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Research Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF DOXYCYCLINE HYCLATE AND TINIDAZOLE IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

High Performance Liquid Chromatography (HPLC) methods are described for determination of drugs as a single or in combined formulations. The objective of the present study was to develop and validate novel, accurate, sensitive, precise, rapid and isocratic Reverse Phase HPLC (RP-HPLC) method for the simultaneous determination of Doxycycline hyclate (DOX) and Tinidazole (TIZ) in bulk and combined tablet dosage form. The separation was achieved on Zorbax C8 column (250mm × 4.6mm, 5µm) with mobile phase consisting of 20mM Potassium dihydrogen ortho phosphate (pH 6, adjusted with triethylamine):Acetonitrile (60:40 % v/v) at a flow rate of 1 ml/min. UV detection at 293nm. DOX and TIZ obeyed linearity in the concentration range of 10-50 µg/ml (r^2 = 0.9993) and 10-50 µg/ml (r^2 = 0.9987) respectively. The asymmetric factors were found to be 1.12 for DOX and 0.97 for TIZ. The developed method was validated as per ICH guidelines. It was concluded that the method can be used for routine analysis of DOX and TIZ in combined formulations.

Keywords: Doxycycline hyclate, Tinidazole, Simultaneous estimation, RP-HPLC.

INTRODUCTION

Doxycycline hyclate (DOX) molecular mass 512.94 g/mol is the hydrochloride hemiethanol hemihydrates of doxycycline. DOX is relatively more soluble than doxycycline monohydrate, which is the main reason for its more frequent use in pharmaceuticals. Doxycycline is preferred to other tetracyclines in the treatment of specific infections because of its fairly reliable absorption and its long half-life, which permits less frequent dosage. It is frequently used to treat chronic prostatitis, sinusitis, syphilis, chlamdydia, and pelvic inflammatory disease. Tinidazole (TIZ) is a 1-[2-(ethyl sulphonyl) ethyl]–2- methyl – 5- nitro – 1H- imidazole, derivative used as antiprotozoal/antibiotic and antibacterial [1-2]. Few methods have been reported for the estimation of these drugs as single drug or in combination with other drugs. Doxycycline hyclate has been reported to be estimated by Spectrophotometry[3], Titrymetry [4], HPLC [5-12] and Tinidazole by Spectrophotometry, HPLC [13-17], HPLC-LCMS [18]. Also one method has been reported for simultaneous estimation of DOX and TIZ by UV spectrophotomery [19]. So far, no method has been reported for simultaneous estimation of DOX and TIZ by HPLC in combined dosage form, hence the present work is aimed to develop an accurate, sensitive and rapid RP-HPLC analytical method. This paper describes validated RP-HPLC method for simultaneous estimation of DOX and TIZ in bulk and combined tablet dosage form on zorbax C-8 column (250mm X 4.6mm i.d., with particle size 5 µm) using 20mM Potassium dihydrogen ortho phosphate (pH 6, adjusted with triethylamine): Acetonitrile (60:40 % v/v), flow rate of 1 ml/min and UV detection at 293nm.

MATERIALS AND METHODS

Materials

HPLC grade acetonitrile (Qualigens), HPLC grade Potassium dihydrogen ortho phosphate and triethylamine were used. Deionised HPLC grade water was used to prepare mobile phase and diluents solutions. Both the drugs; DOX and TIZ were obtained from Shreya Life Sciences Pvt. Ltd., Aurangabad, M.S., India. Dosage form was purchased from local commercial sources.

Equipments

HPLC analysis was performed on a Dionex HPLC system with P680 Pump, automated sample injector, a Zorbax C-8 column (250mm X 4.6mm i.d., with particle size 5 µm) and a programmable variable wavelength UV-visible detector. Data were collected and processed using "Chromeleon version 6.0" software.

Preparation of Potassium dihydrogen orthophosphate buffer pH 6

Approximately weighed 3.16g of crystalline buffer and dissolve in 500 ml of distilled water to get 20mM buffer strength. Then pH of the buffer was adjusted to 6 using Triethylamine [20].

Preparation of Mobile Phase

The two components of mobile phase; Acetonitrile (HPLC grade) and Buffer (prepared previously) were separately filtered through a 0.45µm nylon membrane filter. They were mixed respectively in the ratio of 40:60 % v/v and sonicated for 15min [20].

