CHAPTER-4

Preformulation Studies and Ultra-Violet Method Development For Simultaneous Analysis Of Ornidazole And Doxycycline Hyclate In Pharmaceutical Dosage Form

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4.1. METHODS

4.1.1. Fourier-Transform Infrared (FTIR) analysis

FTIR spectroscopic analysis of both the pure drugs (ornidazole and doxycycline hyclate) individually and their physical mixture (1:1) was done by using pelletization technique (Shimadzu-8400, Japan) to determine any possible interaction between both drugs when present in combination. In this method a small pellet of samples was prepared with potassium bromide using press. Samples were scanned in the region of 4,000-400 cm^{-1} with a resolution of 4 cm^{-1} for 20 scans.

4.1.2. Ultra-violet (UV) Spectrophotometric analysis

Spectrophotometric analysis of drugs was performed on a UV visible double beam spectrophotometer (Shimadzu-1700 Tokyo, Japan) using 1 cm quartz cells with a slit width of 2 nm and a scan rate of 100 nm/min. Absorbance was measured and spectra was plotted against the solvent blank over the wavelength range of 200-400 nm. Spectrophotometric determinations were made using visible spectrophotometer attached to a baseline computer.

4.1.2.1. Preparation of standard and sample solution of OZ and DX

4.1.2.1.1. Preparation of Phosphate buffer

A phosphate buffer solution (PBS) pH 6.8 was prepared according to standard method given in Indian Pharmacopoeia, 2014. Briefly, 6.8 g of KH_2PO_4 and 0.94 g of NaOH was dissolved in 1000 ml Millipore water. The pH of the solution was adjusted

to 6.8 by using dilute orthophosphoric acid or NaOH. Digital pH-meter (Perfit India) was used for adjusting pH of the buffer solution.

4.1.2.1.2. Stock solutions

100 µg/ml stock solutions of drugs were prepared in PBS pH 6.8.

4.1.2.1.3. Standard solutions

Standard solutions of OZ and DX were prepared in the concentration range of 5-30 µg/ml by serial dilution from the corresponding stock solutions using PBS pH 6.8.

4.1.2.1.4. Calibration curve preparation

The resulting standard solutions were scanned individually in the range of 200-400 nm to determine the wavelength of maximum absorbance (λ_{max}) for both the drugs. The calibration curves were plotted between observed absorbance (A) and corresponding concentrations of drugs at their absorption maxima. The zero-order spectra of OZ and DX shows the absorption maxima of OZ and DX (Fig.4.1).

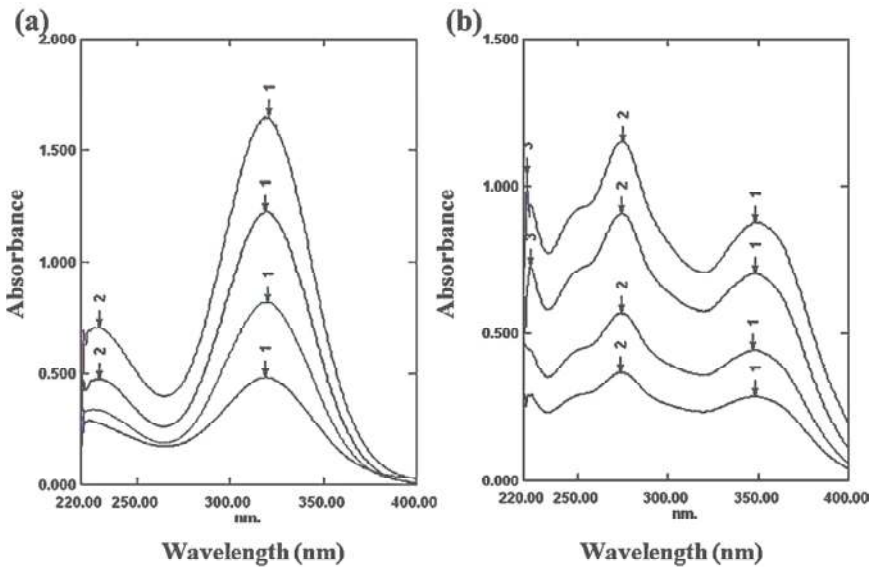

Figure 4.1: Zero order UV spectra; a) Ornidazole b) Doxycycline hyclate.

