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Analytical method development & validation for simultaneous estimation of ofloxacin, ornidazole & racecadotril in pharmaceutical dosage form by HPTLC

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Abstract
A simple, rapid, economical, precise and accurate HPTLC method for simultaneous estimation of Ofloxacin, Ornidazole and Racecadotril has been developed. Chromatographic separation was achieved using silica gel aluminium plate $60F_{254}$ (10*10) as a stationary phase and Methanol: chloroform: Diethylamine (2.5:7.5:0.1) as a mobile phase. The developed plates scanned densitometrically using UV 254 nm Wavelength. The R_f value of OFL, ORN and RAC was found to be 0.73, 0.44 and 0.36 respectively. The method is validated for different validation parameter such as linearity, accuracy, precision, LOD, LOQ and robustness and the result were found to be within the acceptance limit as per the guideline of international conference on harmonization (ICH).

Keywords: Ofloxacin, racecadotril, HPTLC method development, validation parameter, ICH guideline

Introduction
Ofloxacin (OFL) is a second generation fluoroquinolone acting as antimicrobial agent. Chemically it is known as 7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)- 10-oxo-4-oxa-1-azatricyclotrideca-5(13),6,8,11-tetraene-11-carboxylic acid. Ornidazole (ORN) belongs to nitroimidazole class of drugs mainly used as tissue moebicides, Chemically known as 1-chloro-3-(2-methyl-5- nitro-1H-imidazol-1-yl) propan-2-ol. Racecadotril (RAC) is a drug used for the treatment of acute diarrhoea owes its mechanism of action as a true antisecretory agent Chemically it is known as Benzyl [[(2RS)-2-[(acetylsulfanyl) methyl]-3phenylpropanoyl] amino] acetate [3-8]. The chemical structure of Ofloxacin, Ornidazole and Racecadotril are shown in Figure 1, 2 and 3 respectively.

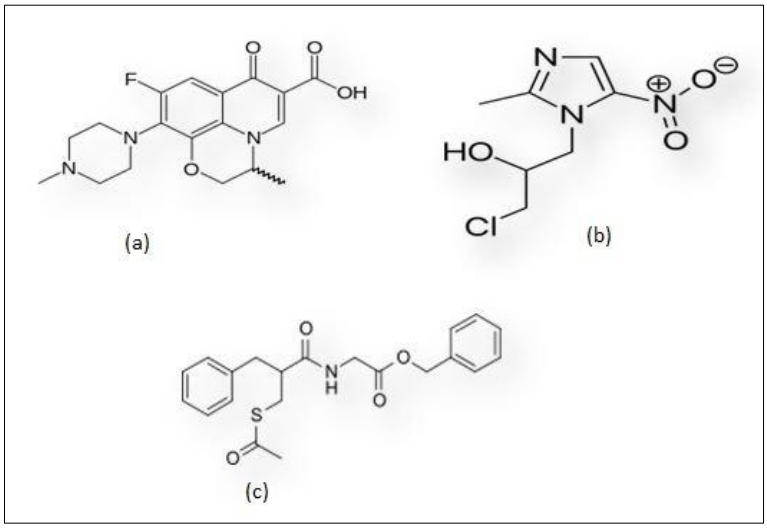

Fig 1: Structure of Ofloxacin (a), Ornidazole (b), Racecadotril (c)

Literature review reveals that only a few analytical methods are reported for estimation of Ofloxacin, Ornidazole and Racecadotril as a single component and in combination with other drugs [10-32] and it also reveals that there is no analytical method reported for estimation of

Ofloxacin, Ornidazole and Racecadotril in combined liquid oral dosage form. In this present work, the aim is Analytical Method Development & Validation for Simultaneous Estimation of Ofloxacin, Ornidazole & Racecadotril in Pharmaceutical Dosage form by HPTLC.

