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Evaluation of analytical method for determination of tetracyclines in their dosage forms by HPLC using different types of chromatographic stationary phases (RP-C_8 and RP-C_{18})

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ABSTRACT

Antibiotics are extensively used in animal husbandry practices for the treatment of diseases. Four tetracyclines are commercially available as veterinary medicines, of which oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DOX). In the present investigation, a novel simple, sensitive, and inexpensive method of analysis was verified for tetracyclines by RP-HPLC system in bulk drug substance and pharmaceutical forms via two stationary phases (C8 & C18). The separation was attained on a 250 mm column length, 4.6 mm internal diameter, 5 µm particle size, C18 and C8 columns with a (55:25:20) 5% glacial acetic acid: acetonitrile: methanolic acid as elution solution at a 355 nm wavelength. The method was performed at a0.8 ml/m inflow rate. This method was applied without an extraction process. The method was validated following ICH and USP guidelines. It was proved to be reproducible and more economical due to the short retention time of less than 6 min by C8 column and 8 min by C18 column which enabled analysis of tetracycline pharmaceutical products with a relatively lower volume of mobile phase. The result of formulation analysis was statistically validated with a high recovery percentage. Furthermore, the developed method was free from the interference of excipients used in the formulation. The validated method was accurate, precise, linear, and reproducible. The data suggest the possibility of using the method in quality control laboratories with better routine analysis of tetracyclines in their dosage forms.

INTRODUCTION

Antibiotics are extensively used in animal husbandry practices for the treatment and diseases (**Moudgil et al. 2019**).Tetracyclines are synthetic antibiotics, highly active against a varied range of Gram-negative and Gram-positive microbes. Four tetracyclines are commercially available as veterinary medications, of which oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DOX); their chemical structures showed in figure 1.This group of antibiotics is most commonly used for therapeutic purposes in veterinary practice due to their low cost, easy over-the-counter availability, and broad-spectrum (**Martins et al. 2015 Prado et al. 2015 and Jank et al. 2017**).

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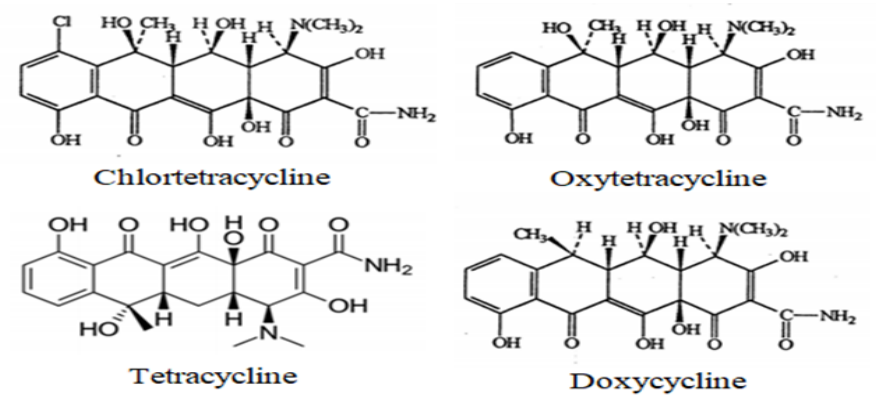

Figure (1): Chemical structure of tetracyclines (**Liu et al. 2018**)

A lot of techniques have been described for tetracycline's determination in various biological matrices and pharmaceutical preparations. While the official methods of assay retain the microbiological approach (**British Pharmacopeia 1993, United State Pharmacopeia 1995**) these techniques are lacking of specificityas they are not distinguish between different types of antibiotics leading to false-negative or false-positive results. A further problem of the bioassay is that the degradation products or contaminants of tetracyclines, as epi-, anhydro-, and epianhydro-tetracyclines, which are present in varying amounts in the raw materials and finished products of tetracyclines, may also have antimicrobial properties. Thus the results of bioassay won't necessarily be a true representation of the antimicrobial potency of the labeled material. Under abnormal environmental conditions (heat, pH, humidity, and light), tetracyclines undergo reversible epimerization at C-4 to form a mixture of epi-, anhydro-, epianhydro and tetracyclines.

The epimer has antibiotic activity only 2–5% of that of the parent tetracycline. These degradation products, especially the epianhydrotetracyclines shown definite toxicity to renal tubular function and initiate a reversible Fanconi-Type syndrome, characterized by polyuria, polydipsia, vomiting, proteinuria, aminoaciduria, and acidosis (**Pena et al. 1998**). From this viewpoint and owing to the toxicity and probable inactivity of the impurities and degradation products, it is very important to determine tetracyclines in dosage forms simply and precisely.

