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Development and validated RP-UPLC method for simultaneous estimation of ciprofloxacin HCl, doxycycline and phenazopyridine HCl in bulk and tablet dosage form.

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ABSTRACT

A Reversed-Phase Ultra Performance liquid Chromatographic (RP-UPLC) method was developed for the simultaneous determination of Ciprofloxacin HCL, Doxycycline Hyclate and Phenazopyridine HCL in tablet dosage form. The analysis was carried out using Acquity UPLC, BEH C–18, 50 X 2.1, 1.7µ column. Mobile phase, containing 0.05 M Ammonium Acetate Buffer: Methanol (50:50) pH adjusted to 4 with Ortho-phosphoric acid was pumped at a flow rate of 1mL/min with UV-detection at 278,350,378 nm Respectively. Retention time was 0.90 ± 0.01 min, 1.60±0.02 min & 4.17±0.01 min. for Ciprofloxacin HCL, Doxycycline and Phenazopyridine HCL respectively. The method was validated for linearity, accuracy, precision, and specificity. The method showed good linearity in the range 20, 50, 80,100, 120, 150, 200 ppm. The % R.S.D for precision and accuracy of the method was found to be less than 2%. The method was validated as per the ICH guidelines. The method was successfully applied for routine analysis of Ciprofloxacin HCL, Doxycycline and Phenazopyridine HCL in combined tablet dosage form.

Keywords: Ciprofloxacin HCL (CIPRO), Doxycycline Hyclate(DOXY) , Phenazopyridine HCL(PHENA) **,**Ultra performance liquid chromatography.

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INTRODUCTION

Ciprofloxacin Hcl, Doxycycline and Phenazopyridine are available in tablet dosage form in the Ratio of 500mg, 100mg, 50mg Respectively. Chemically (ciprofloxacin hydrochloride) fluoroquinolone, is the monohydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid (Figure. 1a). It is a faintly yellowish to light yellow crystalline substance with a molecular weight of 385.8. Its empirical formula is $C_{17}H_{18}FN_3O_3$•HCl•H_2O broad spectrum antimicrobial. Doxycycline hyclate is [4S(4aR,5S,5aR,6R,12aS)]-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,10,12,12a-pentahydroxy-6methyl-1,11-deoxonapthtacene-2-carboxamide monohydrochloride (Figure 1b). Doxycycline is a tetracycline antibiotic. It fights bacteria in the body. Doxycycline is used to treat many different bacterial infections, such as urinary tract infections, acne, gonorrhea, and chlamydia, periodontitis (gum disease). Phenazopyridine hydrochloride is chemically designated 2,6-Pyridinediamine, 3-(phenylazo), monohydrochloride (Figure. 1c). It is a urinary tract analgesic agent for oral administration Literature survey reveals, UV, UPLC methods for analysis of ciprofloxacin Hcl as single and combined dosage forms with other drugs and UV, UPLC methods for analysis of Doxycycline and Phenazopiridine as single component systems. There are no reported methods for analysis of this three drugs in combination. This paper presents simple, rapid, accurate and economical methods for simultaneous analysis of Ciprofloxacin HCL Doxycycline and Phenazopyridine HCL in combined tablet dosage form.

Figure1a:Structure of Ciprofloxacin HCl

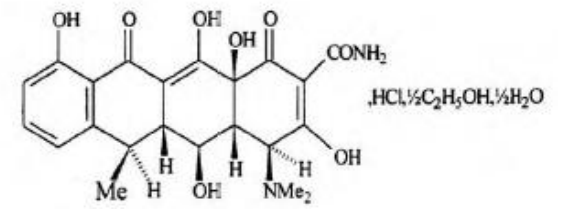

Figure1b:Structure of Doxycycline Hyclate **Figure 1c: Structure of Phenazopyridine**

MATERIAL AND METHODS:

Procurement of drug samples and formulation

Pure drug sample of Ciprofloxacin HCL, Doxycycline Hyclate, Phenazopyridine HCL with % purity 99.27, 95.21 and 99.23 supplied as gift samples by Genpharma International Pvt. Ltd. were used. Tablet formulation manufactured by Genpharma, containing CIPRO 500 mg DOXY 100 mg and PENA 50 mg per tablet were purchased from local market and were used for analysis.