4.1.2.2. Development of UV method

4.1.2.2.1. Method I

Vierordt's method was used which is also known as simultaneous equation method. It is based on measuring the absorbance at two selected absorption maxima wavelengths i.e. 319 nm (λ_{max} of OZ) and 274 nm (λ_{max} = 274 nm of DX) (Fig. 4.1). This method can be applied only when OZ and DX have well-separated absorption maxima. The concentrations of OZ and DX was quantified using following equations (Beckett and Stenlake, 1988).

$$OZ\ cocentration = \frac{A274 \times aDX319 - A319 \times aDX274}{aOZ274 \times aDX319 - aOZ319 \times aDX274} \times 100$$

$$DX\ cocentration = \frac{A319 \times aOZ274 - A274 \times aOZ274}{aOZ274 \times aDX319 - aOZ319 \times aDX274} \times 100$$

Where, A319 and A274 are absorbances of mixture at 319 nm and 274 nm respectively; aOZ319 and aOZ274 are absorptivity coefficients of OZ at 319 nm and 274 nm, respectively; aDX319 and aDX274 are absorptivity coefficients of DX at 319 nm and 274 nm, respectively. Absorptivity at respective wavelengths was calculated using following Beer's Lambert Law.

$$A = Log\ (I_0/I) = \varepsilon c l$$

Where, I_0 = intensity of light incident upon sample cell, I = Intensity of light leaving sample cell, c = molar concentration of solute, l = length of sample cell, ϵ = molar absorptivity.

4.1.2.2.2. Method II

Q-analysis method is modification of Vierordt's method and is also referred as iso-absorptive point or absorbance ratio method. It is based on the principle of iso-absorptive point where compounds obeying Beer's law exhibits constant value of ratio of absorbance's at particular wavelengths. Iso-absorptive point is a constant

value independent of concentration and path length. It was determined by overlapping the UV spectra of both the drugs and is obtained as 291.85 nm ≈ 292 nm (Fig. 4.2).

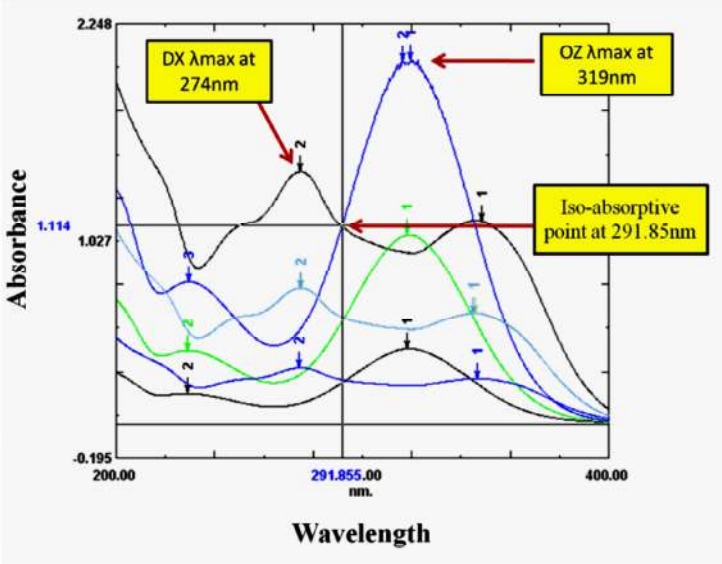

Figure 4.2: Overlay spectra of OZ and DX illustrating λ_{max} of both the drugs and their iso-absorptive point.

For quantitation of drugs in mixture absorbance was measured at two wavelengths. One being λ_{max} of OZ and other being wavelength corresponding to iso-absorptive point. The calibration curve of both drugs was plotted at these wavelengths and the absorptivity values were calculated using Beer's Lambert Law. Following equations were used to calculate the OZ concentration and DX concentration in mixture.

$$OZ\ concentration = \frac{\frac{A319}{A292} - \frac{aDX319}{aDX274}}{\frac{aOZ319}{aOZ292} - \frac{aDX319}{aDX274}} \times \frac{aOZ292}{A292}$$

$$DX\ concentration = \frac{\frac{A319}{A292} - \frac{aDX319}{aDX274}}{\frac{aOZ319}{aOZ292} - \frac{aDX319}{aDX274}} \times \frac{aDX292}{A292}$$

Where, A292 and A319 are absorbance of mixture at 292 nm and 319 nm respectively; aOZ319 and aOZ292 are absorptivity coefficients of OZ at 319 nm and

292 nm, respectively; aDX319 and aDX292 absorptivity coefficients of DX at 319 nm and 292 nm respectively.

