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Development and validation of UV-Spectrophotometric and RP-HPLC method for simultaneous estimation of Metformin and Doxycycline in bulk and synthetic mixture

Rajashree Mashru*, Nirupa Koshti

Faculty of Pharmacy, G. H. Patel Building, Donor`s Plaza, Fateh Ganj, The M.S. University of Baroda, Vadodara, Gujarat, India

Article Info:

Abstract

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A simple, rapid, sensitive, accurate and precise UV spectrophotometric and isocratic RP-HPLC method have been developed for simultaneous estimation of Metformin and Doxycycline in bulk and synthetic mixture. Spectrophotometric estimation was done by two methods. First method was Q-absorbance ratio method, where two wavelengths 236 nm (λ_{max} of Metformin) and 248 nm (Iso-absorptive point) were used. The second method was first derivative method. In this method the zero-crossing point of Metformin was selected at 282 nm and for Doxycycline, it was 232 nm. The solvent used was methanol in both the above UV-spectrophotometric methods. Metformin and Doxycycline showed good linearity in the series of 1-9 µg/ml and 2-20 µg/ml respectively by both the two methods with an excellent correlation coefficient ($r^2 \geq 0.998$). In RP-HPLC method, the chromatographic separation was achieved on Luna Phenomenex C_{18} (150 mmX6 mm, 5 µm) analytical column. A mixture of Acetonitrile: Phosphate buffer (50 mM): Triethylamine (TEA): Tetrahydrofuran (THF) (30:66:2:2) pH adjusted to 2.1 with orthophosphoric acid was used as the mobile phase, at a flow rate of 1 ml/min and at detector wavelength 248 nm. The retention time of Metformin and Doxycycline was found to be 3.561±0.0017 and 5.574±0.0131 minutes respectively. A linear response was observed over the concentration range 4-64 µg/ml of Metformin and 5-80 µg/ml of Doxycycline. All the three methods were validated in accordance to ICH guidelines for linearity, accuracy, precision, LOD and LOQ. The proposed methods were effectively utilized for the concurrent estimation of Metformin and Doxycycline in synthetic mixture.

Keywords: Metformin, Doxycycline, Q-absorbance ratio method, First derivative method, RP-HPLC

INTRODUCTION

Metformin hydrochloride is chemically 3-(diamino methylidene)-1,1-dimethylguanidine; hydrochloride.[1] The structure of Metformin hydrochloride is shown in the Figure 1.[2] Metformin is a widely used antidiabetic drug. It is the recommended treatment for Type II diabetes.[3] Several molecular properties such as the inhibition of reactive oxygen species, mTORC1. ADORA and activation of AMPK have suggested its utility as an antitumor agent. Several population studies have suggested a protective effect of metformin in the cancer of the breast, colon, pancreas, prostate, and liver.[4] Also, the metformin as an adjuvant on periodontal treatment shows potential to reduce needs of additional interventions and also reduces the inflammatory burden in patients.[5]

Doxycycline hyclate is chemically (4S, 4aR,5S,5aR)-4-(dimethylamino)-1,5,10,11,12a-pentahydroxy-6-methyl-3,12-dioxo-4a,5,5a,6-tetrahydro-4H-tetracene-2-carboxamide; ethanol; hydrate; hydrochloride.[6] The structure of Doxycycline hyclate is shown in the Figure 2.[7] Doxycycline is a broad-spectrum antibiotic of tetracycline class.[8] Doxycycline inhibits the synthesis of proteins via preventing aminoacyl-tRNA attachment to the ribosome in bacterial cell. In addition to that, Doxycycline has inhibitory effect on the activity of matrix metalloproteinases (MMPs); according to this, it has the potentiality of being used as anti-neoplastic and also as anti-inflammatory agent.[9] Its anti-neoplastic role has been found to be significant, in vitro and in vivo laboratory trials, in various types of cancer, such as prostate, intestinal, central neural system cancers and osteosarcoma.[10] Short-term use of standard-dose doxycycline is used for treating acute periodontal infections and for eliminating inflammation.[11]
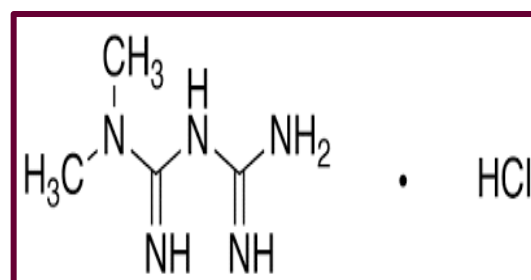

