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Research Article

Characterization of Metronidazole and Doxycycline Therapeutic Formulation by Ultraviolet Spectroscopy

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ABSTRACT

Objectives: To evaluatethe validity and stability of Doxycycline and Metronidazole combination as a pharmaceutical compound.

Materials and Methods: Standard solutions of Metronidazole and Doxycycline drugs were prepared in potassium phosphatebuffer (pH=7.4) at the 10, 15, 20, 25,and 30 mcg/ml concentrations. Absorption of the drugs was measured in spectrum of 200-400 nm both individually, and in the combined form using standard spectroscopy.Linearity, and accuracy (estimated by relative standard deviation (RSD) method) of the procedures were estimated by standard methods. For both individual and combined drugs, limit of detection (LOD) and limit of quantity (LOQ) concentrations were calculated.

Results: Metronidazole and Doxycycline showed maximum absorption (λ max) of 320 nm and 273.5 nm respectively (R2 =0.997 for both drugs). LOD values were 5.48 µg/ml and 1.63 µg/ml, while LOQ were determined as 1.64 µg/ml and 0.49 µg/ml for metronidazole and doxycycline respectively. RSD for both drugs was <4% for both drugs in days one and three. For combined (Metronidazole+ Doxycycline) drug, an additional λ max was observed at 228 nm. Linearity of combined form was comparable with individual drugs (R2=0.989). In addition, RSD of combined drug were <1.2% and <2% at day one, and <1.10% and <1.15% at day three for metronidazole and doxycycline respectively. Recovery percentage for both drugs was 100%.

Conclusion: Spectroscopy seems a simple, accurate, precise, reproducible, and economical procedures for simultaneous evaluation of metronidazole and doxycycline in a combined form.This methodology employs formation and solving of simultaneous equation using 320 nm and 273.5 nm as two analytical wavelengths for metronidazole and doxycycline respectively.

Keywords: Doxycycline, Metronidazole, UV spectroscopy, Simultaneous equations.

INTRODUCTION

Dental caries and periodontitis areas are known as the foremost oral health issues acrossthe world(1). The risk of systemic side effects and development of bacterial resistance are important

disadvantages of using systemic antibiotics. The high flow rate of gingival cervical fluid (GCF) quickly evacuates the released drug from the periodontal pocket to the mouth which leads todepletion ofperiodontal pocket from the drug(2). Given the importance of microorganismsinetiology/progression of periodontitis, treatment regimensmainly involve mechanical removal of plaquetypically in conjunction with topical antimicrobial chemotherapeutics; metronidazole (MTZ) and doxycycline (DOX)(3, 4).MTZ, the most potent tetracycline and a collagenase inhibitor, is effective against a broad-spectrum of microorganisms including gram positive and gram negative organisms, as well as the beta- lactamase producing strains which are frequently encountered in deep periodontal pocket.DOX is used clinically as an independent non-surgical periodontal therapy for treatment of periodontal infections (5). The clinical efficacy of anti-microbial irrigator solutions is low due to the rapid clearance of the solution by the flushing action of the crevicular fluid within the periodontal pocket (6). This efficacy may be improved by using drug delivery systems following insertion into the periodontal pocket, releasingthe antimicrobial agents at a controlled rate into the crevicular fluid (7).The importance of DOX arises from the fact that it has 7–20 times higher stability in gingival crevice when compared to other drugs.Dual mechanism of action of DOX represents it'ssecond most important feature. As an antibiotic agent, it shows more potent action against *A. actinomycetemcomitans*, warranting its efficacyagainst aggressive periodontitis(4-6).

MTZ and DOX, two drugs with separate antibacterial spectrum,have been suggested to be formulated as a combined therapy to renderwider antibacterial effects against aerobic and anaerobic periodontal microflora. Based on this, a periodontal drug transferring system has been developed in our laboratory forMTZ and DOX. In order to extend a controlled emancipation drug transferring system for periodontal space for MTZ and DOX, however, initially is needed to develop an appropriate assay method for validating combination of MTZ and DOX. To our best knowledge, there are no previous reports on a formulation containing combined MTZ and DOX, and so there was no available method for analysis this combination(7-10).