4.1.2.3. Method validation

Validation is an important part of quality assurance program and aims to demonstrate that the analytical method is suitable for the intended proposal and it is safe to run. Method validation was performed following ICH specifications for linearity, accuracy, precision, detection limit, quantitation limit and mixture analysis (ICHQ2(R1), 2005).

4.1.2.3.1. Linearity and range

In analysis, linearity refers to the ability of obtained results to remain directly proportional to the analyte concentration. Mathematically, the concentration range over which Beer's Lambert law is obeyed is linearity. The range of an analytical procedure is the interval between the upper and lower concentration or amounts of analyte in the sample (including these concentrations) for which it exhibits suitable level of precision, accuracy and linearity (ICHQ2(R1), 2005). Linearity and range of analyte at different wavelengths was determined from corresponding calibration curve of drugs.

4.1.2.3.2. Accuracy

ICH guidelines define accuracy as the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the experimental value (ICHQ2(R1), 2005). It is a measure of exactness of analytical method. Accuracy was calculated in terms of % recovery of added known amount of analyte in the sample.

$$\%\, Recovery = \frac{Calculated\ concentration}{Theoretical\ Concentration} \times 100$$

4.1.2.3.3. Precision

Precision is defined by ICH as the closeness of agreement between quantity values obtained by replicate measurements of a quantity under specified conditions. It was evaluated with respect to both repeatability (intra-day precision) and intermediate

precision (inter-day precision). Intra-day precision was determined by repeating the experiments on the same day. Inter-day precision was studied by repetition of the assays on two different days to obtain reproducibility for each method (Kogawa and Salgado, 2012). The precision was expressed in %RSD (relative standard deviation) and calculated by following equation:

$$\%RSD = \frac{Standard\ deviation\ of\ values}{Mean\ value} \times 100$$

4.1.2.3.4. Limit of detection and limit of quantitation

The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily exactly quantified. The limit of quantitation (LOQ) is the lowest amount of analyte in a sample which can be quantitated with precision and accuracy. For each determination, y-intercept was calculated and the standard deviation (SD) of the y-intercept was computed. From these values, LOD and LOQ were calculated on the basis of response and slope (S) of the regression equation obtained from the linearity studies as follows.

$$LOD = 3.3\ (SD/S)$$

$$LOQ = 10\ (SD/S)$$

4.1.2.4. Analysis of synthetic drug mixture of OZ and DX

For verification of developed methods, synthetic mixture of OZ and DX solutions was prepared in the ratio of the mixed standard solutions as 5:10, 10:15, 15:20, 20:25 and were scanned from 200 to 400 nm. The absorbances of final sample solution were measured at 319, 274 and 292 nm and corresponding drug concentrations were determined by Method I and Method II.

4.1.3. Solubility studies

Solubility of OZ and DX were determined in PBS pH 6.8 by shake-flask method. PBS pH 6.8 was chosen for solubility determination to simulate GCF, which is generally used as release media for periodontal pocket medicines (Kassem *et al.,* 2015). In this method, excess amounts of both drugs were separately added to 5 ml of

PBS pH 6.8 contained in glass vials and samples were stirred on magnetic stirrer for 24 h for complete dissolution. The formed suspensions were filtered to remove excess of undissolved drug. The solutions were diluted with PBS pH 6.8 and assayed for drug content using developed UV method.

4.1.4. Statistical analysis

All the experiments were performed in triplicate or six times as per the required method specification and the results are reported as mean±standard deviation (SD).