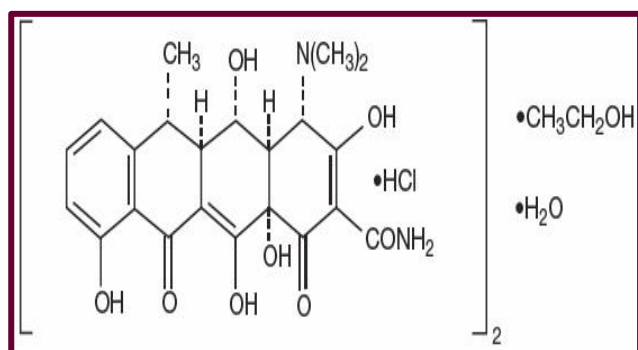**Figure 1: Structure of Metformin Hydrochloride**

Figure 2: Structure of Doxycycline Hyclate

The combination of these two drugs (Metformin and Doxycycline) is under clinical trials for various diseases like head and neck squamous cell carcinoma (Orally), localized breast or uterine cancer (Orally) as well as chronic periodontitis (Topical Gel).[12,13] There are the phase II trial studies which shows that metformin hydrochloride works together in treating patients with localized breast or uterine cancer. Metformin hydrochloride may stop the growth of cancer cells by blocking some of the enzymes needed for cell growth. Doxycycline may stop the growth of bacteria by keeping them from making proteins and minimized the toxic side effects of anti-cancer therapy.[14] The CDSCO has approved the Phase II clinical trials of the FDC of Metformin hydrochloride 1% w/w and Doxycycline hyclate 10% w/w gel for the treatment of Periodontitis in September, 2020.[15]

Various analytical methods are reported for the estimation of individual drug as well as in combination with other drugs.[16-27] Literature survey reveals that there is no any analytical method for simultaneous estimation of Metformin and Doxycycline till date. So, the objective of the proposed work was to develop and validate simple, accurate, precise and reproducible methods for the simultaneous estimation of Metformin and Doxycycline in bulk and synthetic mixture by UV- Spectrophotometric and RP-HPLC method.

MATERIAL AND METHODS:

Instruments and Apparatus

Shimadzu UV-1700 double beam spectrophotometer connected to a computer with Shimadzu UV-Probe 2.10 software installed was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-800 nm. The chromatographic analysis was carried out using Shimadzu HPLC System equipped with UV Detector. Other instruments used were electronic balance, sonicator and pH meter.

Chemicals and reagents

Analytical grade methanol was purchased from Rankem Laboratories. HPLC grade Acetonitrile, Tetrahydrofuran (THF), Triethylamine (TEA), Potassium dihydrogen orthophosphate and Ortho Phosphoric Acid were supplied from Rankem, India. Tablets Bigomet (Metformin

hydrochloride 850 mg) was purchased from local medical store.

Preparation of standard stock solution

For UV-Spectrophotometric method: 10 mg of Metformin and Doxycycline were weighed accurately and transferred into separate 100 ml volumetric flask. To each flask 25 ml of methanol was added and sonicated for 10 minutes. Then volume was made up to the mark with methanol. This will give 100 µg/ml concentration of Metformin and Doxycycline.

For RP-HPLC method: 50 mg of Metformin and Doxycycline was weighed accurately and transferred into separate 50 ml volumetric flask. Then the drug was dissolved in 20 ml of mobile phase and sonicated for 10 minutes and then made up to the mark with mobile phase to obtain the concentration of 1000 µg/ml of Metformin and Doxycycline.

Preparation of test solution for assay

The combined dosage form of Metformin and Doxycycline is not available in the market. The dose of the drugs is also not fixed in the clinical trials. But in most of the clinical trials, the dose of Metformin is 850 mg[28] and of Doxycycline is 200 mg[29], therefore, for the assay, the drugs were used in the ratio of 4.25:1.

For UV-Spectrophotometric method: Twenty tablets of Bigomet were weighed and triturated in a mortar pestle and powder equivalent to 85 mg of Metformin was taken into 100 ml volumetric flask. To this flask, 20 mg of Doxycycline API was added, to make concentration of Metformin and Doxycycline in ratio of 4.25:1. To the flask 25 ml of methanol was added and was sonicated for 10 minutes to dissolve the active ingredients completely. The volume was then adjusted up to the mark with methanol to prepare the stock solutions corresponding to 850 µg/ml of Metformin and 200 µg/ml of Doxycycline. Then the solution was filtered through Whatman filter paper. From this filtered solution, 0.1 ml aliquot was transferred into 10 ml volumetric flask and the volume was made up to the mark with methanol. This test solution containing working concentration of 8.5 µg/ml of Metformin and 2 µg/ml of Doxycycline in the mixture was analysed for assay determination.