Materials and Methods

Instruments and reagents

A double beam UV-visible Spectrophotometer (Lab India, UV-3000+), attached to a computer software UV Win, with a spectral width of 2 nm, wavelength accuracy of 0.5 nm and pair of 1 cm matched quartz cells. The chromatography was performed by a CAMAG HPTLC System with Linomat V Automatic Sample Applicator, Camag TLC Scanner. Precoated Silica Gel Aluminum plate $60F_{254}$, (10×10cm; E. Merck) were used for separation of Components.

Chemical

Ofloxacin, Ornidazole and Racecadotril was received as gift sample from Alicon Pharma private Ltd. The pharmaceutical preparations of combination of Ofloxacin, Ornidazole and Racecadotril that is Floxoday-OR Suspension contains 50mg of Ofloxacin, 125mg of Ornidazole and 15mg of Racecadotril was procured from local market. Methanol, Chloroform, Diethylamine (RAN-KEM LAB) were used.

Preparation of standard stock solution

Ofloxacin stock solution

A stock solution of Ofloxacin (1000µg/ml) was prepared by dissolving 50mg Ofloxacin in 50ml volumetric flask with Methanol. Withdraw 10ml of stock solution and dilute upto 100ml with methanol to prepare 100µg/ml.

Ornidazole stock solution

A stock solution of Ornidazole (1000µg/ml) was prepared by dissolving 50mg Ornidazole in 50ml volumetric flask with Methanol. Withdraw 25ml of stock solution and dilute upto 100ml with methanol to prepare 250µg/ml.

Racecadotril stock solution

A stock solution of Racecadotril (1000µg/ml) was prepared by dissolving 50mg Racecadotril in 50ml volumetric flask with Methanol. Withdraw 3ml of stock solution and dilute upto 100ml with methanol to prepare 30µg/ml.

Preparation of sample solution

Amount equivalent to about 5ml of suspension containing 50 mg of OFL, 125 mg of ORN and 15 mg of RAC was accurately weighed and taken into the 100 ml volumetric flask, methanol was added and the mixture was sonicated for 15 min. The solution was diluted upto mark with methanol, mixed well and filtered through filter paper no. 41 to obtain the sample stock solution containing 500 µg/ml of OFL, 1250 µg/ml of ORN and 500 µg/ml of RAC. From stock solutions 1µl of the filtered solution was applied to get a final concentration of 500 ng/spot of OFL, 1250 ng/spot of ORN and 150 ng/spot of RAC respectively.

Method development

Selection of wavelength for mixture

The concentration of standard mixture solution of Ofloxacin, Ornidazole and Racecadotril (1000 ng/spot) were spotted in form of bands of width 6mm using a 100µl syringe on precoated silica Gel aluminium plate $60F_{254}$ (10×10cm) then all plates are scanned densitometrically at different wavelength using CAMAG TLC Scanner. All the components showed reasonable good response at 254nm.

Selection of mobile phase

Primary trials

Initially prewashing of TLC plate was done using methanol and activated in hot air oven for 5min at 60°C. The standard stock solution of Ofloxacin, Ornidazole and Racecadotril (1000ug/ml) were spotted separately on TLC plate by glass capillary tube and allowed it to dry for 4 to 5 min at room temperature. The mobile phase as displayed in table 1 was taken in CAMAG glass Chamber and allowed it to saturate for 20 min.

The optimized mobile phase consisting of mixture of "Methanol: Chloroform: Diethylamine (2.5:7.5:0.1)".

Table 1: HPTLC mobile phase optimization

Trial	Mobile Phase Ratio (%V/V)	Rf value			Observation
		OFL	ORNI	RACE	
1	Methanol: Chloroform (5.0:5.0)	0.46	0.17	0.16	Peak observed but peak area and baseline was not proper
2	Methanol: Chloroform (2.5:7.5)	0.10	0.28	0.16	Three peak observed but Rf value of OLF and RAC was very low
3	Methanol: Chloroform: Acetic acid (2.5:7.0:0.5)	0.67	0.60	0.47	Peak broadening of RAC
4	Methanol: chloroform: Diethylamine (2:7:1)	0.71	0.45	0.39	Good separation and good peak intensity
5	Methanol: chloroform: Diethylamine (2:7:1)	0.71	0.36	0.35	Rf value of RAC and ORN was too close
6	Methanol: Chloroform: Diethylamine (2.5:7.5:0.1)	0.73	0.44	0.36	Two sharp peak with good separation, no any tailing.