4.2. RESULTS AND DISCUSSION

4.2.1. FTIR spectral analysis

Infrared spectral analysis of OZ and DX was performed to characterize both the drugs. Figure 4.3, shows the characteristics peaks of OZ and DX and their physical mixture. The infrared spectra of OZ exhibited transmittance peaks at 3165 cm^{-1}, 3088 cm^{-1}, 1154 cm^{-1}, and 832 cm^{-1} owing to presence of O-H stretching, aromatic C-H stretching, aliphatic C-H stretching and C-Cl group respectively. Further, peaks at 1540 cm^{-1} and 1361 cm^{-1} corresponds to asymmetric N=O stretching and symmetric N=O stretching. Peaks at 2963 cm^{-1}, 1618 cm^{-1}, 1600 cm^{-1}, 3282cm^{-1} 3454 cm^{-1}, 3300 cm^{-1}, in infrared spectra of DX are attributed to C-H stretching, C-C stretching, aromatic C=C bonds, C-O bond, primary O-H group and N-H group respectively. These characteristic peaks of both the drugs are well preserved in physical mixture of OZ and DX (1:1) indicating absence of any interactions between both the drugs. Therefore, both drugs were found compatible enough to be administered together in a combination.

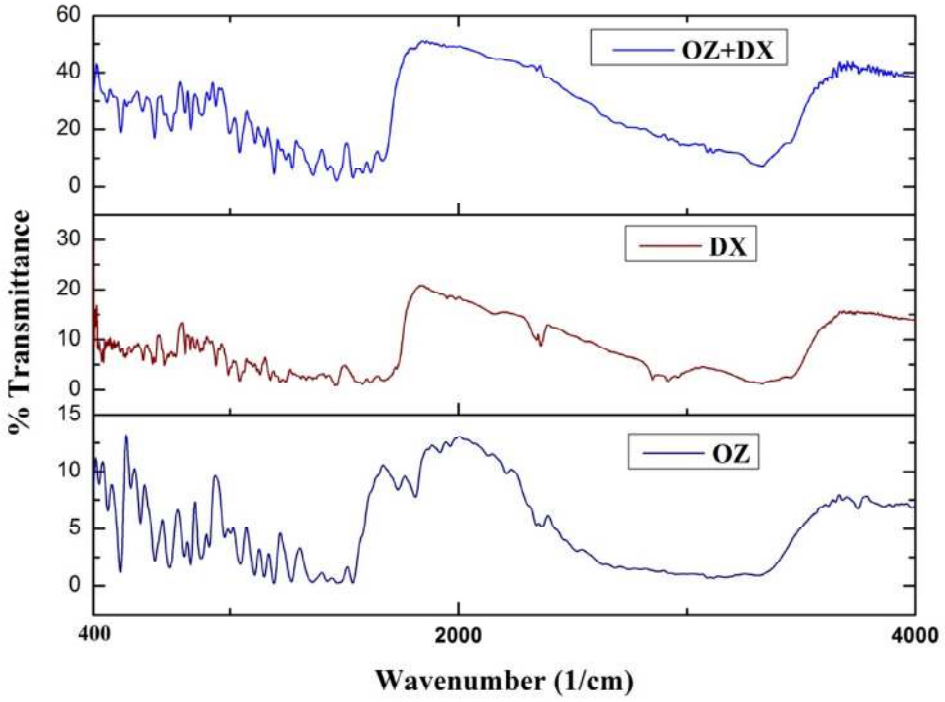

Figure 4.3: FTIR spectra of ornidazole (OZ), doxycycline hyclate (DX), and physical mixture (OZ+DX).

4.2.2. Method development

The individual and overlapping UV spectra of OZ and DX in PBS pH 6.8 are shown in Fig 4.4. UV spectra of OZ showed two absorption peaks at 319 nm and 227 nm while DX exhibited three absorption peaks at 345 nm, 274 nm and 222 nm (Fig.4.1 and 4.4 a and b). Wavelength of maximum absorbance λ_{max} was chosen at 319 nm for OZ and 274 nm for DX which is useful for quantitative estimation of both the drugs when present individually. Nevertheless, OZ and DX are UV active drugs and shows well-defined absorption maxima that are well separated from each other. But, they cannot be determined quantitatively by direct UV method in physical mixture due to masking of λ_{max} of one drug by the other drug peaks.