Chemicals and Reagents:

The Methanol used in the study were of UPLC grade and were procured from RFCL Ltd, (New Delhi). Ammonium Acetate and Ortho Phosphoric acid, also of UPLC grade were obtained from Rankem ltd. The double distilled water was prepared at laboratory scale only by using all glass Double Distillation plant.

Experimental procedures for UPLC Method:

Preparation of Standard Stock Solution:

100 mg of standard CIPRO, 20 mg of standard DOXY and 10 mg of PHENA were accurately weighed individually, transferred to 100 ml volumetric flask and dissolved in 50 ml of methanol and sonicated for 10 mins. Further the volume was make up to the mark by methanol (1000 µg/ml of CIPRO, 200 µg/ml of DOXY and 100 µg/ml of PHENA.)

Further take 10 ml of mixture Solution to 100 ml Volumetric flask, add 50 ml of Mobile Phase sonicated 10 mins , the volume was make up to the mark by Mobile Phase. (100 µg/ml of CIPRO, 20 µg/ml of DOXY and 10 µg/ml of PHENA.)

Instrumentation:

An Aquity Waters Aquity H Class model, employing the using Acquity UPLC, BEH C–18, 50 X 2.1, 1.7µ column, instrument was used for the study. Detection was done using PDA detector. UV spectrophotometer of Varain (Cary 100 model) was used for obtaining the UV Spectra and detector wavelength selection.

Development and Optimization of chromatographic condition:

The UPLC procedure was optimized with a view to develop a simultaneous assay method for CIPRO, DOXY, PHENA respectively. The mixed standard stock solution (100µg/ml for CIPRO and 20µg/ml for DOXY and 10 µg/ml of PHENA) was injected in UPLC. For UPLC method optimization different ratios of different mobile phases were tried in combination with different columns including methanol, water, ACN, Phosphate buffers as the mobile phase and different C8, C18 and amino columns with various dimensions. But it was found that none of these combinations worked. Also Doxycycline showed stability problems in ACN so Methanol was used as solvent. In general it was found that in acidic conditions, there was a co-elution of the

peaks and on the contrary in neutral and basic conditions CIPRO eluted early with retention time about 0.91 min and PHENA was retained too long upto 12-15 min leading to a long method run time. DOXY retained to 1.6. After many trials it was found that Acquity UPLC, BEH C–18, 50 X 2.1., 1.7µ column gives good separation. Further using factorial design of various buffers at different pH ranges from 3 to 10, it was found that acidic conditions gave good resolution but the peak shape was not good. The pH was adjusted to 5.5 by ortho phosphoric acid. Ammonium acetate buffer (pH 4.0) (50 :50 v/v) with a flow rate of 0.2 ml/min in Acquity UPLC, BEH C–18,50 X 2.1., 1.7µ was taken which gave acceptable retention time (t_R), peak shape, plates counts and good resolution for CIPRO, DOXY And PHENA.

Optimized chromatographic conditions:

In the optimized chromatographic conditions, the column used was Acquity UPLC, BEH C–18, 50 X 2.1., 1.7µ column. The mobile phase comprised of 50 volumes of solution containing Ammonium acetate and (pH 4.0 with Orthophosphoric acid) and 50 volumes of Methanol. The flow rate was 0.2 ml/min and the detection wavelength was 278 nm, 350nm, 378nm for CIPRO, DOXY & PHENA respectively. The injection volume was 0.8 µl and the temperature was 15^0 C.