For RP-HPLC method: Twenty tablets of Bigomet were weighed and triturated in a mortar pestle and powder equivalent to 85 mg of Metformin was taken into 100 ml volumetric flask. To this flask, 20 mg of Doxycycline API was added, to make concentration of Metformin and Doxycycline in ratio of 4.25:1. To the flask, 20 ml of mobile phase was added and was sonicated for 10 minutes to dissolve the active ingredients completely. The volume was adjusted up to the mark with mobile phase to prepare the stock solutions corresponding to 850 µg/ml of Metformin and 200 µg/ml of Doxycycline. The solution was filtered through Whatman filter paper. From this filtered solution, 0.4 ml aliquot was transferred into 10 ml volumetric flask and the volume was made up to the mark with mobile phase. This test solution containing working concentration of 34 µg/ml of Metformin and 8 µg/ml of Doxycycline in the mixture was analysed for assay determination.

Method development

UV-Spectrophotometric method
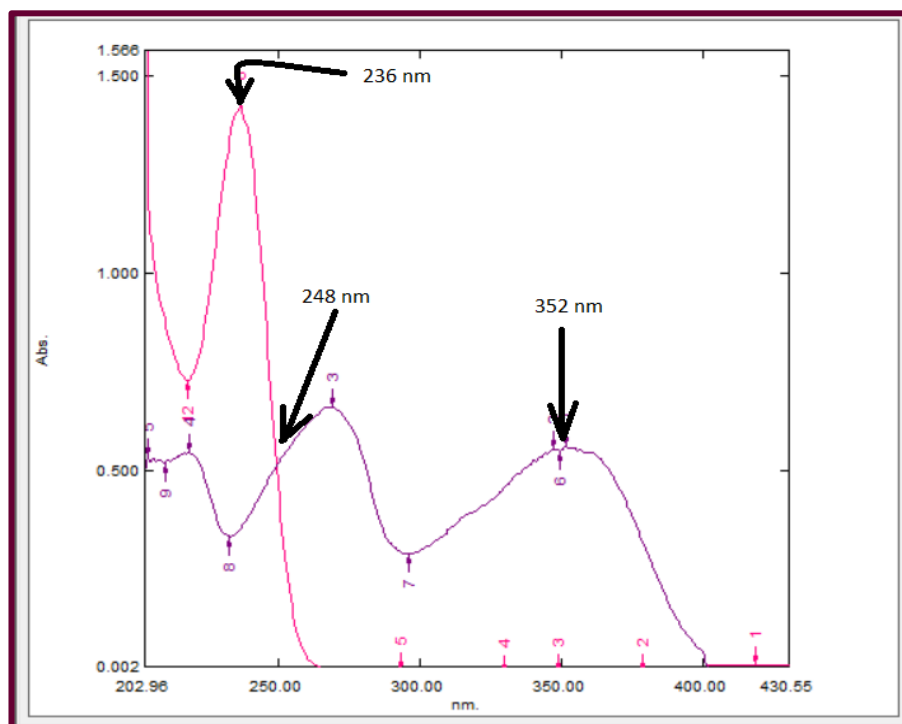
Method I: Q Absorbance Ratio Method

Figure 3: Overlay UV spectra of Metformin and Doxycycline

Absorbance ratio method uses the ratio of absorbances at two wavelengths, one which is an iso absorptive point and other being λ_{max} of one of the two components. From the overlay spectra of two drugs (Figure 3), it is evident that Metformin and Doxycycline show an iso absorptive point at 248 nm. The second wavelength used was 236 nm, which is the λ_{max} of Metformin. Working standard solutions having concentration 1-9 µg/ml of Metformin and 2-20 µg/ml of Doxycycline were prepared in methanol from the respective standard solutions and the absorbances at selected wavelengths (248 & 236 nm) were measured and absorptivities were calculated using equation. The graph of Absorbance Vs Concentration was plotted at each wavelength and regression coefficients were calculated.

The concentration of two drugs in the mixture can be calculated using following equations.

$C_X = [(Q_M – Q_Y) / (Q_X - Q_Y)] \times A_1/a_{x1}$................ (1)

$C_Y = [(Q_M – Q_X) / (Q_Y - Q_X)] \times A_2/a_{y1}$ .... (2)

Where,

A_1 and A_2 are absorbances of mixture at 248 and 236 nm respectively;

a_{x1} and a_{y1} are absorptivities of Metformin and Doxycycline at 248 nm respectively;

a_{x2} and a_{y2} are absorptivities of Metformin and Doxycycline at 236 nm respectively;

$Q_M = A_2 / A_1$,

$Q_X = a_{x2} / a_{x1}$ and

$Q_Y = a_{y2} / a_{y1}$.