Recently, new techniques are applied instead of thetraditional biological assay, high-performance liquid chromatography (HPLC) to achieve the sensitivity, specificity,and reproducibility for identification and quantification of tetracyclines in its pharmaceutical products. Few reverse-phase high-performance liquid chromatographic (RP-HPLC) methods were reported for the estimation of tetracyclines (**Bryan and Stewart 1994, Kazemifard and Moore 1997, Moreno-Cerezo et al. 2001, Hussien 2014**).

In this paper, the LC method for analysis of selected tetracyclines in bulk drug substance and dosage forms using two different types of stationary phases (C8 and C18) are described and validated. This method is applicable for combination use and has been shown to identify oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DOX).

MATERIAL AND METHODS

Chromatography (instrumentation and conditions):

The HPLC system consists of a quaternary pump, model1200, an auto sampler injector, and a UV-Vis detector (Agilent). The chromatographic conditions (**Ruz et al. 2004)** were illustrated in table (1) and quantitative analysis was performed and calculated from the area under curves extrapolated automatically by the software.

Table (1). Chromatographic conditions

Mobile phase	5% glacial acetic acid: acetonitrile: methanol (55:25:20)
Flow rate	0.8 ml/min.
Injection volume	10 µl
Column type	LiChrospher C8 and C18 (250 mm, 4.6 mm i.d., 5 µm particle size)
Column temperature	35ºC
Stop time	10minutes
UV- detector	355 nm

Chemical reagents:

Glacial acetic acid, water, acetonitrile, and methanol of HPLC grade (Honeywell Co, Germany).

The reference standards are Oxytetracycline hydrochloride (100%), Tetracycline hydrochloride (97.1%), Chlortetracycline hydrochloride (102.2%), and Doxycycline hydrochloride (85.7% as doxycycline base); *USP* reference standards, obtained from Twinbrook Pkwy, Rockville, MD, USA.

Pharmaceutical products containing OTC-HCL (Spectropan®5% inject able solution), DOX-HCL (DOXIN® 20% water soluble powder), and CTC-HCL (Chloracline® 20%water soluble powder) were obtained from PHARMA SWEDECo.; TC-HCL (Tetracyclin® WSP Vet 5% water soluble powder) from MSD Animal Health Co.

Standard preparation:

Stock solutions of OTC, TC, CTC, and DOX were prepared by dissolving 10 mg of each compound up to 10 ml methanol to obtain(1 mg/ml for OTC-HCL, 0.97mg/ml for TC-HCL, 1.022 mg/ml for CTC-HCL and 0.857 mg/ml for DOX-base).

The intermediate solution is prepared by taking1 ml from stock solution up to 10 ml of the mobile phase.

The working solutions were set by taking 20, 50, 100, 200, and 500µl from intermediate standard solution up to 1ml of the mobile phase to give a sequence of working solutions that should be freshly prepared. The quality control sample concentrations are10, 9.7, 10.22,and 8.57µg/ml for OTC-HCL, TC-HCL, CTC-HCL, and DOX-base; respectively.

Calibration plots:

The linearity was achieved over the range of (2–50; 1.94-48.5, 2.044-51.1, and 1.714-42.85 µg/ml for OTC-HCL, TC-HCL, CTC-HCL, and DOX-base in progress). Calibration curves were freshly prepared (triplicate injection for each level) and estimation of the amount of the analytes in samples was interpolated from these curves.

Drug preparation:

The pharmaceutical products are prepared by dissolving an accurate amount of drug in methanol to obtain a solution at a concentration of (1 mg/ml); then make variable dilutions in the mobile phase.

Validation of HPLC assay:

Ideal validation features which should be considered are Linearity and Range, Accuracy (standard addition), Precision (Repeatability, Intermediate Precision), Specificity, Detection Limit (DL), Quantitation Limit (QL), Robustness, and System suitability testing (SST) (**ICH 2005 and USP 2019)**.

Linearity and range:

Linearity should be established across the range of the analytical procedure. It appears to be a linear relationship, test results should be proven by appropriate statistical methods (e.g., by calculation of a regression equation and correlation coefficient) to estimate the degree of linearity. The correlation coefficient, the regression lineslope, and y-intercept should be determined. The use of at least five concentrations for the establishing of linearity is recommended by ICH.