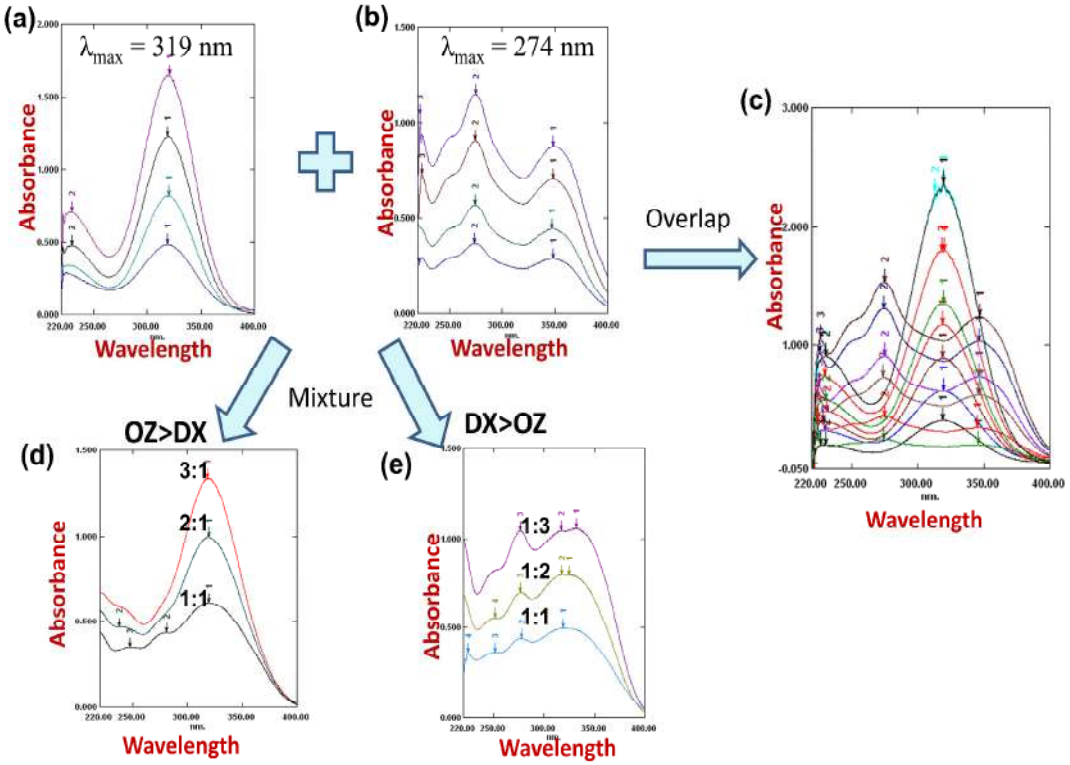

Figure 4.4: Zero order UV spectra; (a) OZ; (b) DX; (c) overlay spectra of OZ and DX. The spectra of mixed drug solutions OZ:DX as (d) 1:1 (black), 2:1 (green), and red (3:1); (e) 1:1 (blue), 1:2 (green), 1:3 (violet).

As obtained in Figure 4.4 (d) and (e), with the increasing concentration of one drug the λ_{max} of second drug in lower concentration starts disappearing. For instance, when OZ concentration was increased, λ_{max} of DX got omitted and OZ absorption maxima peak were more prominently observed giving a perception of absence of DX in the mixed solution (Fig. 4.4 d) (Yadav and Mishra, 2016). This effect can be more clearly observed in Fig. 4.4 e where complete disappearance of λ_{max} of OZ has occurred at the ratio of 1:2 and 1:3 (OZ:DX). Thus, mixed solutions of these two drugs cannot be estimated by direct UV method. Therefore, two indirect UV methods had been developed and validated in PBS pH 6.8.