Assay: To determine the content of OFL, ORN and RAC in Formulation, amount equivalent about to 5ml of suspension containing 50 mg of OFL, 125 mg of ORN and 15 mg of RAC was accurately weighed and taken into the 100 ml volumetric flask, methanol was added and the mixture was sonicated for 15 min. The solution was diluted upto mark with methanol, mixed well and filtered through filter paper no. 41 to obtain the sample stock solution containing 500 µg/ml of OFL, 1250 µg/ml of ORN and 500 µg/ml of RAC. From stock solutions 1 µl of the filtered solution (500 ng/spot of OFL, 1250 ng/spot of ORN and 150 ng/spot of RAC) was applied on the HPTLC plate. The plate was developed and scanned. The analysis was repeated in triplicate. The content of each drug in the Formulation was calculated by putting Respective Response into Regression line equation for OFL, ORN and RAC. The % assay of the drugs was calculated and the results are given in Table-2.

Method validation

The developed HPTLC method was validated as per ICH guidelines.

Specificity

The specificity of the method was ascertained by analyzing

standard drug and sample. The band for OFL. ORN and RAC in the sample was confirmed by comparing the R_f and spectrum of the band with that of standard.

Accuracy

The accuracy of the proposed method was determined by standard addition method by calculating the percentage recoveries of all three drugs. The accuracy was evaluated in triplicates, at three different concentrations levels i.e. 50, 100 and 150 % of the active ingredients, by adding different concentration of OFL, ORN and RAC standard to the known amount of sample and calculating the recovery and % RSD for all the drugs.

Recovery studies were carried out by spiking three different amount of OFL standard (100 ng/spot, 200 ng/spot, 300 ng/spot), ORN standard (250 ng/spot, 500 ng/spot, 750 ng/spot), and RAC standard (30ng/spot, 60 ng/spot, 90 ng/spot) by standard addition method. The results of the recovery studies are given in Table - 8,9,10.

Precision

Precision of the developed method was evaluated by performing repeatability, intraday and inter day precision studies. Intraday precision was carried out by analyzing three replicates of three different concentrations (300 ng/spot, 500 ng/spot, 700 ng/spot for OFL; 750 ng/spot, 1250 ng/spot, 1750 ng/spot for ORN and 90 ng/spot, 150 ng/spot, 210 ng/spot for RAC, respectively). The peak area measured was measured and % RSD was calculated at each concentration level. Intraday precision studies were carried out on the same day at different time intervals whereas Inter day studies were carried out on three different consecutive days using mentioned concentrations for all three drugs in triplicate.

Limit of detection (LOD) and limit of quantitation (LOQ)

As per ICH guideline, limit of detection and limit of quantitation of the developed method were calculated from the standard deviation method i.e. from standard deviation of response (y- intercept) and slope of the calibration curve of drugs using the formula,

Limit of detection = $3.3 \times \sigma/S$

Limit of quantitation = $10 \times \sigma/S$

Where, "σ" is standard deviation of response (y- intercept) and "S" is Slope of calibration curve

Calibration curves were plotted in the range of 100-800 ng/spot for OFL, 250-2000ng/spot for ORN and 30-240 ng/spot for RAC.