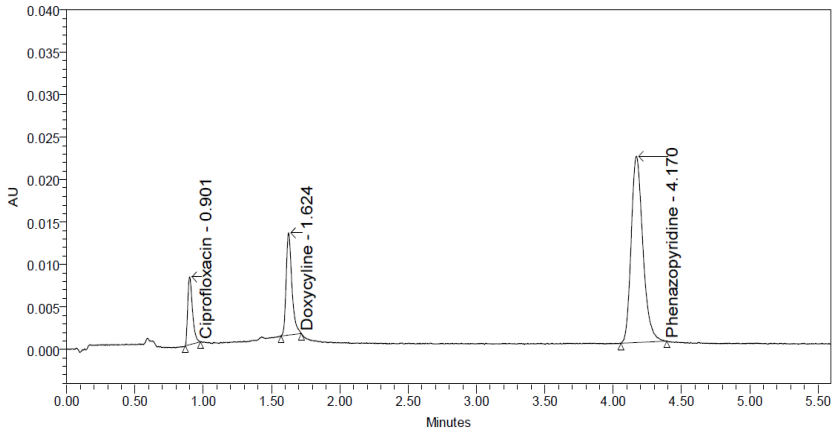

Figure 2: Chromatogram of CIPRO, DOXY & PHENA for UPLC method

RESULTS AND DISCUSSION:

System suitability parameters:

To ascertain resolution and reproducibility of proposed chromatographic system for estimation of CIPRO, DOXY and PHENA in tablets, system suitability parameters like tailing factor (T), resolution (Rs) and column efficiency (number of theoretical plates, N) were studied. Standard stock solution containing CIPRO (100 µg/ml) DOXY (20 µg/ml) and PHENA (10 µg/ml) was used for analysis. The filtrate (0.8 µl) was injected into the column and chromatographed using optimized chromatographic conditions. The system suitability test was performed from six

replicate injections of mixed standard solution. The corresponding chromatograms were recorded at 378 nm. Typical chromatogram obtained, is given in System suitability parameters were calculated and are presented in Table 1.0.

Table 1: System suitability parameters (n=6)

Sr. No.	Parameter	CIPRO	DOXY	PHENA
1.	Retention time (t_R)	0.91	1.62	4.17
2.	USP Resolution[a] (R_S)	-	10.36	22.08
3.	Tailing factor [a] (T_f)	1.45	1.37	1.26
4.	No. of theoretical plates[a] (N)	3385	7799	11360
5.	Capacity factor (k)	0.13	1.21	1.27

Solution stability:

To ascertain the stability of CIPRO, DOXY and PHENA in the selected solvent system i.e. methanol, solution stability study was done by injecting a solution of CIPRO, DOXY and PHENA in the optimized chromatic conditions after regular interval of time. The results of solution stability studies are given in Table 2.0

Table 2: Solution Stability of CIPRO, DOXY & PHENA

Sr.No	Solution Stability of CIPRO		Solution Stability of DOXY		Solution Stability of PHENA	
	Area					
	0 hour stability	24 hour stability	0 hour stability	24 hour stability	0 hour stability	24 hour stability
1.	2287358	2287411	71206	71217	133760	133777
2.	2287417	2287359	71236	71226	133729	133770
3.	2287361	2287371	71208	71200	133756	133775
4.	2287355	2287385	71213	71219	133749	133773
5.	2287354	2287513	71228	71213	133756	133764
6.	2287357	2287501	71221	71201	133742	133756

The solution stability studies showed that there is no significant change in the solutions up to a period of 24 hrs and all the drugs had enough stability up to 24 hrs after preparation in amber coloured flasks

METHOD VALIDATION:

Linearity:

The results of linearity studies are shown in the following tables and graphs.