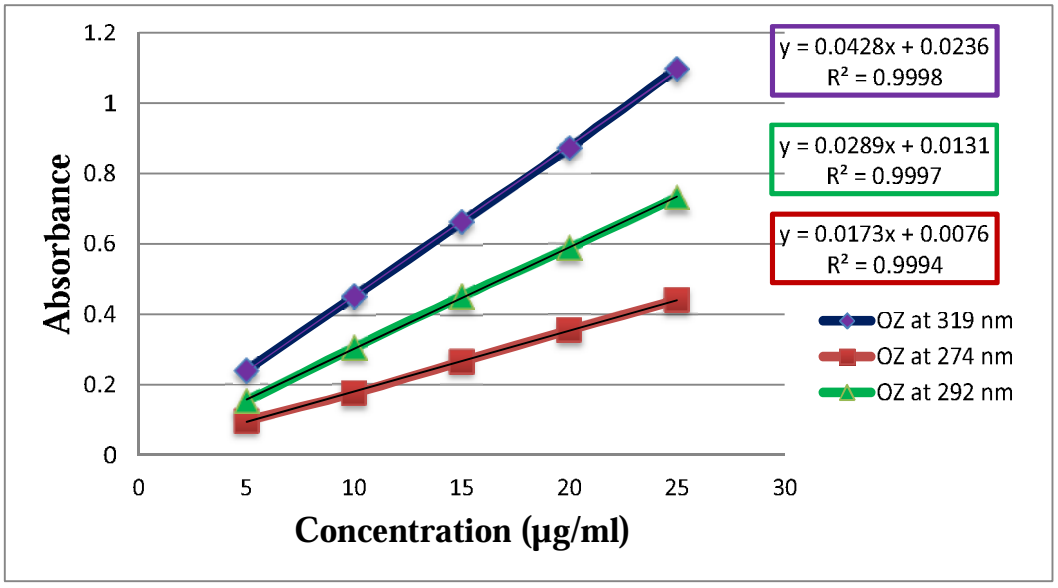

Figure 4.5: Calibration curve of OZ at 319, 274 and 292 nm.

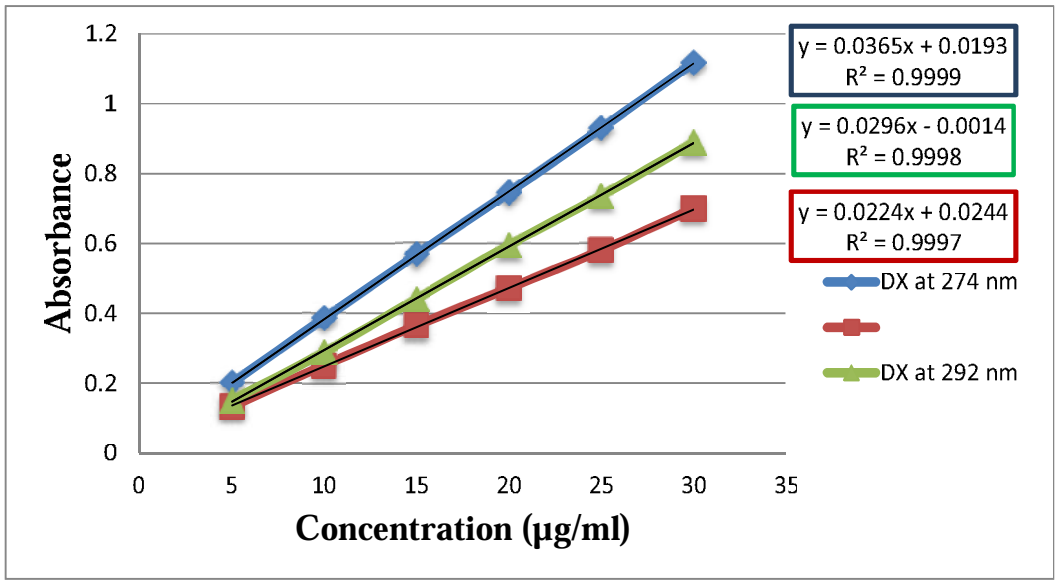

Figure 4.6: Calibration curve of DX at 319, 274 and 292 nm.

Calibration curves of both drugs were plotted at 319 nm, 274 nm and 292 nm (Fig. 4.5 and 4.6). The drugs obeyed Beer's Lambert law and showed linearity in the concentration range of 5-25 µg/ml and 5-30 µg/ml for OZ and DX respectively at all wavelengths.