Preparation of Standard Laboratory Mixture Solution

Accurately weighed quantity of 10 mg of DOX and 50 mg of TIZ were transferred to 100 ml volumetric flask, dissolved in mobile phase and volume was made up to mark with same solvent. From stock solution suitable aliquot was transferred to 10 ml volumetric flask and diluted to mark with the mobile phase, to obtain the concentration of 10 µg/ml of DOX and 50 µg/ml of TIZ. A volume of 10 µl of solution was injected. All measurements were repeated three times for each concentration.

Preparation of Sample (Tablet) Solution

Twenty tablets were weighed accurately; the average weight was determined and then triturated to a fine powder. A quantity equivalent to 10 mg of DOX and 50 mg of TIZ was weighed and transferred to a 100 ml volumetric flask. The contents were sonicated for 20 min with mobile phase to dissolve the active ingredients and the volume was made up to 100 ml with same solvent and filtered through a 0.22µm nylon membrane filter. Suitable aliquots of the solution were further diluted with mobile phase concentration range for the two drugs. Then 10µl volume of each sample solutions were injected into sample injector of HPLC three times under the chromatographic conditions as described above. The area under the curve of each peak was measured at 293nm.

RESULT AND DISCUSSION

Under the stated chromatographic conditions, the retention time of drugs was 3.3min for DOX and 4.0min for TIZ. A model Chromatogram for pure laboratory mixture and tablet sample is shown in Fig. 1 and 2 respectively.

Method Validation: [21-27]

The proposed method was validated as per ICH parameters and guidelines.

Linearity

Appropriate aliquots of the standard stock solutions of DOX and TIZ were pipette out and transferred to a series of 10 ml volumetric flasks respectively. The volume was made up to the mark with mobile phase to obtain working standard solutions of each drug separately of concentrations 10, 20, 30, 40, and 50µg/ml. From these solutions, 10 µl injections of each concentration of the drug were injected into the HPLC system three times separately and chromatogram was obtained under the conditions as finalized by developed method. The peak areas were recorded.

The standard calibration curves for DOX and TIZ were plotted separately as peak area Vs the respective concentration.