In the present work, MTZ and DOXwith different antibacterial spectra have been formulated as combinedtherapeuticwhich is expected to have a broader antibacterial effect, and be effective against both aerobic and anaerobic periodontal microflora. Accordingly, simultaneous use of metronidazole and doxycycline is suggested to be effective against wide range of periodontal pathogens.

MATERIALS AND METHODS

1. Validating the method of analysis

Common performance criteria that must be taken in the validation the methods of analysis include linearity, range, linearity, selectivity, limit of detection, limit of quantitative accuracy.

1.1. Linearity:

The linearity of a method of analysis is needed to show direct proportionality between the results. The concentration of the analytic in the tested sample is a whole domain. The linearity of the calibration curve by plotting the results should be confirmed, and then calculated by statistical methods such as regression line and by the least squares method(11).

First, maximum wavelength (λmax) for each drug in phosphate buffered solution (pH = 7.4) was measured using a single beam of UV. Different concentrations of the drugs were made by preparation, and diluting the standard solution.Absorption of the drug solution was measured at λ max for each concentration. In order to eliminate random errors and improve the accuracy of the results, three samples of each drug concentration were prepared. The graphswere depicted in terms of the absorption against the drug concentration.

The results of this instruction are strongly influenced by the quality of calibration function. Therefore, it is necessary to carefully evaluate the linearity range and linearity calibration function. Linearity of the calibration function is judged by

the regression coefficient of R2 ≥0.985 as acceptable.

Linear range of amethod of analysis is presented as the gap between the upper and lower concentrations of the analytic (including the two extremes). This is normally derived from linearity results. Precision, accuracy and linearity across the entire range should be fixed.

1.2. The limit of detection (LOD):

LOD of an analytic in the sample method is the lowest detectable concentration of the analytic that is needed to be quantified. There are different ways to measure the LOD. One of the most common methods is using the results of the calibration curve. By definition, a LOD is calculated as three times the standard deviation (SD) of the control sample divided by the slope of the calibration curve.

1.3. Limit of quantitative accuracy (LOQ): The lowest amount of analyticdetectable in the sample, which is ten standard folds in some cases. This parameter is calculated by dividing the response control sample over the slope of the calibration curve.

1.4. Accuracy:

Aindicator of reproducibilityof methods and analysis on a sample. One way to obtain accuracy is using absolute or relative standard deviation (RSD) based on standard deviation (SD). By repeatingthe tests in one day or in several days, comprehensive information can be obtained on the accuracy. Accuracy measurement can be based on RSD ≤% 4 or RSD ≤ 2% when there is a need to repeat the test conditions (equation 1).

Equation 1:

$$SD=\sqrt{\frac{(x_1-\bar{x})^2+(x_2-\bar{x})^2+(x_3-\bar{x})^2+\dots}{n-1}}$$

$$RSD=(SD/\bar{X})*100$$

The accuracy deviation is calculatedbased on the result of the actual values. Accuracy of the method can also be evaluatedbased on parameters of the recovery percentage of ≥110% or ≤90%. To calculate these parameters, the procedure is analyzed at known concentrations, and the measured valuesare compared with actual values, and represented as relative error percentage or recovery percentage (equation 2).

Equation 2:

Recovery percentage = (measured concentration / true concentration) * 100

2. Materials

UV-VIS spectrophotometer Shimadzu UV-1650 (Japan), pH meter Metrohm 781pH/ion meter, Switzerland and single pan electronic balance, Shimadzu AR 220, Japan, Oven, STERICELL, Medcenter, Germany were used for the experimental purpose. MTZand DOX were obtained from Sigma Aldrich with C16H19N3O5S, 3H2O (Construction formula).Potassium dihydrogen phosphate (KH2PO4, company Merck, Germany).Sodium hydroxide (NaOH, company Merck, Germany).