Linearity

Standard solutions of OFL, ORN and RAC were prepared using methanol as solvent having concentration 1000 µg/ml respectively. Volumes of standard solutions (10 ml of OFL, 25 ml of ORN, and 3 ml of RAC) was taken from stock solution and makeup with 100 ml methanol. Different volumes of standard solutions were spotted on the HPTLC plate to obtain the concentration range, for all three drugs. Linear relationship between peak area and concentration for all the drugs were evaluated over the concentration range expressed as ng/spot by making three replicates measurements in the concentrations range of 100-800 ng/spot for OFL, 250-2000 ng/spot for ORN and 30-240 ng/spot for RAC at 254 nm was determined in terms of correlation coefficient.

Plot a graph of peak area versus concentration (On X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results were shown in Table-6.

Robustness

The robustness of the method was evaluated by varying method parameters such as saturation time by ±0.2% and mobile phase by ± 2%. Each parameter was varied at a time. It was assessed by using the three replicates of one standard concentration (500 ng/spot of OFL, 1250 ng/spot of ORN and 150ng/spot of RAC) and calculating the values of mean area and % RSD. No significance change was observed.

Result and Discussion

Selection of wavelength for mixture

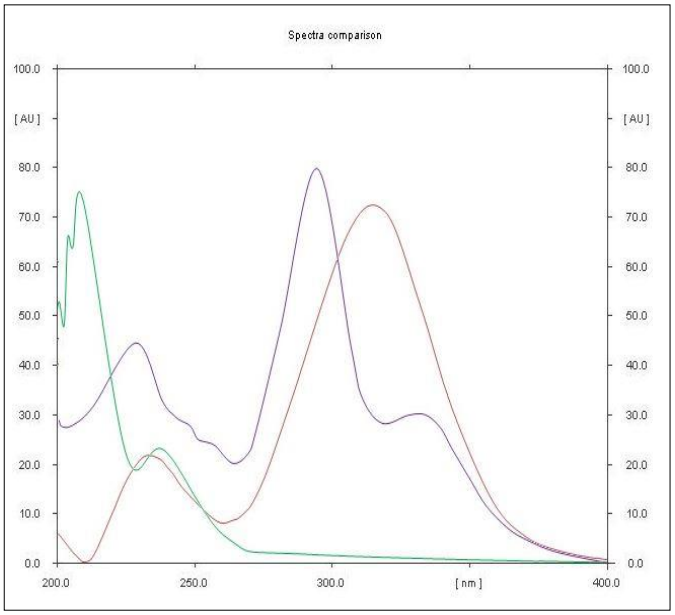

Fig 2: Overlain spectra of OFL, ORN and RAC standard

Selection of mobile phase

The optimize mobile phase was selected after number of trial using different reagents. The optimize mobile phase consist mixture of "Methanol: Chloroform: Diethylamine (2.5:7.5:0.1)"

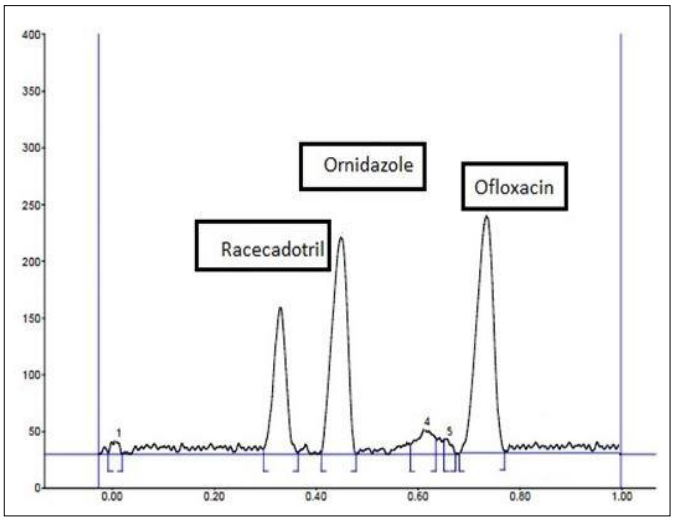

Fig 3: Methanol: chloroform: Diethylamine (2.5:7.5:0.1)