Method II: First order derivative method

Working standard solutions having concentration 1-9 µg/ml of Metformin and 2-20 µg/ml of Doxycycline were prepared in methanol from the respective standard solutions and were scanned in the UV range 200-400 nm. The overlain zero order spectra of Metformin and Doxycycline were obtained. These absorption spectra of Metformin and Doxycycline were converted to first-order derivative spectra by using instrument mode ($\Delta\lambda$ = 5 nm and scaling factor = 5). From the overlain first-order derivative spectra (Figure 4), zero crossing points of drugs were selected for the analysis of another drug. The first wavelength selected was 232 nm (zero crossing point of Doxycycline), where Metformin showed considerable absorbance. The second wavelength selected was 282 nm (zero crossing point of Metformin), where Doxycycline showed considerable absorbance. Thus, the absorbances of the working standard solutions of Metformin and Doxycycline were measured at 232 nm (ZCP of Doxycycline) and 282 nm (ZCP of Metformin), respectively. The graph of absorbance Vs concentration was plotted at each wavelength and regression coefficients were calculated.
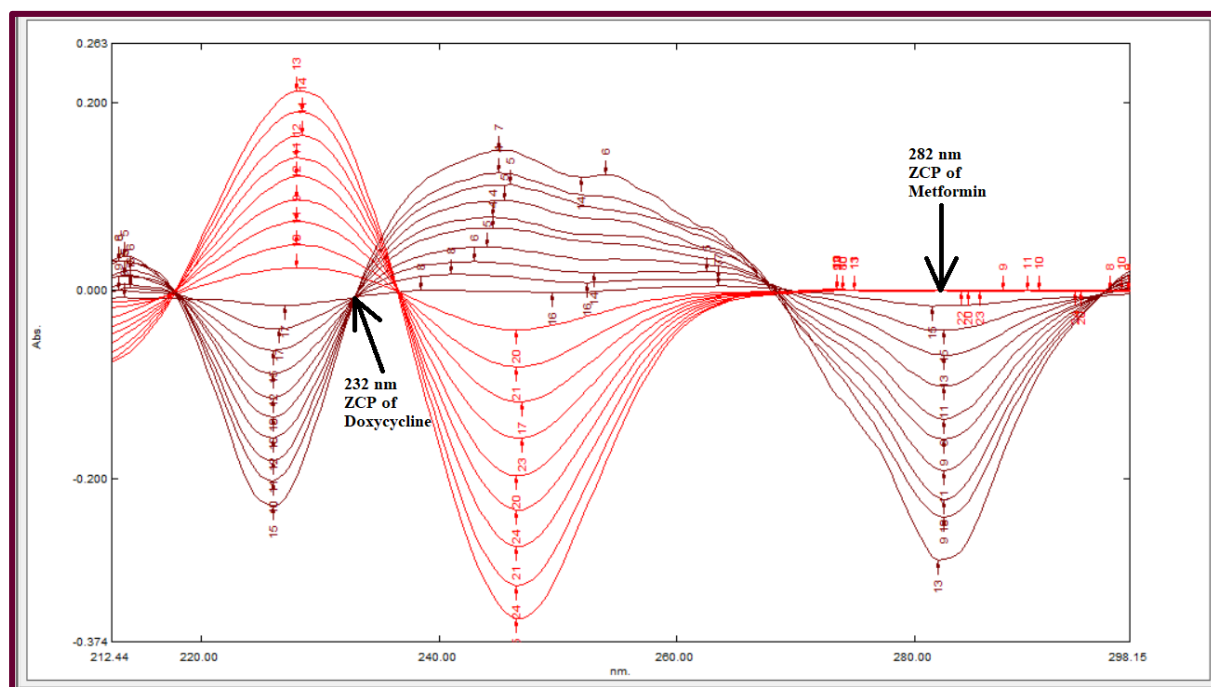
Figure 4: First Derivative Overlay Spectra of Metformin and Doxycycline

RP-HPLC method:

Chromatography was performed on Shimadzu HPLC System equipped with UV Detector. Data acquisition and integration was performed using Spinchrome software. A manual Rheodyne 7725 Injector valve with a fixed injection volume of 20µL was used. The chromatogram was recorded at 248 nm as both the drugs show good sensitivity at their isosbestic point. Several attempts were performed in order to get satisfactory resolution and peak symmetry of Metformin and Doxycycline. During the optimization of the separation method, two columns HyperChrome ODS-BP C18, 250 mm X 6 mm, 5µm and Luna Phenomenex C18 column (150 X 6 mm, 5 µm) and the mobile phase composed of acetonitrile and 0.05 M phosphate buffer solution adjusted to different pH values (2-7.2) with and without TEA and THF were tested. Of the stationary phases experienced, Luna

Phenomenex C18 column was chosen as the most suitable separation factors were obtained with it. After trying several mobile phases containing acetonitrile with various buffer proportions, the mobile phase consisting of acetonitrile: 0.05 M KH_2PO_4: TEA (2%): THF (2%) was proved to be useful for better resolution and peak symmetry. To optimize this mobile phase, proportions of acetonitrile and 0.05 M KH_2PO_4 buffer were systemically changed whilst percentage of THF and TEA remained unchanged. After, so many trials, the pH and the ratio of mobile phase presented in the Table 1 was found to give symmetric peak, high column performance, shorter running time and good resolution among two drugs. Typical chromatogram obtained with the final condition is shown in the Figure 5. The elution order was Metformin (RT= 3.563 min) and Doxycycline (RT=5.57 min), at a flow rate of 1 ml/min.