Table 3: Calibration curve of CIPRO

Standard Conc. Replicates	20 µg/ml	50 µg/ml	80 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml
	Peak area					
1	451372	1143679	1828885	2287358	3441039	4574617
2	452375	1193577	1829880	2287356	3451035	4574820
Mean	451874	1168628	1829383	2287357	3446037	4574719

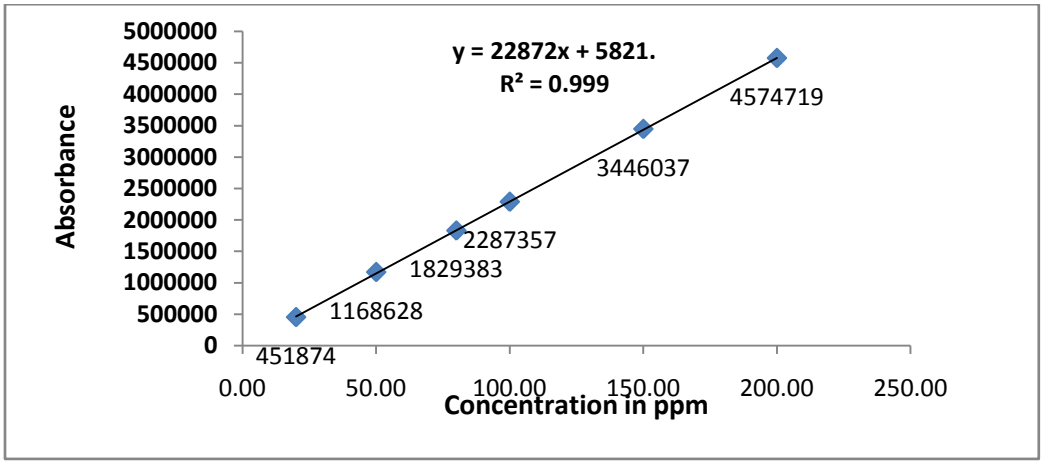

Figure. 3 : Standard calibration curve of CIPRO for UPLC method

Table 4Calibration curve of DOXY (n = 3)

Standard Conc. Replicates	20 µg/ml Peak area	50 µg/ml	80 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml
1	14242	35610	56966	71206	106810	142415
2	14244	35622	56970	71210	106822	142425
Mean	14243	35616	56968	71208	106816	142420