3. Preparation of buffer used for standard solution

In accordance with the Pharmacopoeia (USP30), 8.6 grams of KH2PO4 was dissolved in 800 ml of twice-distilled water (solution 1). The amount of 2 g of NaOH was dissolved in 100 ml twice-distilled water(solution 2). The two solutions were admixed, pH was set to 7.4, and the volume was reached to 1000 ml.

4. Preparation of standard solution

Standard solutions were prepared with concentration of 250 µg/ml. For this, 0.025 g of each drug was initially weighed and dissolvedin 100 ml of KH2PO4 solution buffer (pH= 4.7, net concentration of 0.05 M). A serial dilution was prepared from standard solution in order to draw the calibration curve.

5. Determination of λ max

An initial 100 mcg/ml concentration of both MTZ, and DOX were prepared as the stock solution, and this was diluted to a 45 mcg/ml concentration for λ max measurements. Absorptions of both drugs were determined in spectrum of 200-400 nm by spectroscopy. MTZ demonstrated an absorbance peak at 320 nm, whilstDOX peak absorbance was at 273.5 nm. In the combined formulation of MTZ and DOX, two iso-absorptive peaks were also observed at 289 nm and 346 nm.

6. **Simultaneous equation method:** The wavelengths selected for our method were320 nm and 273.5 nm asmaximum absorptions of MTZ and DOX respectively. Standard stocksolution(s) of MTZ and DOX wereprovided in phosphate bufferedsolution (pH= 7.4). The stocksolutions of the drugs were diluted with the buffer for obtaining a series of standardsolutions with 10-30 mcg/mL concentrations (10-15-20-25-30 mcg/mL). Theabsorptions were measured at selected wavelengths.

RESULTS

1. MTZ

For the analysis of MTZ, the first solution as the standard solution was prepared at 45 ppm, with considering λ = 230nm as the λmax (figures 1, and 2, table.1).

Table 1.Data of calibration curve of metronidazole phosphate buffer at pH = 7.41

Density(µg/ml)	1	2.5	5	7	10	15	45
The average absorption	0.0313	0.0523	0.0906	0.134	0.197	0.2686	0.9063

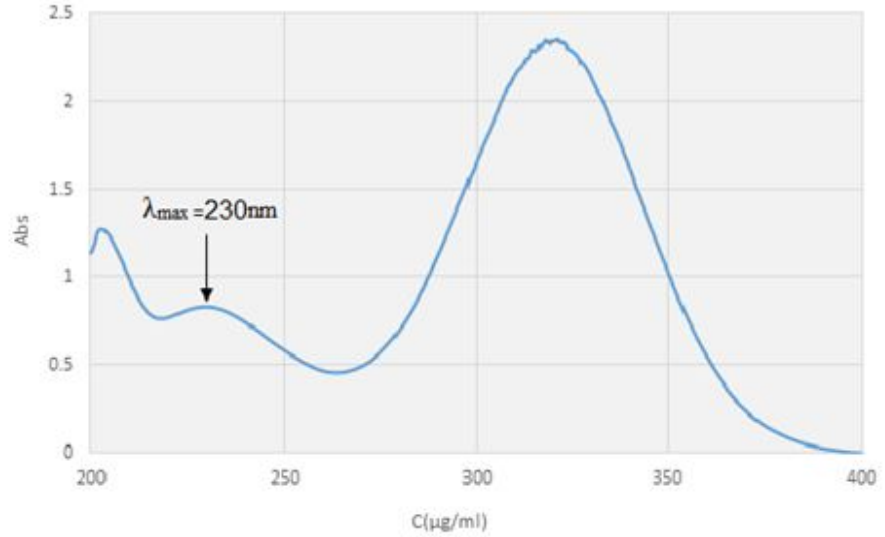


Figure 1: The whole UV standard solution metronidazole phosphate buffer at pH = 7.4.