Table 1: Optimized chromatographic conditions

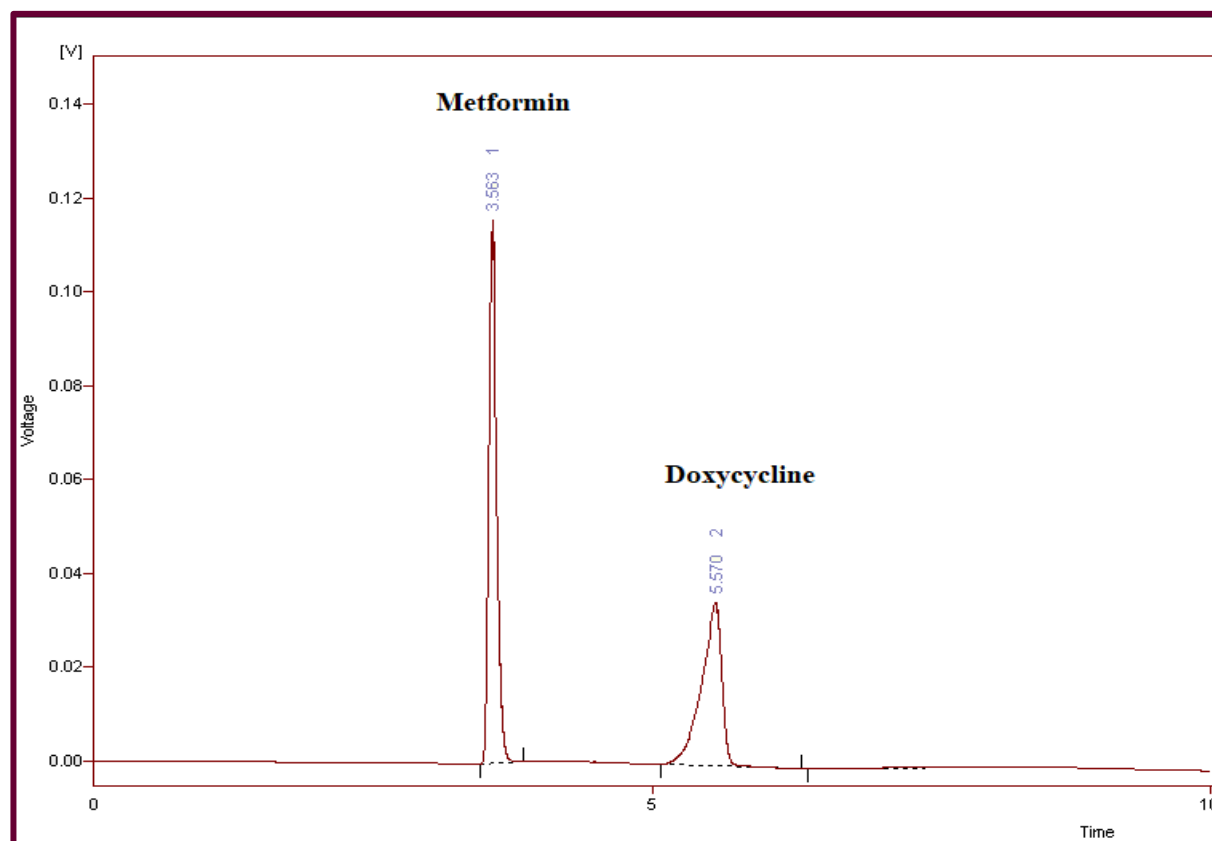| Parameter	Chromatographic condition
Instrument	Shimadzu LC20A
Column	Luna Phenomenex C18 column (150 X 6 mm, 5 µm)
Flow rate	1 mL /min
Detection wavelength	248 nm
Injection volume	20µl
Run time	8 min
Temperature	Ambient
Mobile phase	Acetonitrile (ACN): Phosphate buffer (50 mM): TEA: THF (30: 66: 2: 2) pH adjusted to 2.1 with orthophosphoric acid
Diluent	Mobile phase

Figure 5: Chromatogram of Standard drug solutions

RESULT AND DISCUSSION:

Method Validation[30]

All the developed three methods were validated according to the validation of analytical procedures provided in the ICH guidelines.

Linearity:

For both the UV spectrophotometric methods, Metformin was linear within the concentration range of 1-9 µg/ml and Doxycycline showed the linearity in the range of 2-20 µg/ml. A calibration curve was plotted of Absorbance Vs Concentration. The plot was found to be linear. Also, the statistical data are shown in the table 2 for both the methods.