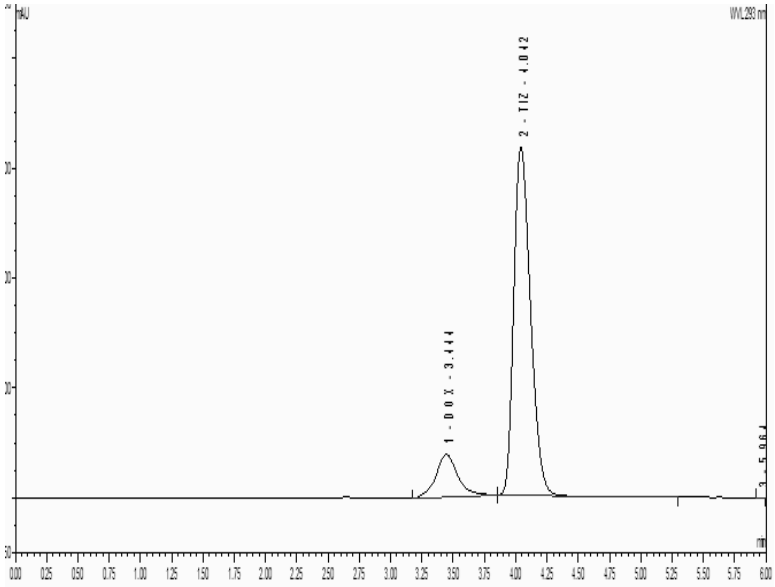

Fig. 1: Chromatogram for Pure Drug Mixture

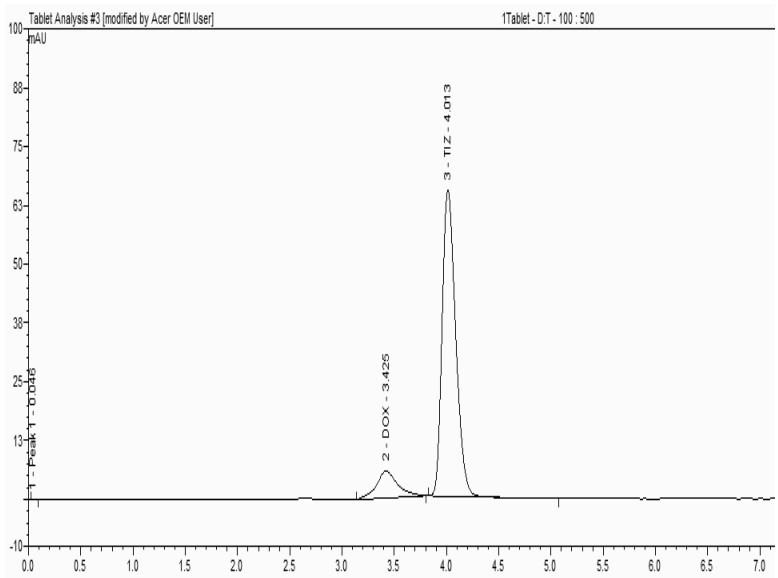

Fig. 2: Chromatogram for Sample Solution of Tablet

Table 1: Linearity Study of DOX at 293nm

S. No.	Concentration of DOX [µg/ml]	Peak area Mean ± S.D. [n = 3]	% R.S.D.
1	10	1.4488 ± 0.020	1.3952
2	20	2.8034 ± 0.020	0.7292
3	30	4.6520 ± 0.076	1.6505
4	40	6.2864 ±0.026	0.4291
5	50	8.1482 ±0.021	0.2615
			Avg. % RSD 0.8931

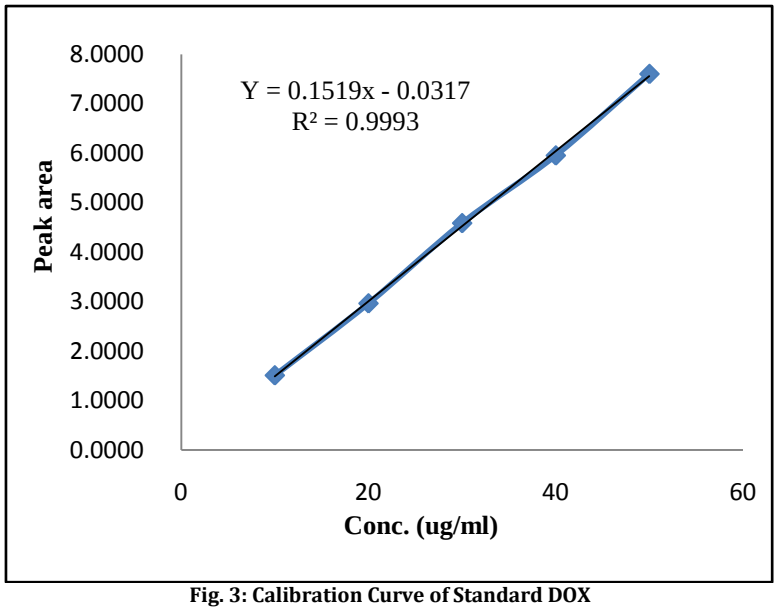

Fig. 3: Calibration Curve of Standard DOX

Table 2: Linearity Study of TIZ at 293nm

S. No.	Concentration of TIZ [µg/ml]	Peak area Mean ± S.D. [n = 3]	% R.S.D.
1	10	1.5082 ±0.005	0.3799
2	20	2.9641 ± 0.029	1.0018
3	30	4.5872 ± 0.052	1.1412
4	40	5.9558 ±0.075	1.2727
5	50	7.6054 ±0.060	0.7958
			Avg. % RSD
			0.9183

