, unused therapeutic drugs should not be flushed down the drain and physicians must be made aware that antibiotics are not completely metabolized by patients. On the contrary, antibiotics and other pharmaceuticals are often excreted largely unchanged.

Table 1 Summary of the target analytes, limits of detection (LOD) and retention time		
Compound (Abbrev.)	**LOD (µg/l)**	**Retention time t_R (min.)**
Oxytetracycline (oxy-TC)	0.040	10
Tetracycline (TC)	0.030	14
Chlortetracycline (chlor-TC)	0.075	23
Doxycycline	0.037	27

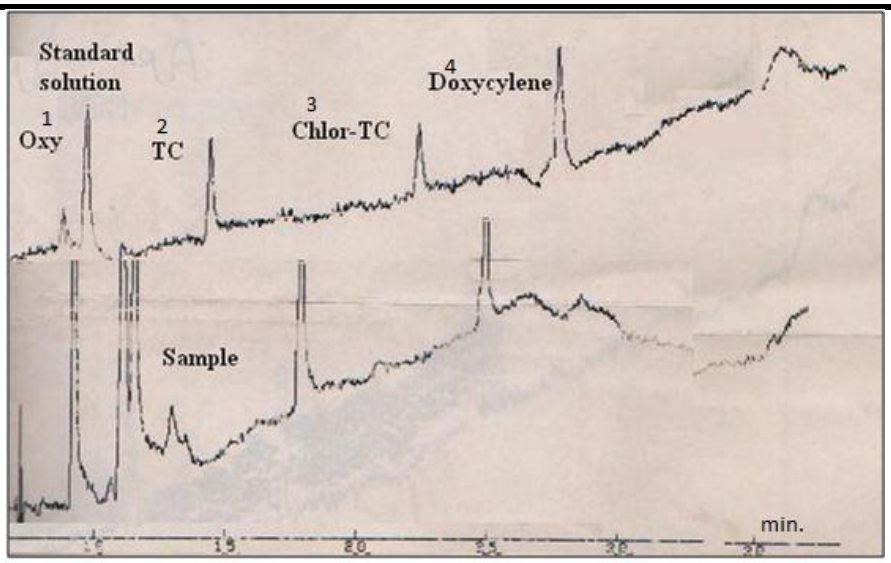

Fig. 1. High-performance liquid chromatogram of the standard solution. Peaks 1= oxy-TC; 2=TC; 3=chlor-TC, and 4= Doxycyclene having retention time of about 10, 14, 23, and 27 min respectively, and a chromatogram of NDF industrial effluent.