Accuracy (Standard addition):

Accuracy is calculated as recovery percentage by assaying the known added amount of the analyte in the drug at 3 levels (50, 100, and 150%) by triple injection.

Precision:

The analytical procedure precisions typically expressed as the relative standard deviation (RSD %) of a sequence of measurements. It may be a measure of the degree of frequency (interday precision) or the average accuracy that expresses variances within the laboratory, as is the case on different days (interday precision), or with different person sin the same laboratory.

Precision should be evaluated using at least nine determinations cover the stated range of procedure (i.e., three different levels and triplicates for each level).

Specificity*:*

In the case of analysis, proof of specificity requires that it can be demonstrated that the assay is not affected by the occurrence of impurities or recipients. Practically, this can be done by fortifying the drug or product substance with appropriate concentrations of impurities or excipients and demonstrating that the result of the examination is not affected by the presence of these foreign substances.

Detection and quantification limits (DL and QL):

DL is determined by analyzing samples with known quantities of the analyte and determining the lowest level at which the analyte can be dependably detected; It was determined using the SD deviation (S) of the intercept and the slope (a) of the lowest concentrations of a standard plot. [DL = 3.3 * S / a;

QL = 10 * S /a).

Robustness*:*

Robustness may be determined during the development of the analytical procedure to ensure the ability of liquid chromatography assay to stand up to slight variations in chromatographic conditions as the wavelength (± 2 nm), column temperature (± 2°C), and mobile phase composition; the following adjustment limits apply to minor components of the mobile phase (specified at 50% or less). The quantity of these constituents can be attuned by ±30% relative. Nevertheless, the variations in any constituent cannot exceed ±10% absolute (to the total mobile phase), consequently, mixture ranges of 47.5:32.5:20 – 62.5:17.5:20 or 61:25:14 – 49:25:26 while the original mixture is 55:25:20 (**USP 2019**).

System suitability testing (SST):

System suitability testing is run every time a method is used either before or during analysis. The results of each system suitability test are compared with defined acceptance criteria and if they pass, the method is deemed satisfactory on that occasion. The primary SST parameters considered are retention time (t_R), theoretical plate number of column (N), and tailing factor (Tf).

Statistical Assessment:

The obtained results were analyzed using SPSS Inc., version 22.0, Chicago, IL, USA to calculate mean, standard deviation (SD), and relative standard deviation (RSD) (**Morgan et al. 2019)**.

RESULTS

Linearity and range:

Linearity results for both columns are illustrated in table (2) and figure (2-5) showing that an excellent correlation between the analyte concentration and the peak area.

Table (2) Results of linearity and range

Parameters	Column	OTC	TC	CTC	DOX
Linearity range (µg/ml)		2.00-50.0	1.940-48.50	2.044-51.10	1.714-42.85
Correlation coefficient	C8	0.9999	0.9999	0.9997	0.9993
	C18	0.9999	0.9999	0.9999	0.9997
Slope	C8	6.2173	4.528	1.509	4.6455
	C18	6.4937	4.8007	1.696	5.3587
Intercept	C8	0.0292	0.6003	0.2703	2.2023
	C18	- 0.2041	- 0.2939	0.127	1.877