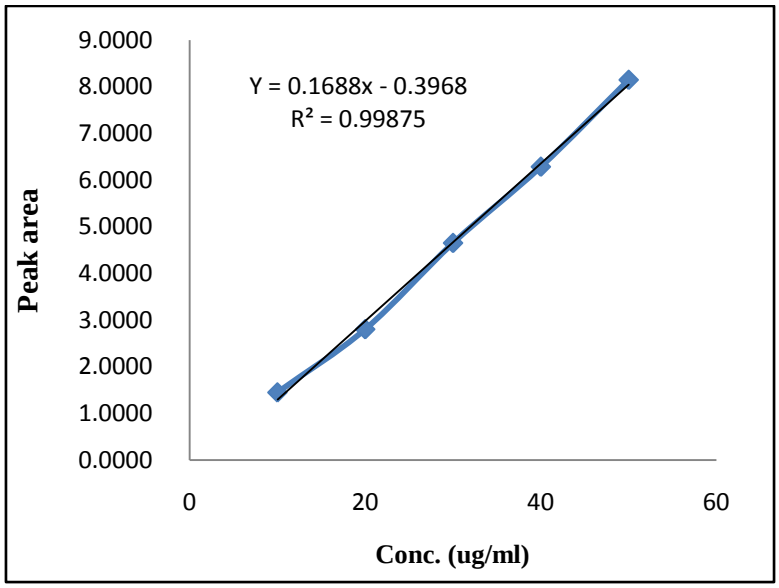

Fig. 4: Calibration Curve of Standard TIZ

Accuracy

Accuracy of proposed method has been carried out by recovery studies. Recovery studies were carried out by applying the method to drug content analysis present in sample to which known amount of standard DOX and standard TIZ was added at 80 %, 100 % and 120 % levels. The technique involves addition of standard drug solution to preanalysed sample solution. The resulting sample solutions were injected onto HPLC system and chromatogram was recorded. The results are shown in Table 3.

Table 3 : Results of Recovery Studies

Pre-Analysed Sample Solution [µg/ml]	% Level of Addition	Excess Drug Added [µg/ml] [n = 3]	Amount Recovered [µg/ml]	Average %Recovery	Average %RSD
	80	8	17.80	98.88	0.312
DOX(10)	100	10	20.12	100.60	0.316
	120	12	21.99	99.95	0.308
	80	40	90.12	100.13	0.061
TIZ(50)	100	50	99.99	99.99	0.975
	120	60	110.00	100.00	0.021

Precision

It is expressed as ± S.D. of series of measurements. Precision was carried out by two methods according to ICH guidelines:

1) Repeatability studies
2) Intermediate precision – variation in days and analyst.

Repeatability

It is measured by multiple injections of a homogenous sample of 10 µg/ml of DOX and 50 µg/ml of TIZ that indicates the performance of the HPLC instrument under chromatographic conditions. Results are shown in Table 4.

Table 4: Results of Repeatability Studies

Drug	Amount taken [µg/ml] [n = 6]	Amount found [µg/ml]±S.D.	%R.S.D.
DOX	10	9.623 ± 0.015	0.1558
TIZ	50	49.816 ± 0.180	0.3632

Intermediate precision

1) Variation in days : Intra–day and Inter–day Precision

In the intra-day studies, 3 replicates of 3 different concentrations (5, 10, 15 µg/ml) of DOX and (25, 50, 75 µg/ml) of TIZ were analyzed in a day and percentage RSD was calculated. For the inter day variation studies, 3 replicates of different concentrations were analyzed on 3 consecutive days and percentage RSD were calculated. Results shown in Table 5, 6.

Table 5: Results of Intra-Day and Inter-Day Precision of DOX

Drug	Conc.[µg/ml]	Intra-day Peak area found [µg/ml]		Inter-day Peak area found [µg/ml]	
		Mean±S.D [n = 3]	%R.S.D.	Mean±S.D. [n = 3]	%R.S.D.
DOX	5	0.727±0.004	0.551	0.7367±0.009	1.22
	10	1.448±0.009	0.625	1.4616±0.0078	0.53
	15	2.169±0.017	0.786	2.181±0.010	0.45
			Avg. % RSD=0.653		Avg. % RSD=0.733

Table 6: Results of Intra-Day and Inter-Day Precision of TIZ

Drug	Conc.[µg/ml]	Intra-day peak area found [µg/ml]		Inter-day peak area found [µg/ml]	
		Mean±S.D [n = 3]	%R.S.D.	Mean±S.D. [n = 3]	%R.S.D.
TIZ	25	3.792±0.015	0.395	3.812±0.017	0.445
	50	7.6595±0.024	0.313	7.691±0.036	0.468
	75	11.56±0.068	0.588	11.710±0.07	0.597
			Avg. % RSD=0.432		Avg. % RSD=0.503

2) Variation in Analyst

Table 7: Result of Precision after Variation of Analyst

Drug	Amount taken [µg/ml] [n=3]	Analyst I Avg. amount found [µg/ml]	%R.S.D.	Analyst II Avg. amount found [µg/ml]	%R.S.D.
DOX	10	9.87	0.139	9.99	0.228
TIZ	50	49.9	0.816	49.87	0.552

Specificity and Selectivity

The analytes should have no interference from other extraneous components and be well resolved from them. Specificity is a procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix.