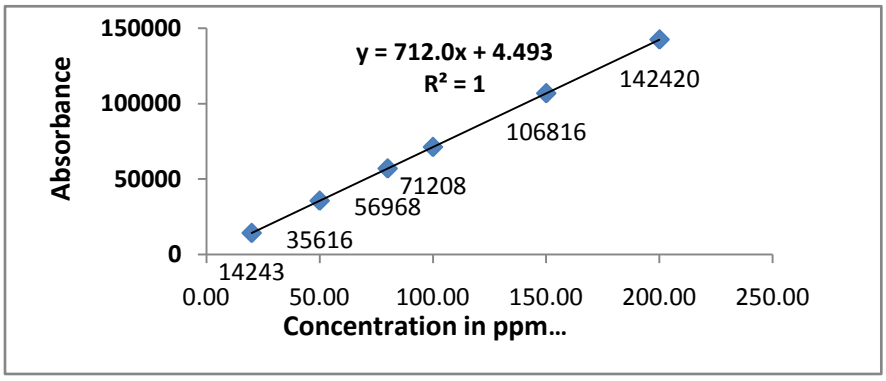

Figure. 4: Standard calibration curve of DOXY for UPLC method

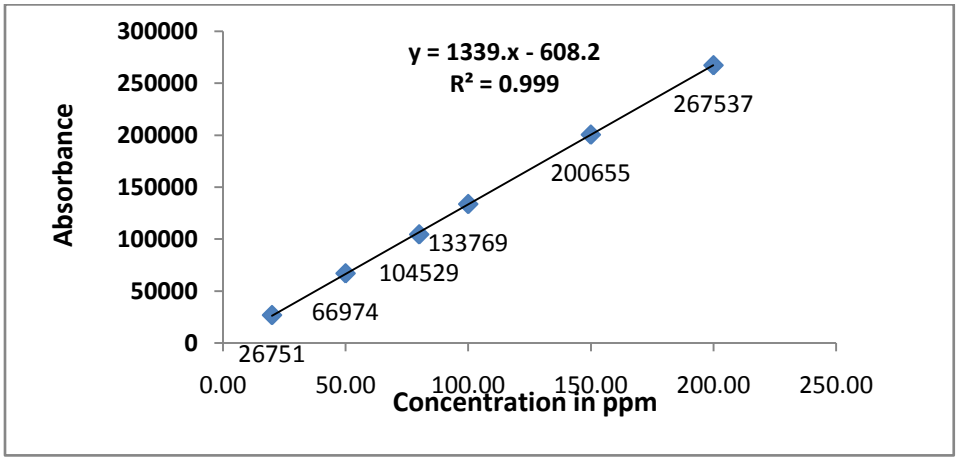

Figure. 5 Standard calibration curve of PHENA for UPLC method

Table 5: Calibration curve of PHENA

Standard Conc.	20 µg/ml	50 µg/ml	80 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml
Replicates	Peak area					
1	26744	66989	102008	133760	200670	267550
2	26758	66959	107050	133777	200640	267523
Mean	26751	66974	104529	133769	200655	267537

The linearity studies showed that both the drugs had a linear response in the stated range of 20-200 µg/ml for CIPRO, 20 – 200 µg/ml for DOXY and PHENA 20-200 µg/ml the co-efficient of correlation was 0.999 for CIPRO, DOXY, PHENA Respectively which indicate a good linearity.

ii) Precision:

The intra-day and the inter-day precision results show that there is not much variation in the analysis results and the %RSD was always less than 1.00. (Ideally should be less than 2.0). The results of intra-day and inter-day precision are shown in following tables and graphs. The system precision parameter is perform to validate the system used for analysis. The method precision is perform to check whether the developed method is precise for the sample or not.

Table 6: Intraday and Inter day precision of CIPRO (n=6)

Sr. No.	Standard Area of CIPRO (100 mcg/ml)	Retention time of CIPRO (Minute)	Theoretical Plates of CIPRO	Tailing Factor of CIPRO	Standard Area of CIPRO (100 mcg/ml)	Retention time of CIPRO (Minute)	Theoretical Plates of CIPRO	Tailing Factor of CIPRO
	Intra day precision				**Inter day precision**			
1	2287358	0.901	3172	1.82	2287401	0.903	3163	1.81
2	2287417	0.902	3176	1.82	2287422	0.901	3180	1.82
3	2287361	0.901	3173	1.83	2287378	0.904	3169	1.80
4	2287355	0.905	3170	1.82	2287406	0.901	3159	1.83
5	2287354	0.900	3174	1.84	2287409	0.903	3181	1.82
6	2287357	0.901	3175	1.82	2287402	0.900	3179	1.81
% RSD	**0.00**	**0.19**	----------	-------	**0.00**	**0.17**	---------	---------

Table 7: Intraday and Inter day precision of DOXY (n=6)

Sr. No.	Standard Area of DOXY (20mcg/ml	Retention time of DOXY (Minute)	Theoretical Plates of DOXY	Tailing Factor of DOXY	Standard Area of DOXY (20mcg/ml	Retention time of DOXY (Minute)	Theoretical Plates of DOXY	Tailing Factor of DOXY
	Intraday precision				**Inter day precision**			
1	71201	1.623	7662	1.60	71201	1.621	7660	1.66
2	71246	1.625	7663	1.69	71246	1.622	7661	1.68
3	71201	1.628	7660	1.68	71201	1.623	7666	1.66
4	71222	1.627	7689	1.66	71222	1.620	7679	1.64