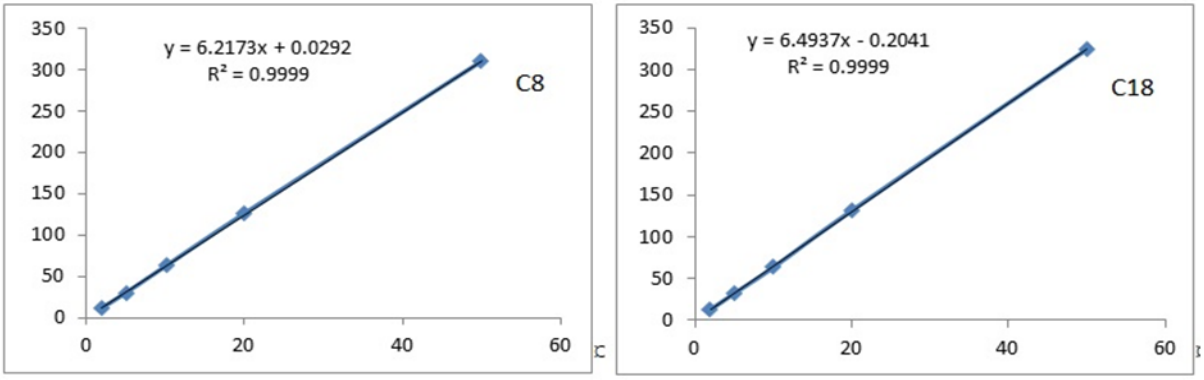

Figure (2). Calibration curves of OTC-HCL using C8 & C18 columns

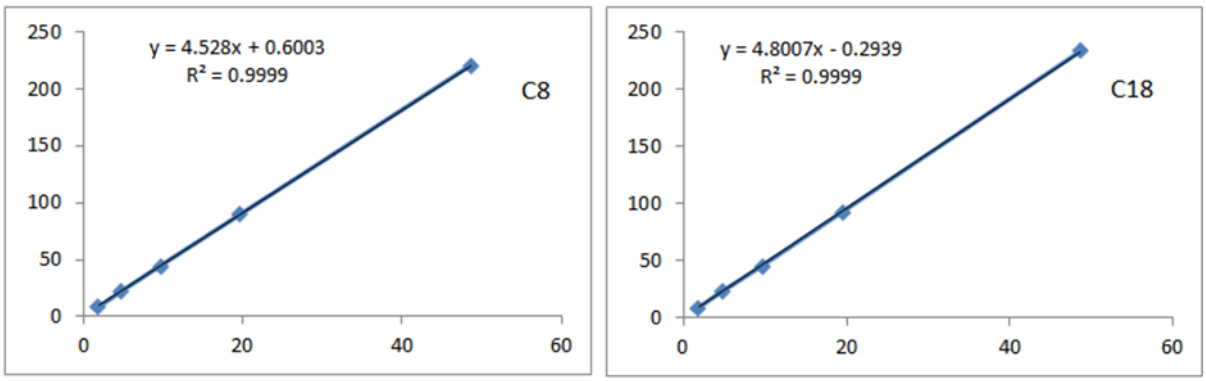

Figure (3). Calibration curves of TC-HCL using C8 & C18 columns

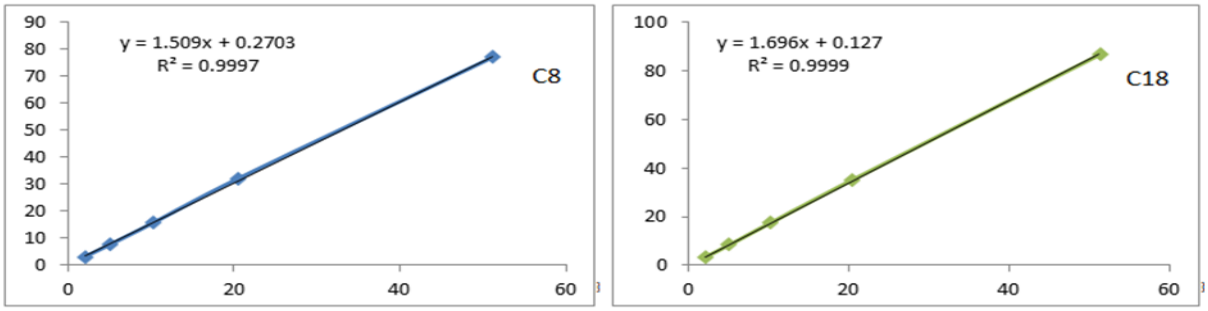

Figure (4). Calibration curves of CTC-HCL using C8 & C18 columns

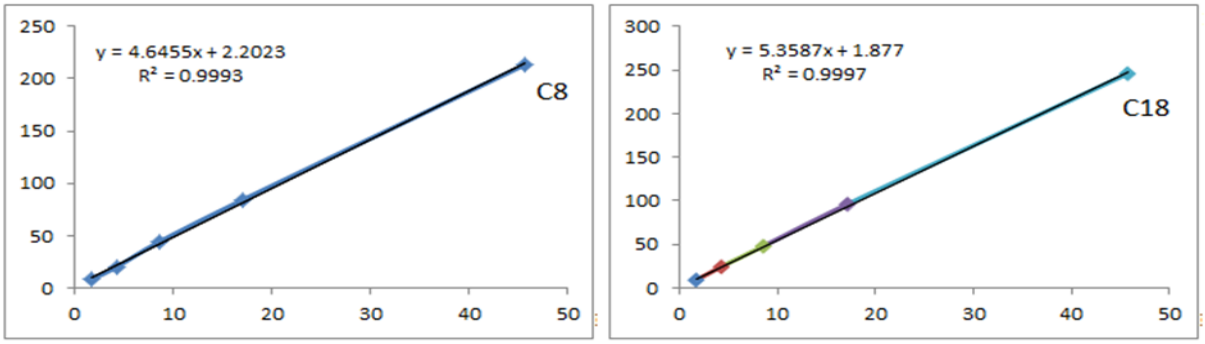

Figure (5). Calibration curves of DOX-HCL using C8 & C18 columns

Accuracy:

The percentage recoveries for both columns were analyzed and tabulated in table (3).

Table (3). Results of accuracy (%)

Level (%)	Column	OTC	TC	CTC	DOX	Acceptance criteria
50	C8	98.7	99.1	99.1	99.3	98- 102 %
	C18	99.5	99.2	98.8	99.5	
100	C8	100.1	101.5	100.5	100.01	
	C18	100.5	100.2	101.1	100.1	
150	C8	101.1	99.1	101.2	100.4	
	C18	101.2	98.7	99.05	101.1	

Precision:

The results of precision are summarized in table (4).Relative standard deviations (RSD) were calculated and assured that both columns were found to be precise. The Intra-day Precision ranged from 0.51- 0.92 % and the Intermediate Precision ranged from 0.8- 1.6 % for all tested compounds.

Table (4). Results of precision (RSD %)

Parameters		Column	OTC	TC	CTC	DOX	Acceptance criteria
Repeatability (Intra-day Precision)		C8	0.74	0.61	0.82	0.55	≤ 1%
		C18	0.71	0.92	0.66	0.51	
Intermediate Precision	Inter-day Precision	C8	0.82	0.81	0.94	0.89	≤ 2%
		C18	0.9	0.88	1.2	1.1	
	Different persons	C8	1.2	1.4	1.1	1.6	≤ 2%
		C18	0.8	1.1	0.92	1.5	

Specificity:

As shown in fig (6-9), HPLC Chromatograms of four tetracyclines pure standard at a concentration of QC and their dosage forms at a concentration of QC. No matrix interferences were observed on the intended chromatograms at the same retention time (T_R).