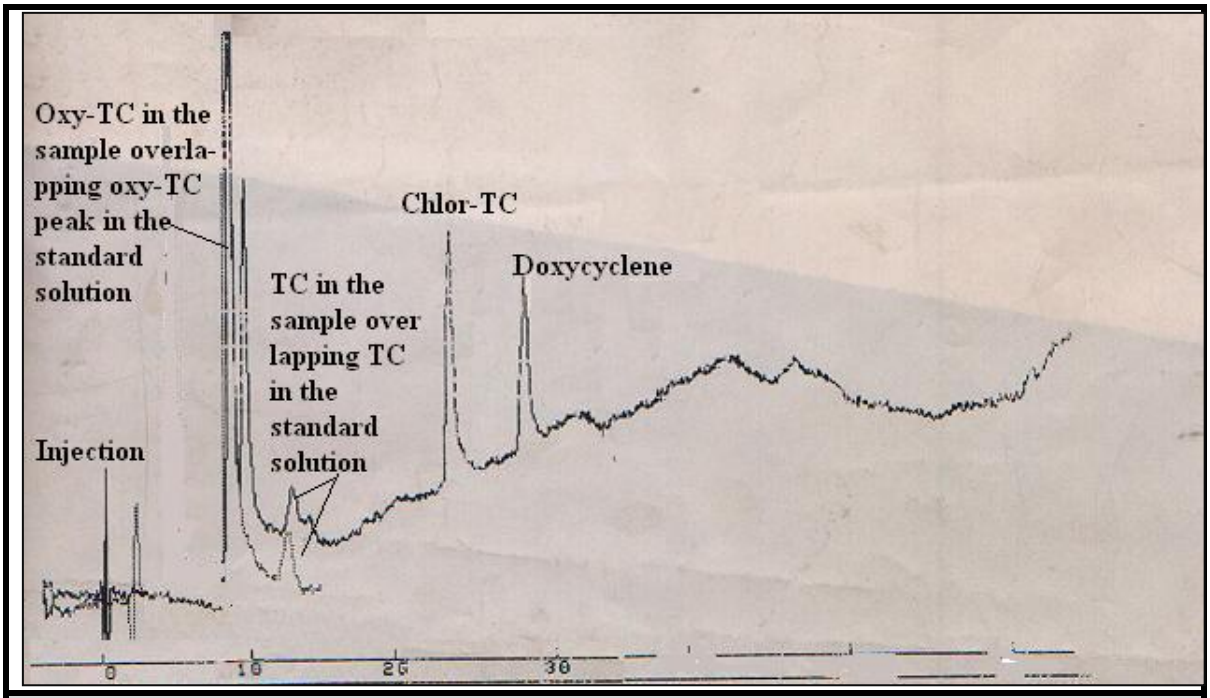

Fig.2 Detection of oxy-TC and TC antibiotics in effluent wastewater

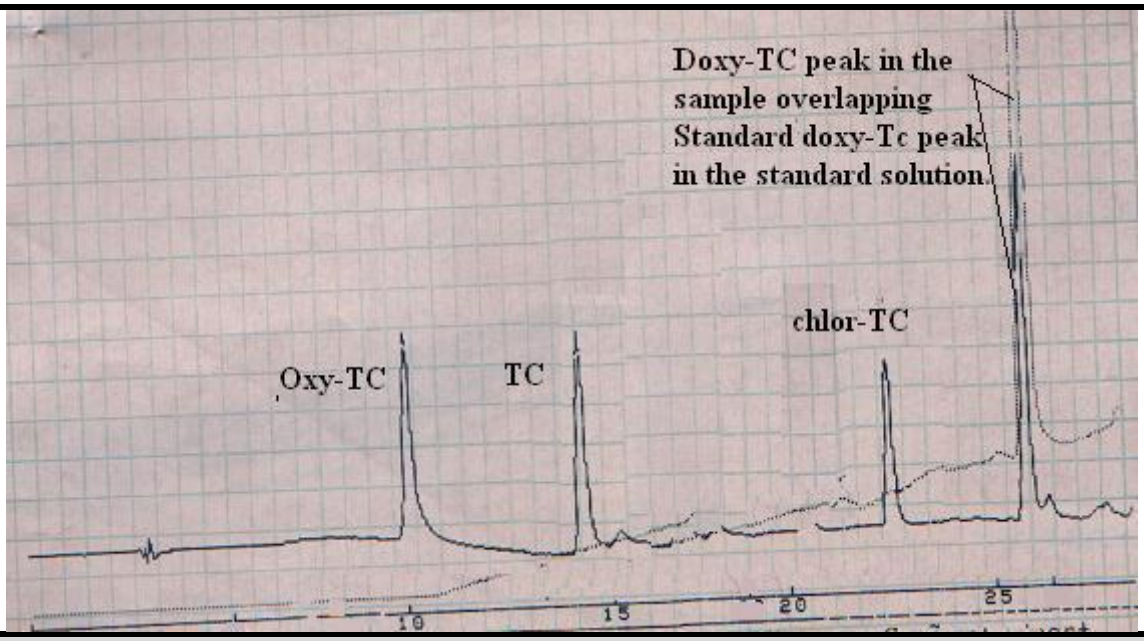

Fig. 3 Detection of doxycycline in NDF industrial effluent

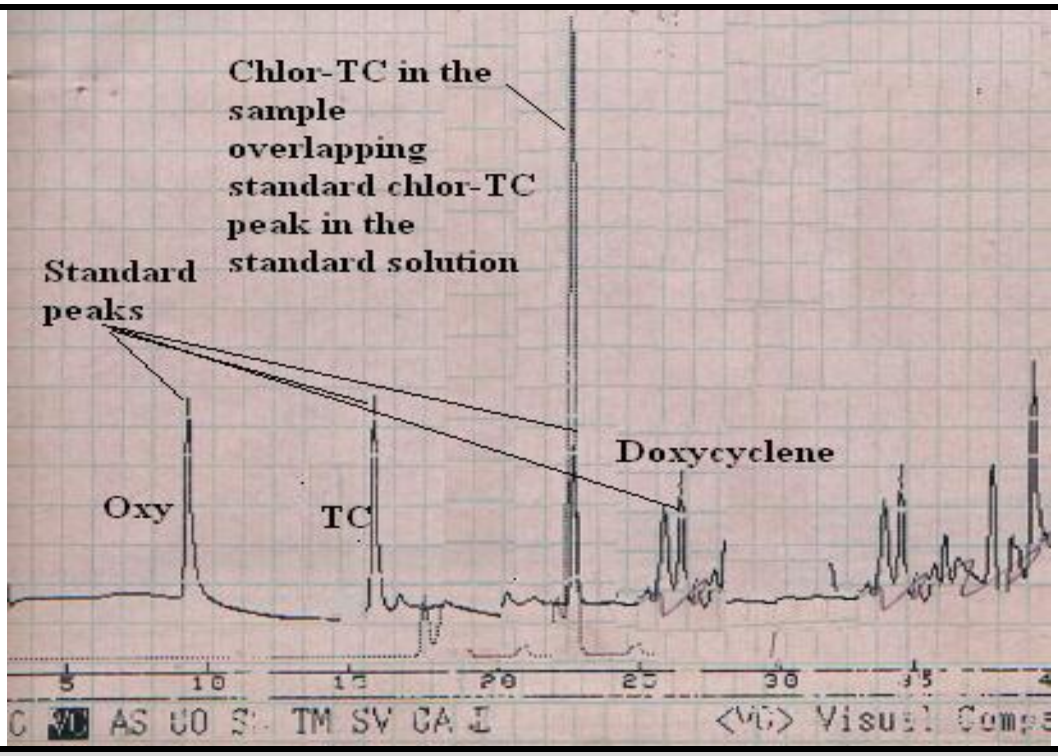

Fig. 4. Detection of chlor-tetracycline in NDF industrial effluent

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التحري عن المضادات الحيوية نوع تتراسايكلين ودوكسي سايكلين وكلوروتتراسايكلين واوكسي تتراسايكلين في مياه التصريف الصناعية لمعمل ادوية نينوى

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الخلاصة:

يصف البحث طريقة سريعة للكشف عن التتراسايكلين (TC) و مشتقاته الدوكسي سايكلين (الدوكسي سام) والكلورتتراسايكلين ,(chlor-TC) والاوكسي تتراسايكلين (oxy-TC) في المياه الصناعية المصرفة عن وحدة انتاج المضادات الحيوية في معمل ادوية الموصل (NDF) وذلك باستخدام طريقة الاستخلاص بالطور الصلب يتبعها عملية فصل بطريقة