5	71231	1.625	7671	1.67	71231	1.624	7670	1.65
6	71227	1.625	7677	1.64	71227	1.621	7678	1.62
% RSD	**0.02**	**0.11**	---	---	**0.02**	**0.09**	---	---

Table 8: Intraday and Inter day precision of PHENA (n=6)

Sr. No.	Standard Area of PHENA (10mcg/ml)	Retention time of PHENA (Minute)	Theoretical Plates of PHENA	Tailing Factor of PHENA	Standard Area of PHENA (10mcg/ml)	Retention time of PHENA (Minute)	Theoretical Plates of PHENA	Tailing Factor of PHENA
	Intra day precision				**Inter day precision**			
1	133760	4.170	11231	1.29	133750	4.160	11251	1.23
2	133729	4.173	11235	1.28	133751	4.171	11245	1.29
3	133756	4.178	11236	1.26	133750	4.172	11237	1.24
4	133749	4.172	11234	1.27	133762	4.170	11254	1.25
5	133756	4.173	11257	1.26	133755	4.176	11250	1.26
6	133742	4.170	11255	1.27	133747	4.171	11253	1.23
%RSD	**0.01**	**0.07**	--------	-------	**0.00**	**0.13**	--------	---------

Table 9: Method Precision for CIPRO, DOXY and PHENA

Observation no.	Assay % of Ciprofloxacin 100 mcg/ml	Assay % of Doxycycline 20mcg/ml	Assay % of phenazopyridine 10 mcg/ml
Sample I	100.98 %	101.25 %	99.83 %
Sample II	101.85 %	101.23 %	100.29 %
Sample III	101.52 %	99.87 %	100.78 %
Sample IV	102.3 %	101.87 %	99.78 %
Sample V	100.87 %	100.67 %	100.23 %
Sample VI	100.99 %	102.35 %	100.12 %
Mean	101.42 %	101.21 %	100.17 %
% RSD of six determinations	**0.57**	**0.86**	**1.05**

Table 10.0: Results of the recovery analysis of CIPRO, DOXY and PHENA (n=3)

Compound	Recovery Level (%)	Base level amount (µg/ml)	Qty. spiked (µg/ml)	Qty. recovered (µg/ml)	Recovery (%)	R.S.D (%)
	80	500	159.76	157.56	98.62	0.127
CIPRO	100	500	199.60	199.52	99.95	0.358
	120	500	239.64	239.36	99.95	0.124
	80	100	159.76	159.59	99.89	0.176
DOXY	100	100	199.60	199.63	100.01	0.173
	120	100	239.64	241.58	100.80	0.17
	80	50	159.76	159.11	99.59	0.358
PHENA	100	50	199.60	199.90	100.15	0.243
	120	50	239.64	240.70	100.44	0.125

iii) Accuracy:

The results of recovery studies of the marketed formulation are shown in the following table. It indicates that there is no interference in the analysis of the drug from the excipients in the tablet formulation. The results of recovery studies at various levels shows that the recovery is between 98.62 to 100.80 % (Ideally should be between 98-102%).

Twenty tablets were weighed accurately, powdered and a quantity of tablet powder equivalent to 500 mg of CIPRO, 100 mg DOXY and 50 mg of PHENA (Label Claimed 650mg, Average Weight 996mg) was weighed and dissolved in the 50 ml of methanol with the aid of ultrasonication for 10 min and solution was filtered through Whatman paper No. 41 into a 100 ml volumetric flask and volume was made up to the mark with methanol.

Further take 10 of this solution to 10 ml volumetric flask with mobile phase. Appropriate dilution was done in methanol to get a solution of 1000 µg/ml and further 100 µg/ml of solution. From this solution appropriate dilutions were made and injected in to the system to get the chromatogram.