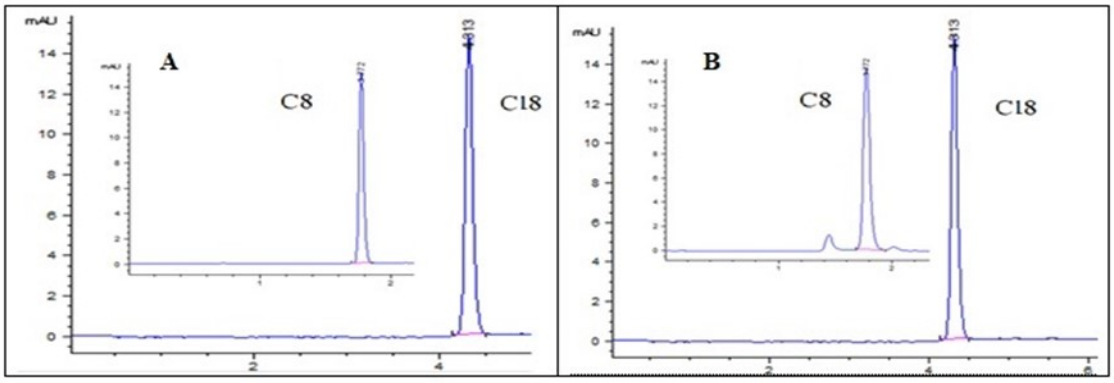

Figure (6). Chromatograms of OTC-HCL (10µg/ml) in pure [A] and dosage [B] forms

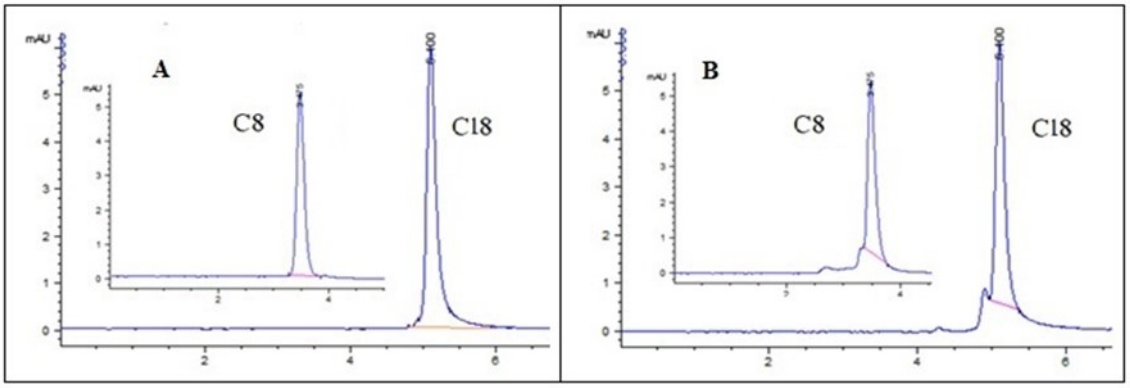

Figure (7). Chromatograms of TC-HCL (9.7µg/ml) in pure [A] and dosage [B] forms

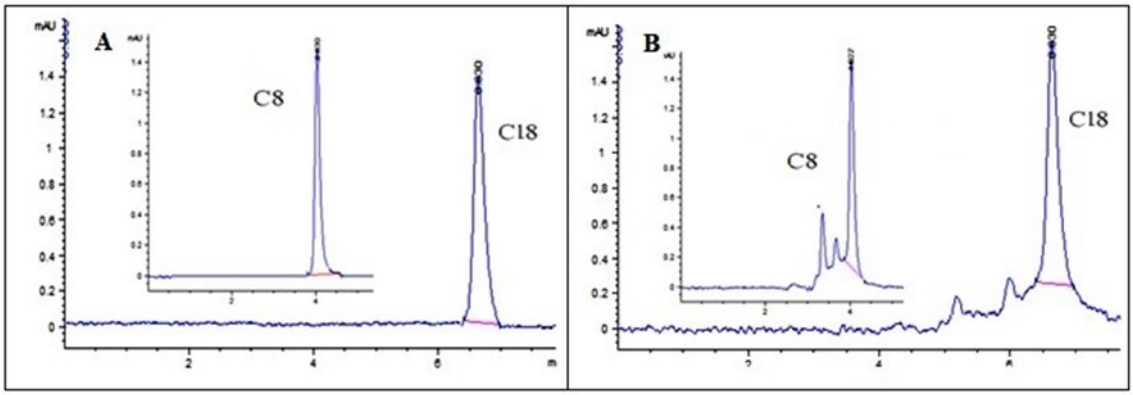

Figure (8). Chromatograms of CTC-HCL (10.22µg/ml) in pure [A] and dosage [B] forms

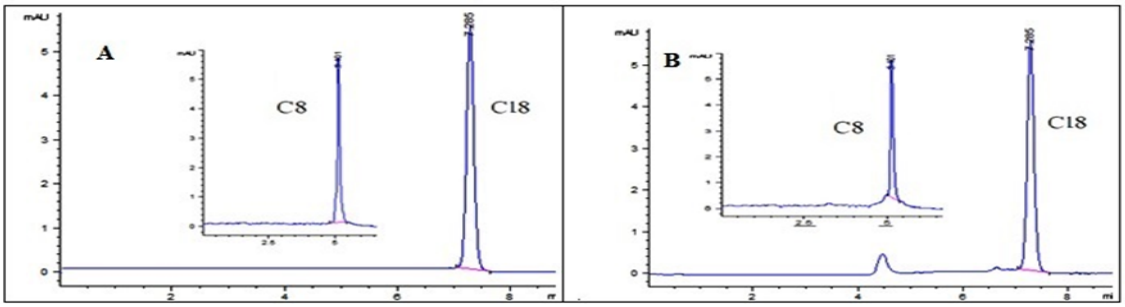

Figure (9): Chromatograms of DOX-base (8.57µg/ml) in pure [A] and dosage [B] forms

DL and QL:

The DL and QL values were summarized in table (5); the obtained results indicate that the developed method is sensitive.

Table (5):. Results of DL and QL (µg/ml)

Parameters	Column	OTC	TC	CTC	DOX
DL	C8	0.13	0.05	0.25	0.25
	C18	0.09	0.03	0.19	0.15
QL	C8	0.38	0.16	0.76	0.76
	C18	0.26	0.09	0.56	0.46

Robustness:

The robustness results are summarized in table (6). Small changes in the chromatographic conditions have no significant effect on the peak area of the peak of specified drugs. Hence, the developed method was found to be robust.