Assay of marketed formulation

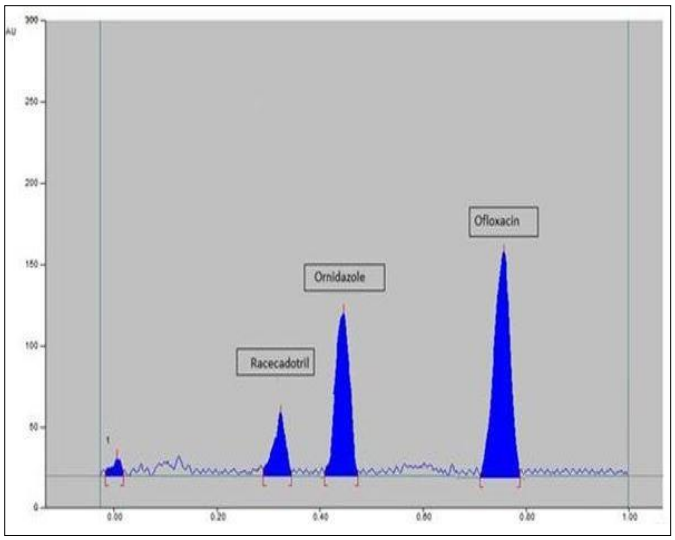

Fig 4: Chromatogram of marketed formulation

Assay results of marked formulation

Table 2: Assay results of marked formulation

Drug	Actual conc. of drug (ng/spot)	Amt. of drug found (ng/spot)	% of Drug found	Avg of % Drug found	SD	%RSD
OFL	500	498.00	99.60	99.8	0.2000	0.2004
	500	499.00	99.80			
	500	500.00	100.00			
ORN	1250	1230.98	98.47	98.4	0.1281	0.1301
	1250	1232.17	98.57			
	1250	1229.00	98.32			
RAC	150	151.66	101.10	101.1	0.0305	0.0302
	150	151.69	101.12			
	150	151.75	101.16			

Validation of the proposed method
Linearity and range

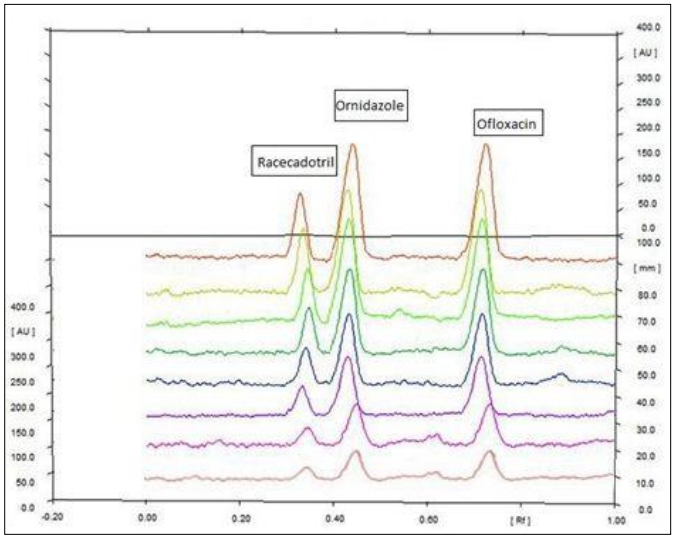

Fig 5: Chromatogram for calibration of ofloxacin, ornidazole and racecadotril

Linearity for ofloxacin

Table 3: Linearity for ofloxacin

Conc. (ng/spot)	Mean area ± S.D (n=3)	% RSD
100	1322.1±15.4	1.16
200	1827.3±7.8	0.42
300	2606.5±10.3	0.39
400	3161.1±13.6	0.43
500	3743.5±11.9	0.31
600	4347.9±12.6	0.29
700	4962.3±31.6	0.63
800	5648.7±42.8	0.75