The tablets, purchased from local market, were analysed and the results were obtained in the range of 101.72-104.36 % compared to the label claim. The %RSD for six tablets was found to be 0.201, 0.116 and 0.243 for CIPRO, DOXY, PHENA respectively. From these data it can be concluded that the proposed method is suitable and specific the estimation of CIPRO, DOXY and PHENA in the tablet dosage form. There is no interference in the estimation of CIPRO, DOXY and PHENA by the excipients in the tablet dosage form.

Table 12.0: Analysis of Tablet formulation :

Name of Drug	Label claim	Recovered (%)	Amount found (mg/tab)	% of Label claim determined	% RSD
	500	80	522.26	100.39	0.125
CIPRO	500	100	502.45	100.50	0.356
	500	120	501.02	100.20	0.122
	100	80	50.25	100.86	0.175
DOXY	100	100	49.98	99.96	0.174
	100	120	50.68	101.36	0.170
	50	80	100.49	100.49	0.343
PHENA	50	100	101.11	101.11	0.256
	50	120	100.66	100.66	0.130

The peaks of excipients do not co-elute with the CIPRO, DOXY and PHENA peaks. In peak purity analysis with photo diode array detector, purity angle was always less than purity threshold for the analytes. The above discussion leads to a conclusion that the method can be easily applied to the estimation of CIPRO, DOXY and PHENA as an industrially applicable

method. The results of analysis of marketed formulation are shown in the following table:

Robustness :

The robustness studies show that after deliberate changes in the various parameters there is not much change in the system suitability parameters and the assay values were in the range of 99.12 – 101.81 %. The results of robustness studies by change in various parameters are shown in the following tables.

Table 13: Results of robustness study (n=3) CIPRO Wavelength:

Wavelength + 3 nm (281nm)		Wavelength - 3 nm(275 nm)	
Sample Area	**Retention time**	**Sample Area**	**Retention time.**
2168355	0.903	2158385	0.903
2167258	0.902	2157158	0.902
2168112	0.902	2168102	0.903
2168552	0.904	2158549	0.904
2167879	0.903	2157350	0.903
2167189	0.901	2185120	0.901
% RSD 0.03	0.12	0.51	0.11

Table 14: Results of robustness study (n=3) DOXY Wavelength:

Wavelength + 3 nm (347nm)		Wavelength - 3 nm(353 nm)	
Sample Area	**Retention time**	**Sample Area**	**Retention time.**
70201	1.641	70201	1.612
70246	1.620	70723	1.655
70201	1.625	70593	1.642
69899	1.629	70828	1.619
70562	1.622	69892	1.625
70419	1.624	70855	1.668
% RSD 0.32	0.47	0.55	1.34

Table 15: Results of robustness study (n=3) PHENA Wavelength :

Wavelength + 3 nm (381nm)		Wavelength - 3 nm(375 nm)	
Sample Area	**Retention time**	**Sample Area**	**Retention time.**
123920	4.140	125896	4.150
124552	4.123	125896	4.102
123596	4.158	124346	4.153
119966	4.123	122379	4.125
126335	4.189	120258	4.115
125896	4.118	123658	4.145
% RSD 1.83	0.66	1.76	0.50

CONCLUSION:

UPLC method was developed and validated as per ICH guidelines. The method is specific for simultaneous estimation CIPRO, DOXY and PHENA in pharmaceutical dosage form. The method has linear response in stated range of 20-200 µg/ml for CIPRO, DOXY, PHENA and is

accurate and precise. The %RSD during precision were always less than 2 and recovery studies at various levels i.e. 80, 100, 120 % were in the range of 98-102%. The assay value was in the range of 98-102 %. Robustness studies did not show any significant change in the various system suitability parameters nor were the assay values significantly changed by minute changes. Statistical analysis proves that the method is suitable for the analysis of CIPRO, DOXY and PHENA as bulk drugs and in pharmaceutical formulations without any interference from the excipients. All the above discussions and statistics prove that the method is excellent and has a wide industrial application and thus can be used for in-house estimation of CIPRO, DOXY and PHENA simultaneously and thus saving the time, energy and money.

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