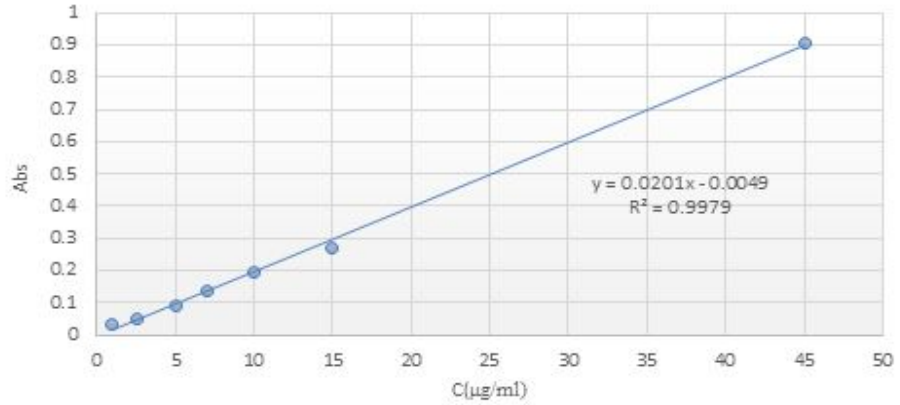


Figure 2: The calibration function metronidazole phosphate buffered at pH = 7.4

Between 1-45 ppm calibration function and the polynomial equation of y= 0.0201x-0.0049, R2 value wasmeasured as 0.9979 confirming the linearity of the calibration function. The LOD and LOQ values were calculated based on the "LOD=(3* SD_{stock})/Slope" and "LOQ=(10* SD_{stock})/Slope" equations. In this analysis, LOD and LOQ of MTZ were 5.48 µg /mland 1.644 µg / ml respectively (table 2).

Table 1 Analysis of the limit of detection(5.48 µg /ml) and Limit of quantitative accuracy (1.644 µg / ml) for Metronidazole.

	C=1	C=2.5	C=5	C=7	C=10	C=15	C=45
n=1	0.03	0.054	0.091	0.131	0.196	0.265	0.895
n=2	0.031	0.053	0.091	0.136	0.197	0.268	0.907
n=3	0.029	0.05	0.09	0.135	0.198	0.273	0.917
Ave	0.0313	0.0523	0.090667	0.134	0.197	0.2686	0.906333
SD	0.001	0.002082	0.000577	0.000646	0.001	0.004041	0.011015
RSD	3.194888	3.980241	0.636783	1.974441	0.507614	1.504636	1.215352

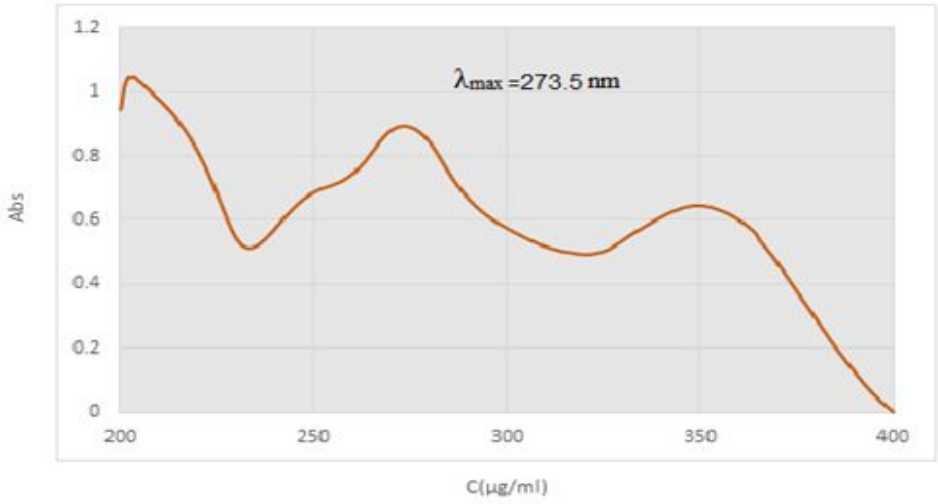
Regarding accuracy estimation, RSD values were less than 4% for all concentrations at days 1 and 3demonstrating the acceptable accuracy of the analysis method (table 3).