The method is quite selective. There was no other interfering peak around the retention time of DOX and TIZ; also the base line did not show any significant noise.

Sensitivity (LOD and LOQ)

Sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). LOD was found to be 4.7µg/ml and 3.43µg/ml for DOX and TIZ respectively. LOQ was found to be 7.5µg/ml and 10.4µg/ml for DOX and TIZ respectively.

Robustness

Robustness of the proposed method was assessed by making deliberate changes in flow rate, pH and proportion of mobile phase which was performed by injecting sample solution containing 10 µg/ml of DOX and 50 µg/ml of TIZ and chromatographic resolution between two drugs were evaluated. No significant change was observed during the study. Results are shown in Table 8.

Table 8: Result of Robustness of DOX and TIZ for HPLC

Parameters	DOX (10 µg/ml)		%RSD	TIZ (50 µg/ml)		%RSD
	RT	Peak area		RT	Peak area	
Mobile Phase Composition(v/v) (ACN:Phosphate Buffer pH 6)						
45:55	3.40	1.445	0.169	4.0	7.667	0.975
40:60	3.40	1.448		4.04	7.669	
35:65	3.39	1.446		4.02	7.665	
Different Flow Rate of Mobile phase(mL/min)						
0.9	3.40	1.447	0.168	4.0	7.665	0.975
1.0	3.38	1.451		3.99	7.671	
1.1	3.39	1.450		4.01	7.664	
Different pH of the Mobile Phase						
5.5	3.40	1.451	0.166	4.0	7.658	0.544
6	3.40	1.448		4.01	7.669	
6.5	3.41	1.445		4.04	7.659	

Table 9: Analysis of Tablet Formulation

Drug	Labeled amount [mg]	Amount taken [µg/ml] [n = 6]	Amount found [µg/ml]±S.D.	%R.S.D.
DOX	10	10	9.72 ± 0.020	0.205
TIZ	50	50	49.91 ± 0.085	0.170

Table 10: Summary of Validation Parameter

Parameters	DOX	TIZ
Range (µg/ml)	10-50	10–50
Linearity		
Regression eq.[Y = mx±C] and Coefficient of regression	Y = 0.1519x - 0.0317 R^2 = 0.9993	Y = 0.1688x - 0.3968 R^2 = 0.99875
LOD	4.7	3.43
LOQ	7.5	10.4
Recovery (% R.S.D.)	0.312	0.352
Precision (% R.S.D.)		
• Repeatability (n = 6)	0.1558	0.3632
• Intra- day (n = 3)	0.653	0.432
• Inter- day (n = 3)	0.733	0.503
Ruggedness (% R.S.D.)		
• Analyst I (n = 3)	0.139	0.816
• Analyst II (n = 3)	0.228	0.552
Sensitivity	Sensitive	Sensitive
Robustness	Robust	Robust

CONCLUSIONS

The research work concluded that the formulation containing Antibacterial drugs (DOX and TIZ) has been simultaneously analyzed with modern tools and techniques like HPLC. It can be further concluded that the RP-HPLC method being developed is reproducible, sensitive, precise, specific, and accurate and can be employed for the routine quality control tests for the formulation. The mobile phase is simple to prepare and the sample recovery in formulation was in good agreement with their respective label claims.

Developed RP-HPLC method for simultaneous determination of Doxycycline hyclate and Tinidazole in Pharmaceutical formulation is costly as compared to Spectrophotometric method but it is more sensitive, specific, rugged and robust. Statistical analysis proves that the developed method can be used for routine analysis of Doxycycline hyclate and Tinidazole in their respective pharmaceutical formulations.

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