Table 2: Statistical data for the regression equation of the proposed method

Parameter	Method I				Method II	
	Metformin		Doxycycline		Metformin	Doxycycline
Analytical wavelength (nm)	236	248	236	248	232	282
Linearity range (µg/ml)	**1-9**	**1-9**	**2-20**	**2-20**	**1-9**	**2-20**
Regression equation	y = 0.1121x + 0.0173	y =0.0526x + 0.0068	y = 0.0538x + 0.0141	y = 0.047x + 0.0161	y = 0.0183x + 0.0016	y = 0.015x - 0.0183
Slope	0.1121	0.0526	0.0538	0.047	0.0183	0.015
Intercept	0.0173	0.0068	0.0141	0.0161	0.0016	0.0183
Correlation coefficient (R^2)	**0.9997**	**0.9993**	**0.9988**	**0.9987**	**0.9995**	**0.999**
Limit of detection (µg/ml)	**0.0164**	**0.0316**	**0.0308**	**0.0954**	**0.0374**	**0.171**
Limit of Quantification (µg/ml)	**0.0497**	**0.0959**	**0.0934**	**0.2892**	**0.113**	**0.519**

For RP-HPLC method, the linearity of the method was investigated by using concentrations in the range 4-64 µg/ml for Metformin and 5-80 µg/ml for Doxycycline. The calibration curve was constructed by plotting peak area versus concentration of Metformin and Doxycycline, and the regression equations were calculated. Retention time for

Metformin and Doxycycline was found to be 3.561±0.0017 and 5.574±0.0131 min respectively. The plot obtained from linear regression is given in Figure 6 for Metformin and Figure 7 for Doxycycline. Figure 8 shows chromatograms for linearity.
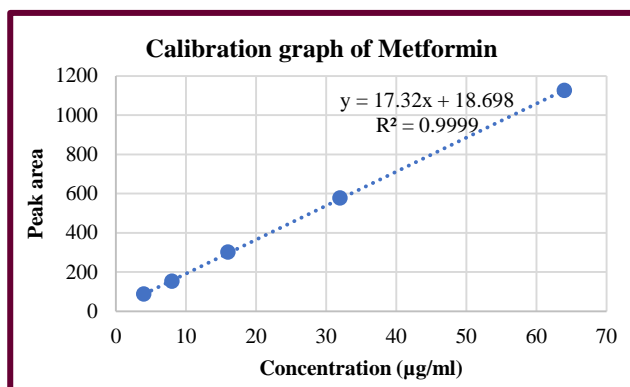

Figure 6: Calibration graph of Metformin
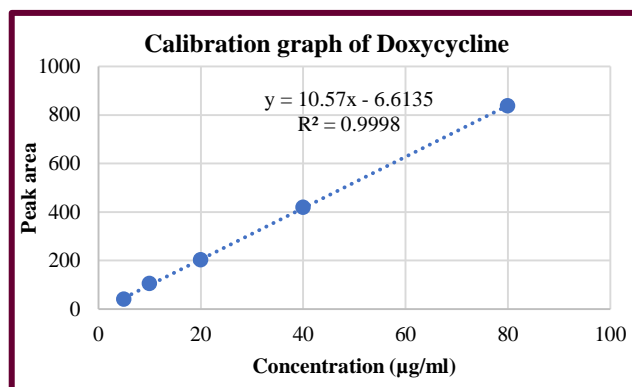
Figure 7: Calibration graph of Doxycycline
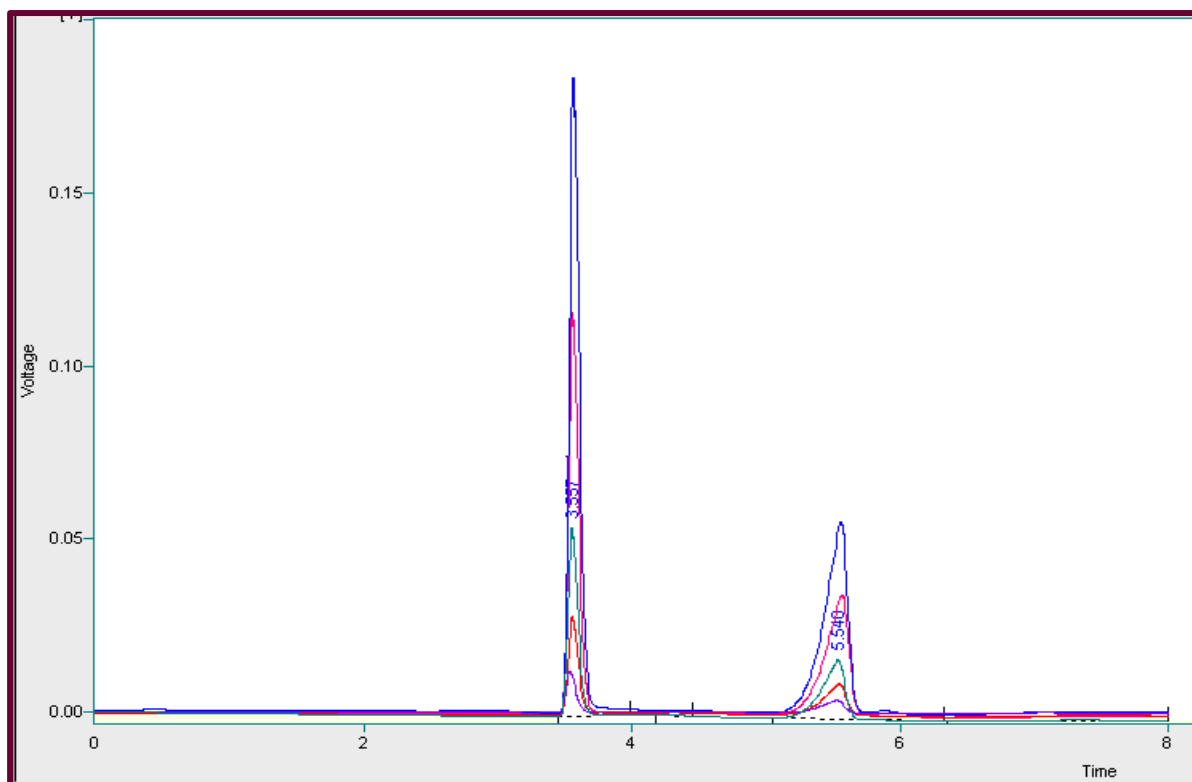
Figure 8: Overlay chromatographs of the different concentration used in linearity