The values of regression coefficients (R^2) and linear equations at the corresponding wavelengths were determined from the calibration curve. The regression equation for OZ was calculated as $y = 0.0428x+0.0236$ ($R^2 = 0.9998$), $y = 0.289x+0.0131$ ($R^2 = 0.9997$) and $y = 0.0173x+0.0076$ ($R^2 = 0.9994$) at 319 nm, 292 nm and 274 nm respectively. The regression equation for DX was calculated as $y = 0.0365x+0.0193$ ($R^2 = 0.9999$), $y = 0.0296x+0.0014$ ($R^2 = 0.9998$) and $y = 0.0224x+0.0244$ ($R^2 = 0.9997$) at 274 nm, 292 nm and 319 nm respectively (Fig. 4.5 and 4.6). The values of R^2 were more than 0.999 indicating very good linearity. The absorptivity values A (1%, 1 cm) and molar absorptivity for OZ and DX were determined at selected wavelengths from the corresponding calibrations curves and are shown in Table 4.1.

Table 4.1: Absorptivity and Molar absorptivity of drugs at respective wavelengths.

Optical parameters	Ornidazole			Doxycycline hyclate		
	319nm	**274nm**	**292nm**	**274nm**	**292nm**	**319nm**
Absorptivity	468.88	142.84	299.73	372.17	233.17	296.33
Molar absorptivity (L/mole/cm)	1.02×10^4	3.14×10^4	1.02×10^4	1.65×10^4	1.04×10^4	1.31×10^4

For simultaneous equation method, wavelengths (λ_{max}) selected for quantitation were 319 nm for OZ and 274 nm for DX. However for Q-analysis method iso-absorptive point at 292 nm and 319 nm (λ_{max} of OZ) was selected for analysis. The overlay spectra of both the drugs showed iso-absorptive point at 292 nm, which falls in between the absorption maxima of both drugs.

Table 4.2: Data of Intraday recovery and precision studies by Method I and Method II.

Theoretical drug concentration (µg/ml)		Calculated concentration (µg/ml±SD, n = 6)		Intraday % recovery		Intraday Precision (%RSD)	
		Method I	Method II	Method I	Method II	Method I	Method II
OZ	5	4.91±0.09	5.04±0.07	98.21	100.80	1.835	1.428
	10	10.18±0.18	9.95±0.13	101.80	99.50	1.739	1.376
	15	14.89±0.12	15.04±0.11	99.26	100.26	0.826	0.751
DX	5	4.95±0.07	4.89±0.07	99.00	97.80	1.333	1.411
	10	10.13±0.09	10.19±0.14	101.30	101.70	0.839	1.403
	15	15.1±0.19	14.71±0.13	100.67	98.07	1.232	0.891

Table 4.3: Data of Interday recovery and precision studies by Method I and Method II.

Theoretical concentration (µg/ml)		Calculated Concentration (µg/ml±SD, n = 6)		Interday % recovery		Interday Precision (%RSD)	
		Method I	Method II	Method I	Method II	Method I	Method II
OZ	5	5.06±0.09	5.05±0.08	101.2	101.00	1.83	1.6237
	10	9.82±0.18	9.88±0.16	98.2	98.80	1.77	1.6093
	15	14.87±0.16	14.94±0.16	99.13	99.60	1.09	1.0910
DX	5	4.96±0.0.1	5.10±0.08	99.2	102.00	1.80	1.6078
	10	10.17±0.13	10.01±0.09	101.7	100.10	1.34	0.9190
	15	15.16±0.21	15.29±0.14	101.07	101.93	1.41	0.8894

Validation of the proposed methods was carried out in accordance with the ICH guidelines. Intra-day and inter-day accuracy was calculated and reported in terms of % recovery of drugs from prepared mixture (Table 4.2 and 4.3). In all cases the % recovery was more than 99%, which indicates high accuracy of both the methods.

The intra-day and inter-day precisions were determined and expressed as %RSD. The intra-day precisions of six replicates on the same day for all concentrations were < 2.00 for both the methods. The inter-day precisions on 3 consecutive days were ≤ 1.3% for all concentrations for both the methods. Thus, both precision and accuracy were within acceptable limits for routine drug analysis (15%) for both methods. The obtained % RSD indicates good precision and sufficient sensitivity for the analysis of OZ and DX using both the methods. For UV Spectrophotometric method the LOD and LOQ values obtained indicate drugs in microgram concentration can be quantified accurately. Table 4.4 gives the summary of validation parameters for both methods I and II.