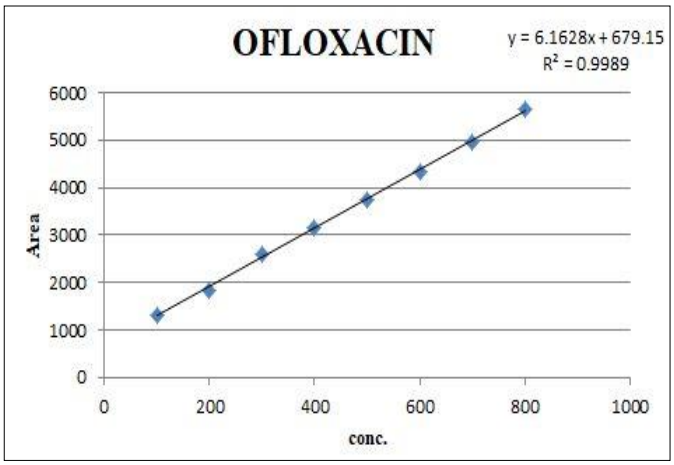

Fig 6: Calibration curve of ofloxacin

Linearity for ornidazole

Table 4: Linearity for ornidazole

Conc. (ng/spot)	Mean area ± S.D (n=3)	% RSD
250	1048.0±15.87	1.49
500	1518±13.5	0.88
750	2336.4±22.4	0.96
1000	2841.4±22.5	0.79
1250	3439.9±19.2	0.56
1500	4087.4±37.4	0.91
1750	4661.1±33.9	0.72
2000	5544.6±25.5	0.46

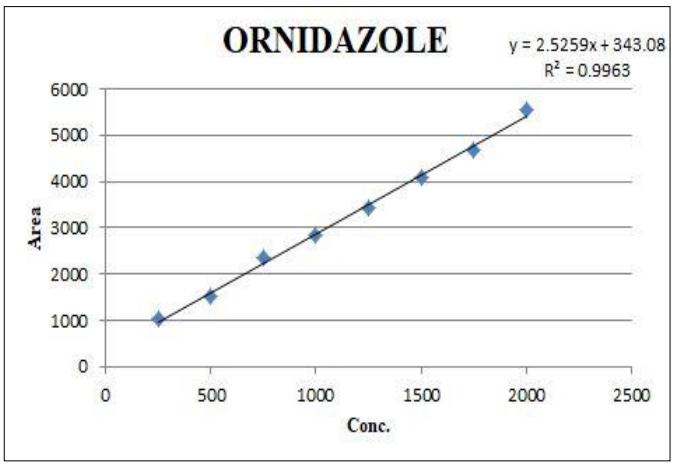

Fig 7: Calibration curve of Ornidazole

Linearity for racecadotril

Table 5: Linearity for racecadotril

Conc. (ng/spot)	Mean area ± S.D (n=3)	% RSD
30	873.3±8.61	0.98
60	1713.9±12.2	0.71
90	2721.8±23.2	0.85
120	3561.9±16.3	0.45
150	4621.9±13.2	0.28
180	5328.4±18.5	0.34
210	6326.5±26.9	0.45
240	7851.1±51.8	0.66

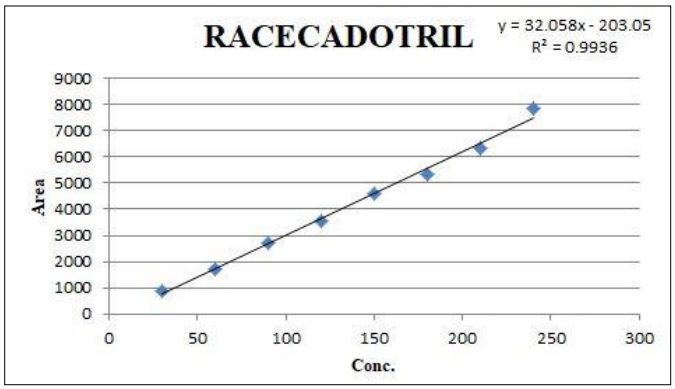

Fig 8: Calibration curve of racecadotril

Stastistical data of ofloxacin, ornidazole and racecadotril

Table 6: Stastistical data of OFL, ORN and RAC

Parameters	Result		
	Ofloxacin	Ornidazole	Racecadotril
Linearity range (ng/spot)	100-800	250-2000	30-240
Slope	6.162	2.525	32.058
Intercept	679.15	343.08	203.05
Rf value	0.73	0.44	0.36
Correlation Coefficient (r^2)	0.998	0.996	0.993