Table 2 Check the accuracy of the measurement method with UV linear in the range of concentration of metronidazole

UV absorption values for the unknown concentration solution	0.256	0.332	0.746
	0.255	0.331	0.745
	0.254	0.332	0.743
Average absorption	0.255	0.3316	0.7446
The measured concentration of calibration equation	12.93	16.74	37.2
The actual concentration	12	18	35
Recovery	%107.75	%93	%106.5

2. DOX

For the analysis of DOX drug, the standard solution was prepared at 50 ppm concentration,and λ = 273.5 nm was determined as the λmax(Figures 3, and 4, table 4).The calibration function was conducted at 1-50 ppm concentrations, and the polynomial equation was y = 0.0282x + 0.00142. R2 value was equal to 0.9978 indicating a linear function.The values LOD and LOQ were 1.637 µg/ml and 0.491 µg/ml respectively (table 5). Table 6 showsaccuracy and checking results for DOX.



Figur.3: UV spectrum of standard solution of phosphate buffer at pH = 7.4 drug doxycycline.

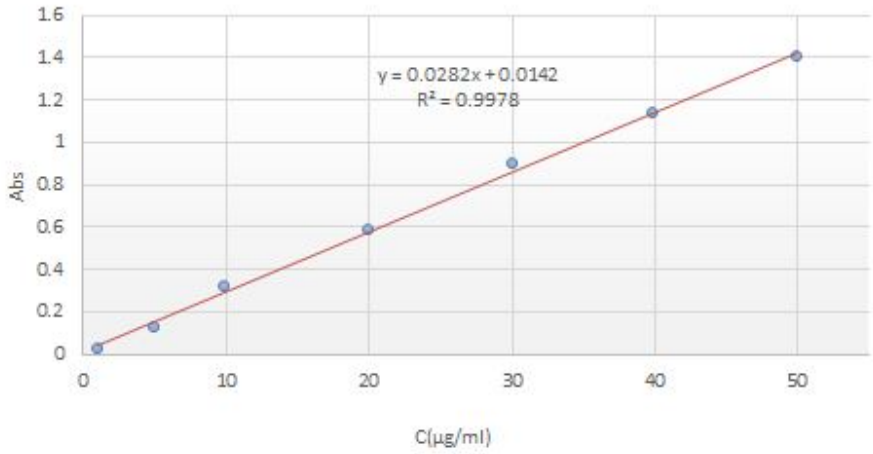


Figure 4: The calibration function doxycycline drug in phosphate buffer pH = 7.4

Table 3 Concentrations of doxycycline in phosphate buffer (pH = 7.4) used for creating the calibration curve.

Density (µg/ml)	1	5	10	20	30	40	50
The average absorption	0.0266	0.1286	0.3212	0.5903	0.8983	1.1373	1.4033

Table 4 Analysis of the limit of detection(1.637 µg /ml) and Limit of quantitative accuracy **(**0.491 µg / ml) for Doxycycline.