Analysis of prepared drug solution mixture was performed at different concentration ratios of OZ and DX. The % recovery and %RSD were calculated and are shown in Table 4.5. All the values were within limit indicating suitability of both methods for the simultaneous estimation of OZ and DX.

Table 4.4: Summary of validation parameters for Method I and Method II.

Methods	Parameters	Ornidazole	Doxycycline hyclate
Method I	Intraday Precision	0.9678	1.2522
	Interday Precision	1.2022	1.3890
	Linearity range (µg/ml)	5-25	5-30
	Accuracy (%Recovery)	Intra-99.96 Inter-99.84	100.20 100.15
	LOD (µg)	0.1635 at 319 nm 0.1014 at 274 nm	0.4062 at 319 nm 0.1023 at 274 nm
	LOQ (µg)	0.4956 at 319 nm 0.3072 at 274 nm	1.2311 at 319 nm 0.3099 at 274 nm
Method II	Intraday Precision	0.7670	1.0715
	Interday Precision	1.0698	1.2029
	Linearity range (µg/ml)	5-25	5-30
	Accuracy (%Recovery)	Intraday-100.63 Interday-100.44	Intraday-100.50 Intraday-100.75
	LOD (µg)	0.1460 at 292 nm	0.1563 at 292 nm
	LOQ (µg)	0.4431 at 292 nm	0.4738 at 292 nm

Table 4.5: Analysis of synthetic mixture of OZ and DX.

Composition (µg)		Amount recovered (µg±SD)				% Recovery				% RSD			
		Method I		Method II		Method I		Method II		Method I		Method II	
OZ	DX	OZ	DX	OZ	DX	OZ	DX	OZ	DX	OZ	DX	OZ	DX
5	10	5.07±0.09	10.19±0.07	4.97±0.084	9.87±0.10	101.40	101.90	99.40	98.70	1.755	0.716	1.690	1.064
10	15	10.10±0.17	15.2±0.23	10.17±0.13	14.86±0.13	101.00	101.33	101.70	99.07	1.643	1.493	1.248	0.915
15	20	14.87±0.12	19.56±0.21	15.17±0.10	19.89±0.17	99.13	97.80	101.13	99.45	0.813	1.078	0.678	0.864
20	25	19.68±0.11	24.89±0.18	19.78±0.13	25.09±0.11	98.40	99.56	98.90	100.36	0.538	0.743	0.677	0.418

4.2.3. Solubility

Solubility in water and physiological fluids is an important issue to be taken into account for pharmaceuticals. For dissolution of the solid drug, the forces of attraction between solute and solvent molecules must overcome the internal attractive forces of intact solid drug and solvent-solvent molecules together. Generally the solubility of ionized and protonated forms are higher than free acid and free base respectively (Brittain, 2007). Due to presence of some acidic and basic functional groups the solubility is dependent on pKa of drug and pH of medium (Bogardus and Blackwood, 1979). The observed solubility was 3.88 mg/ml and 0.493 mg/ml for OZ and DX respectively (Table 4.6).

OZ has shown approximately eight folds higher solubility than DX. Nevertheless, both drugs has shown acceptable and good solubility for use in drug delivery formulations. It is highly water soluble drug which supports their release and absorption in physiological environment (GCF) for effective treatment of bacterial environment in the periodontal cavity (Lund, 1994).

Out of three existing forms of doxycycline (hyclate, monohydrate and hydrochloride), the solubility follows the trend hyclate>monohydrate>hydrochloride (Kogawa and Salgado, 2012). The high solubility of DX is attributed to its aggregation or dimer forming behaviour (Bogardus and Blackwood, 1979). DX exhibits three pKa values 3.4, 7.7 and 9.3 because of presence of both acidic and basic group in the molecule. Following this DX exists as zwitterionic molecule at pH 5.5 (Giovagnoli *et al.,* 2010). DX is an amphoteric drug which has ability to dissolve in physiological fluids and lipid surfaces, which increases its efficiency in drug delivery for the treatment of cellular, epidermal and periodontal infections.

Table 4.6: Solubility of ornidazole and doxycycline in Phosphate buffer pH 6.8.

Parameters	Ornidazole (mean±SD, n = 3)	Doxycycline hyclate (mean±SD, n = 3)
λ_{max}	319	274
Solubility (mg/ml)	3.88±0.67	0.493±0.17