Specificity

The specificity of the method was ascertained by analyzing standard drugs and sample of OFL, ORN and RAC. The results suggested that proposed method is specific, the excipients present in the formulation does not affect the result. The chromatogram taken by running with mobile phase, Ofloxacin Api, Ornidazole Api and Racecadotril Api, Std. mixture, market formulation.

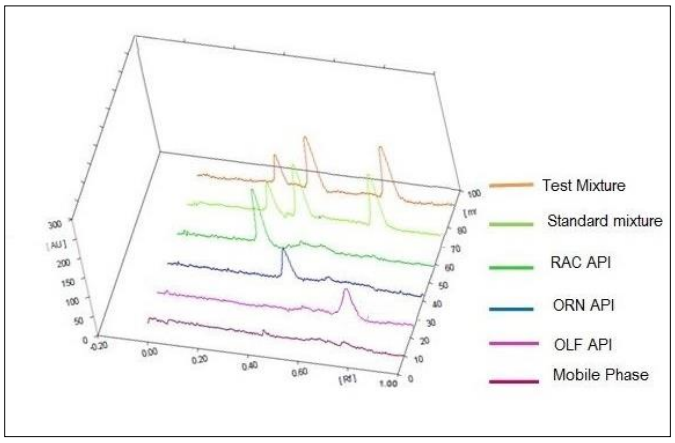

Fig 9: 3-D Chromatogram of Specificity

Limit of detection (LOD) and limit of quantitation (LOQ)

Table 7: LOD and LOQ data for OFL, ORN and RAC

Parameter	Ofloxacin	Ornidazole	Racecadotril
L.O.D (ng/spot)	4.02	13.0	2.56
L.O.Q(ng/spot)	13.4	39.5	8.55

Accuracy

Table 8: Accuracy data for Ofloxacin

% Recovery level	Target Conc. (ng/spot)	Spiked Conc. (ng/spot)	Final Conc. (ng/spot)	Conc. Obtained (ng/spot)	% recovery
50%	200	100	300	294.5	98.1
	200	100	300	304.4	101.4
	200	100	300	302.8	100.9
100%	200	200	400	407.4	101.8
	200	200	400	400.2	100.0
	200	200	400	392.8	98.2
150%	200	300	500	501.9	100.3
	200	300	500	497.5	99.5
	200	300	500	491.5	98.3

Table 9: Accuracy data for Ornidazole

% Recovery level	Target Conc. (ng/spot)	Spiked Conc. (ng/spot)	Final Conc. (ng/spot)	Conc. Obtained (ng/spot)	% recovery
50%	500	250	750	751.9	100.2
	500	250	750	748.1	99.7
	500	250	750	744.0	99.2
100%	500	500	1000	1000.2	100.0
	500	500	1000	999.2	99.9
	500	500	1000	1012.7	101.2
150%	500	750	1250	1274.0	101.9
	500	750	1250	1240.9	99.2
	500	750	1250	1255.0	100.4

Table 10: Accuracy data for racecadotril

% Recovery level	Target Conc. (ng/spot)	Spiked Conc. (ng/spot)	Final Conc. (ng/spot)	Conc. Obtained (ng/spot)	% recovery
50%	60	30	90	90.2	100.2
	60	30	90	89.4	99.4
	60	30	90	88.4	98.2
100%	60	60	120	121.6	101.3
	60	60	120	118.6	98.8
	60	60	120	119.9	99.9
150%	60	90	150	152.4	101.6
	60	90	150	149.3	99.5
	60	90	150	151.0	100.6