كروموتوغرافيا السائل . اعتمدت عملية كشف المضادات الحيوية المذكورة اعلاه على مبدأ مقارنة قمم المخططات الكروموتوغرافية للعينات مع نظيراتها القياسية باستخدام جهاز الفصل الكروموتوغرافي ذو الاداء العالي (HPLC). وقد اظهرت التجارب ان احسن فصل يمكن الحصول عليه بدلالة وضوح وتباين القمم واستقرار منحى الفصل stable base (line) **كان باستخدام المحلول الثنائي اسيتونايترايل –حامض الفورميك كطور متحرك (الطور الناقل). كما تم استخدام فلتر من نوع Sep-Pak C18 لتركيز المضادات الحيوية وعمود فصل ابتدائي (حارس) لضمان سلامة عمود الجهاز وطول موجي 280 نانومتر. بينت النتائج وجود مادة التتراسايكلين في اغلب العينات فقد ظهر في خمسة من اصل ثمانية وبلغ اعلى تركيز له 0.412µg/l بينما ظهر الدوكسي سايكلين في اربعة نماذج من اصل ثمانية وبلغ اعلى تركيز له 0.349µg/l ، وكذلك تم الكشف عن الكلورتتراسايكلين ولكن في نموذج واحد من اصل ثمانية وبتركيز قدره 0.358µg/l ، ولم يظهر الفحص اي اثر للمركب اوكسي تتراسايكلين.**