Table (6). Results of robustness (RSD%)

Altered condition	Column	OTC	TC	CTC	DOX	Acceptance criteria
Column temperature	C8	0.53	0.22	0.26	0.63	≤ 2%
	C18	0.35	0.43	0.42	0.24	
Wavelength	C8	1.08	0.37	0.60	0.40	≤ 2%
	C18	0.61	0.59	0.48	0.89	
Mobile phase composition	C8	1.47	0.58	0.48	1.02	≤ 2%
	C18	0.96	0.50	0.65	0.93	
Pooled RSD%	C8	1.03	0.51	0.53	0.82	≤ 6%
	C18	0.65	0.51	0.51	0.75	

System Suitability test:

The system suitability test was performed using six replicate injections of standards before analysis of samples. Relative standard deviations of the retention time, tailing factor, and number of theoretical plates were illustrated in table (7).

Table (7). Results of SST (mean ± SD)

Parameters	Column	OTC	TC	CTC	DOX	Acceptance criteria
Retention time (t_R)	C8	1.8±0.01 (0.6 %)	3.5±0.01 (0.3 %)	4.0±0.03 (0.8 %)	5.10.04± (0.8 %)	RSD ≤ 1%
	C18	4.3±0.01 (0.2 %)	5.1±0.03 (0.6 %)	6.5±0.02 (0.3 %)	7.50.02± (0.3 %)	
Tailing factor (Tf)	C8	1.1±0.01	1.2±0.01	1.2±0.01	1.1±0.04	Tf ≤ 2
	C18	1.1±0.03	1.1±0.003	1.4±0.01	1.1±0.03	
Theoretical plates (N)	C8	4558±91	9151±80	6580±172	9050±81	N > 2000
	C18	13187±154	12086±178	10101±88	10098±172	

DISCUSSION

Excellent separation of four tetracyclines (oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DOX)) was achieved, and no overlapping peaks were detected using two stationary phases (RP-C8 &RP-C18). The calibration plots were linear; the relationship between concentration and peak area shows its suitability inan in-vitro investigation. The method precision and accuracy were found to be satisfactory. The results of this study were acceptable and the method was robust to be stable against slight changes in the chromatographic conditions.

The linearity was developed at five concentration levels. The prepared standard solutions were freshly prepared before injection into HPLC system as tetracyclines are highly affected by variations in temperature, time, humidity, light (**Okerman et al. 2007 Berendsen et al. 2011**). The correlation coefficient was greater than 0.999 recommended by **ICH (2005)**. The obtained results of accuracy indicate that the developed assay was found to with high recovery for both columns was found to be in the range of 98.7-101.5 % which was within the acceptable range set by **ICH (2005**). It was proved to be reproducible and more economicaldue to the short retention time retention time of less than 6 min by C8 column and 8 min by C18 column which enabled analysis of tetracycline pharmaceutical products with a relatively lower volume of mobile phase. There were no interferences of excipients in the examined pharmaceutical products; consequently extraction and purification are not required to be analyzed (**Sivakumar et al. 2007**).

This is in line with the theory of green analytical chemistry which is part of the concept of sustainable development. The miniaturization of analytical devices and shortening of the time elapsed between conducting analysis and obtaining reliable analytical results are important aspects of green analytical chemistry. Solvent less extraction techniques and the application of alternative solvents are considered to be the main approaches complying with green analytical chemistry principles (**Tobiszewski et al. 2010**).

The results of the study of the two columns are close together with slight differences in retention time as the four tetracyclines were separated approximately two minutes earlier by column RP-C8 from RP-C18. This is because column C8 is less hydrophobic, and has a lower density, but column C18 is more hydrophobic and has a higher density. Therefore, column C8 has less retention time (**Gritti et al. 2019**).

CONCLUSION

In the current investigation; a new simple, sensitive and economical analytical method for tetracyclines has been developed with RP-HPLC technology. The developed RP-HPLC method has been validated for accuracy, precision, linearity, and reproducibility following ICH and USP guidelines. Studies indicate that the developed methods were free from the interference of excipients used in the preparation. The limits of detection and the low quantities achieved indicate that the method is very sensitive. The data suggest the possibility of using the method in quality control laboratories and routine analysis of tetracyclines in their dosage forms using RP-C8 & RP-C18.

CONFLICT OF INTEREST:

None of the authors have any conflict of interest to declare.

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