Limit of Detection and Limit of Quantification

The Limit of Detection and Limit of Quantification was calculated using the series of calibration curves plotted. The LOD and LOQ values were determined using the following equations.

LOD = 3.3 σ/ S

LOQ = 10 σ/ S

Where, σ = The standard deviation of the responses,

S = The slope of the calibration curve

The LOD and LOQ data for both the UV-spectrophotometric methods is represented in table 2 and for RP-HPLC is represented in Table 3.

Table 3: LOD and LOQ data of Metformin and Doxycycline by RP-HPLC method

Drug	Limit of detection (µg/ml)	Limit of quantitation (µg/ml)
Metformin	0.0431	0.0664
Doxycycline	0.1306	0.2012

Precision

The precision of an analytical method expresses the closeness of agreement between a series of measurements which are obtained by performing multiple samplings of the same homogenous sample under the given conditions of the method. Here, the intra-day (Repeatability) and inter-day precision was determined. For that three-concentration having lower, upper and middle limits of both the drugs were taken and analysed three times on the same day for intra-day precision and on 3 different days for inter-day precision at the same concentration level. The % RSD (Relative Standard Deviation) of the results was calculated.

Table 4: Precision data of Metformin by UV-Spectrophotometric method

		Method I				Method II	
	Conc.	Mean ± SD (n=3)		%RSD		Mean ± SD (n=3)	%RSD
		236 nm	248 nm	236 nm	248 nm	232 nm	232 nm
Intra day	1	0.131±0.00058	0.0593±0.00057	0.439	0.973	0.0182±0.00029	1.589
	5	0.571±0.00265	0.274±0.0015	0.463	0.558	0.0937±0.00058	0.616
	9	1.029±0.00551	0.484±0.0030	0.535	0.631	0.1683±0.00115	0.686
Inter day	1	0.131±0.001	0.0583±0.00058	0.763	0.989	1.0182±0.00034	1.903
	5	0.572±0.0038	0.272±0.0015	0.662	0.562	0.094±0.001	1.063
	9	1.028±0.0065	0.484±0.0032	0.633	0.664	0.1687±0.0015	0.906

Table 5: Precision data of Doxycycline by UV-Spectrophotometric method

		Method I				Method II	
	Conc.	Mean ± SD (n=3)		%RSD		Mean ± SD (n=3)	%RSD
		236 nm	248 nm	236 nm	248 nm	282 nm	282 nm
Intra day	2	0.1117±0.0006	0.1233±0.0021	0.517	0.0557	0.0161±0.00017	1.076
	12	0.6633±0.0032	0.5723±0.0051	0.485	0.0296	0.1613±0.0023	1.431
	20	1.093±0.0051	0.9723±0.0025	0.469	0.0085	0.288±0.001	0.347
Inter day	2	0.109±0.001	0.1257±0.0021	0.917	0.0546	0.0162±0.00029	1.786
	12	0.6643±0.0035	0.5713±0.0065	0.528	0.0376	0.1633±0.003	1.87
	20	1.0933±0.0066	0.975±0.003	0.609	0.0101	0.288±0.002	0.694