	C=1	C=5	C=10	C=20	C=30	C=40	C=50
n=1	0.027	0.127	0.319	0.582	0.893	1.12	1.349
n=2	0.026	0.13	0.322	0.594	0.901	1.143	1.428
n=3	0.027	0.129	0.323	0.595	0.901	1.149	1.433
Ave	0.02666	0.1286	0.3213	0.5903	0.8983	1.1373	1.4033
SD	0.000577	0.001528	0.002082	0.007234	0.004619	0.015308	0.04712
RSD	2.165605	1.187811	0.647889	1.225509	0.514171	1.345991	3.357829

Table 5.checking the UV method of measuring drug concentrations in the linear range of doxycycline

UV absorption values for the unknown concentration solution	0.088	0.466	0.757
	0.077	0.465	0.76
	0.077	0.465	0.762
Average absorption	0.0806	0.4653	0.7596
The measured concentration of calibration equation	2.35	15.99	26.43
The actual concentration	2.5	15	25
Recovery	%94	%106.6	%105.72

3. Combined MTZ and DOX formulation

MTZ and DOX exhibited λmax at 320 nm and 273.5 nm respectively. Additionally, an iso-absorptive peak absorbance was observed at 228 nm. Standardcalibration curves for the combined formulation of MTZ and DOX were linear with correlation coefficients (r2) values 0.9816-0.9939 at all the selected wavelengths. The method was repeated for the same day and RSD was found to be <1.21% for MTZ and <2% for DOX. Similarly, the method was repeated for different days and RSD was found to be <1.10 % for MTZ and <1.151% for DOX. The accuracy of the method was confirmed by recovery studies from synthetic mixtures at three different levels.

DISCUSSION

The objective of this study was to determine and analysis of spectroscopiccharacteristicsof combined MTZ and DOX pharmaceutical

formulation. Because simultaneous using of these drugs can significantly reduce microbial biofilm formation in the mouth, this combinationis effective in treatment of gum illnesses, and in drug delivery implants. The spectroscopic analysis is beneficial to measure and monitor the drug concentration during therapeutic procedures.The ultraviolet spectroscopyis a simple, accurate, precise, economical and rapid method for estimation of MTZ and DOX concentrations in a mixed formulation. In the applied method in present study, percentage recovery was found to be 100% and RSD to be less than 2% for the both medications. These strategies can be utilized for routine investigation in quality control at laboratories(12).While λmax of MTZ and DOXwere 320 nm and 273.5 nm respectively, when these two drugs werein a combined formulation, the λ max of the two drugs were inseparable(13).Instead, a λ max wave length of 228 nm was detected for combined formulation that can be used to estimate the dose of the two drugs in combined form(14).Condeet al in 2007 investigated theantimicrobial effects of combined treatment of MTZand other oral antibiotics, and showed that the formulation is highly effective in treating rosacea. Preservation treatment with topical MTZdecreased relapses and resulted in longer diseased-free intervalsof flares(15).Heinonen et al in 1986 worked on treatment efficacy ofcombinedDOX and MTZ or penicillin and MTZin pelvic inflammatory disease. The researchers had identified clarithromycin derivatives by infrared and mass spectroscopy. This methodology was the first study for the concurrent designation of omeprazole, tinidazole, doxycycline and clarithromycin in a a combined therapeutic formulation(16).Fowler et al in 2007 investigated thesynergisticanti-inflammatory effects of therapeutic doses ofDOX (40-mg DOX, as monohydrate controlled-release capsules) and MTZ1% topical gel in treatment of rosacea. Combination therapy was shown to significantly reducethe number of inflammatory lesions counts as early as 4 weeksthrough week 12, While this was not observed in cases who received only metronidazole 1% topical gel. This combined formulated therapy appeared effective and well-tolerated(17).We prepared different concentrations of the drugs, and plotted the calibration curve. Correlation coefficient (r2) as the parameter indicatinglinearity of the data rangedbetween 0.9816 to 0.9939. For theaccuracy parameters, absorptionsfor three different concentrations were determined at three different times of a day and for three consecutive days.The average absorbancewas used to interpolate RSD. For all these concentrations,RSD less than 5% indicated that the method was acceptable.

CONCLUSION:

Spectroscopy representsareproducible, and economical procedure for evaluation of metronidazole and doxycycline in a combined formulation.

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Conflicts of interest

There are no conflicts of interest.

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