Precision

Repeatability study

Table 11: Repeatability data for OFL, ORN and RAC

Drug	Conc.(ng/spot)	Mean area± S.D (n=6)	%RSD
OFL	500	3749.1±28.9	0.77
ORN	1250	3447.7±10.1	0.29
RAC	150	4633.5±30.1	0.65

Intraday

Table 12: Intradaydata for OFL, ORN and RAC

Conc. (ng/ spot)	Ofloxacin		Conc. (ng/ spot)	Ornidazole		Conc. (ng/ spot)	Racecadotril	
	Mean area ± SD (n=3)	% RSD		Mean area ± SD (n=3)	% RSD		Mean area ± SD (n=3)	% RSD
300	2643.1±8.0	0.30	750	2339.3±15.0	0.64	90	2727.9±10.6	0.39
500	3753.8±36.5	0.97	1250	3461.6±11.0	0.31	150	4660.0±27.1	0.58
700	4964.9±27.6	0.55	1750	4681.8±26.0	0.55	210	6339.5±18.9	0.29

Interday

Table 13: Interday data for OFL, ORN and RAC

Conc. (ng/ spot)	Ofloxacin		Conc. (ng/ spot)	Ornidazole		Conc. (ng/ spot)	Racecadotril	
	Mean area ± SD (n=3)	% RSD		Mean area ± SD (n=3)	% RSD		Mean area ± SD (n=3)	% RSD
300	2610.8±22.7	0.86	750	2341.8±15.9	0.68	90	2731±10.8	0.39
500	3746.8±20.0	0.53	1250	3442.2±19.4	0.56	150	4651.9±31.2	0.67
700	4937.7±21.8	0.44	1750	4682.7±25.9	0.55	210	6336.3±22.5	0.35

Robustness

Table 14: Robustness for Ofloxacin

Sr. No.	Ofloxacin (ng/spot)			
	Saturation time		Mobile phase	
	(+0.2 Unit)	(-0.2 Unit)	(+2 %)	(-2 %)
1	3593.8	3955.8	3496.2	3886.8
2	3563.5	3980.5	3475.2	3889.9
3	3540.5	3977.5	3460.8	3871.2
SD	26.73	13.47	18.86	10.0
Mean area	3565.9	3971.2	3478.0	3882.6
% RSD	0.74	0.33	0.54	0.25

Table 15: Robustness for Ornidazole

Sr. No.	Ornidazole (ng/spot)			
	Saturation time		Mobile phase	
	(+0.2 Unit)	(-0.2 Unit)	(+2 %)	(-2 %)
1	3453.5	3552.8	3338.9	3656.8
2	3465.9	3587.9	3358.9	3689.5
3	3430.2	3575.6	3392.6	3681.6
SD	18.15	17.80	27.13	17.65
Mean area	3449.8	3572.1	3363.4	3669.3
% RSD	0.52	0.49	0.80	0.48

Table 16: Robustness for Racecadotril

Sr. No.	Racecadotril (ng/spot)			
	Saturation time		Mobile phase	
	(+0.2 Unit)	(-0.2 Unit)	(+2 %)	(-2 %)
1	4995.8	4672.8	4355.9	4906.7
2	4975.6	4645.8	4380.2	4897.5
3	4922.5	4639.2	4411.1	4859.1
SD	37.86	17.80	27.6	24.7
Mean area	4964.6	4652.6	4382.4	4886.7
% RSD	0.76	0.38	0.63	0.50

Summary and Conclusion

The proposed study describes HPTLC method for the estimation of OFL, ORN and RAC in bulk drugs as well as in Pharmaceutical formulation. The method was validated according to the ICH guidelines. Hence, it can be concluded that the developed HPTLC method is accurate, precise, and selective and it can be employed successfully for the estimation of OFL, ORN and RAC in their bulk drugs and pharmaceutical formulation in routine analysis.

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