Table 6: Precision data of Metformin and Doxycycline by RP-HPLC method

Drug	Conc.	Intraday		Interday	
		Mean ± SD (n=3)	%RSD	Mean ± SD (n=3)	%RSD
Metformin	4	86.143±0.713	0.827	85.947±0.723	0.841
	16	302.453±1.382	0.457	302.557±1.215	0.401
	64	1125.626±1.015	0.090	1126.011±1.4044	0.125
Doxycycline	5	40.671±0.370	0.911	40.598±0.39	0.962
	20	202.961±1.333	0.657	202.413±1.053	0.5202
	80	837.914±1.256	0.15	838.678±1.253	0.149

Accuracy

The accuracy of the method was determined by recovery experiments. A known quantity of the pure drug was added to the pre-analysed sample mixture at 80%, 100 % and

120% levels. The recovery studies were carried out and the percentage recovery and percentage relative standard deviation of the percentage recovery were calculated and given in Table 7 for UV-Spectrophotometric method and in Table 8 for RP-HPLC method.

Table 7: Accuracy data of Metformin and Doxycycline by UV-Spectrophotometric method

Drug	% Spiked	Conc. from formulation	Standard conc. added	Conc. recovered		%recovery ± SD (n=3)		% RSD	
				Method I	Method II	Method I	Method II	Method I	Method II
Met-formin	80	8.5	6.8	6.722	6.798	98.903± 0.200	100.503±0.464	0.202	0.461
	100	8.5	8.5	8.501	8.546	100.23± 0.277	100.546±0.643	0.276	0.639
	120	8.5	10.2	10.192	10.186	100.125±0.485	99.86±0.535	0.484	0.536
Doxy-cycline	80	2	1.6	1.603	1.587	99.341± 0.867	98.472±1.203	0.460	0.872
	100	2	2	1.992	2.02	100.968±0.697	99.889±1.924	0.403	0.690
	120	2	2.4	2.393	2.353	100.323±0.578	98.979±1.605	0.286	0.576

Table 8: Accuracy data of Metformin and Doxycycline by RP-HPLC method

Drug	% Spiked	Conc. from formulation	Standard conc. added	Conc. recovered	%recovery ± SD (n=3)	% RSD
Metformin	80	34	27.2	27.084	100.141±0.5344	0.534
	100	34	34	33.884	99.943±0.8024	0.803
	120	34	40.8	41.377	100.707±0.7881	0.782
Doxycycline	80	8	6.4	6.30	99.204±0.665	0.671
	100	8	8	7.91	99.264±0.742	0.747
	120	8	9.6	9.69	100.722±727	0.722

System suitability parameters

For RP-HPLC method, the system suitability test was performed to verify the suitability of chromatographic system for intended analysis. The test was performed by three replicate injections of standard solution of 20 µg/mL of

Metformin and Doxycycline and system suitability parameters were determined, for their retention time, theoretical plate, asymmetric factor and resolution. The results are given in the Table 9 which are within the acceptable limits.

Table 9: System suitability parameters

Parameter	Result	
	Metformin	Doxycycline
Retention time ± SD	3.561 ± 0.0017	5.574 ± 0.0131
Theoretical plate ± SD	10113 ± 5.196	5081.667 ± 25.324
Asymmetric factor ± SD	1.447 ± 0.0456	0.378 ± 0.0062
Resolution ± SD	3.456 ± 0.51	

Analysis of Metformin and Doxycycline in test solution

The developed methods were applied to sample solution of synthetic mixture. The % Assay of Metformin and

Doxycycline found by UV- Spectrophotometric methods and RP-HPLC method are shown the Table 10 and 11, respectively and were within the acceptance criteria (98-102%).

Table 10: Assay results of Metformin and Doxycycline by UV-Spectrophotometric method

Drug	Conc. (µg/ml)	Conc. found (µg/ml)		% Recovery ± SD (n=6)		% RSD	
		Method I	Method II	Method I	Method II	Method I	Method II
Metformin	8.5	8.47	8.44	99.65±0.1	99.39±0.674	0.1	0.678
Doxycycline	2	2.02	2.02	101±0.23	100.287±0.687	0.228	0.685

Table 11: Assay results of Metformin and Doxycycline by RP-HPLC method

Drug	Conc. (µg/ml)	Conc. found (µg/ml)	% Recovery ± SD (n=6)	% RSD
Metformin	34	33.981	99.94 ± 0.802	0.803
Doxycycline	8	7.941	99.26 ± 0.741	0.746

CONCLUSION

In the present work, two UV-Spectrophotometric methods namely Q-absorbance ratio method and first derivative method and RP-HPLC method were developed for the simultaneous estimation of Metformin and Doxycycline. The developed methods were validated as per ICH guidelines Q2 (R1). The results of the study indicate that the methods are accurate, precise, reproducible, rapid and sensitive. The methods were successfully applied for the simultaneous estimation of Metformin and Doxycycline in synthetic mixture and can be used for the combination formulations of these two drugs in the future.

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