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Developing and validation of impurity and the simultaneous quantity determination methods for tablet forms containing Ciprofloxacin HCl and Ornidazole

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Abstract: In this study simple, effective, fast and economic reverse-phase HPLC methods have been developed for the purposes of identifying impurities and quantities of the two active ingredients in the tablet dosage products containing Ciprofloxacin HCl and Ornidazole together. These methods have been validated according to the parameters of *International Conference on Harmonization* (ICH) and Centre for Drug Evaluation and Research (CDER) to prove reliability and applicability of the methods. In the validation studies, all of the parameters defined in ICH as selectivity, precision, accuracy, linearity, LOD, LOQ, recovery, robustness and solution stability were tested. In the impurity method new developed, Inertsil ODS-3 HPLC column, buffer solution with a pH of 3 and acetonitrile mobile phase mixture in isocratic elution were used. Mobile phase flow rate was 1.0 ml/min and detector wavelength were adjusted to be 330 nm. In the quantity determination method, Inertstil C_8 HPLC column, a buffer solution having a pH of 3, methanol and acetonitrile mobile phase mixture in gradient elution was employed. In this method also, mobile phase flow rate was preferred to be 1.5 ml/min and detector wavelength as 330 nm.

Keywords: Ciprofloxacin hydrochloride, Ornidazole, RP-HPLC method, degradation product (impurity).

INTRODUCTION

Ciprofloxacin (CF) is employed in treatment of the infections caused by gram+ and gram- bacteria. It is an antibiotic belonging to wide spectrum quinolone group. Its chemical formula is 1-cyclopropyl-6-fluoro-4-oxo-7-(1-piperazinyl)-1,4-dihydro-3-quinolinecarboxylic acid hydrochloride (1:1). The Food and Drug Administration (FDA) approved CF in 1987 for Bayer Healthcare under the name Cipro (USP, 2016; EP, 2015; CDR CH, 2011; IDI, cipro).

Ornidazole (OR) is an antiprotozoal and antibiotic being derivative of nitroimidazole. Its chemical formula is 1-chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl)-2-propanol. It is active against protozoan and bacterial infections and might be administered orally, parenteral or intravaginal. OR is partially connected to plasma in addition to having radiation sensitizing effect (PILS, 2012; Pharma B, 2015; CDR O, 2011).

The medicinal combination containing CF and OR is effectively used in treatment of such infectious diseases as sexually transmitted Chlamydia, mycoplasmosis, a urogenital infectious disease, gonorrhea, flixy and contagious disease of reproduction organs and trichomoniasis, being a urinary tract infection (Pharma O, 2015).

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In the literature survey related to CF and OR combination, we have encountered UV-spectrophotometric methods (Grewal *et al.,* 2012; Natraj *et al.,* 2013; Krishna *et al.,* 2014; Patel *et al.,* 2006; Amornath *et al.,* 2013; Wankhede *et al.,* 2008) and chromatographic methods (Krishna *et al.,* 2014; Damerakonda and Bindu, 2015; Thoppil and Amin, 2000; Aksoy *et al.,* 2007; Lacroix *et al.,* 1996; Vega *et al.,* 1999; Dhandapani *et al.,* 2010; Pande and Chandorker, 2009; Sani *et al.,* 2011; Nimila *et al.,* 2011; Ranjit *et al.,* 2009; Patel, 2011; Patel and Patel, 2011; Anbarasi *et al.,* 2011; Rote and Saudagar, 2015; Sirisha *et al.,* 2014), in these binary tablet combination products, no previous study was observed with regard to determining impurity through reverse-phase HPLC. In this study, it was also aimed to develop a new method for the determination of impurities and to develop a new, short-term, economical and effective reverse-phase HPLC method which can be an alternative to the existing methods for the quantification of OR with CF. In addition, both of these methods have been validated according to ICH Text and Methodology Q2 (R1) (ICH, 1994) and CDER's Validation of Chromatographic Methods (CDER, 1994) guidelines.

MATERIALS AND METHODS

Chemicals and reagents

The EDQM certified CF reference standard used in the studies was provided from European Pharmacopeia (EP) while CF Impurity C (CFIC) reference standard

consisting of the known impurities of CF substance was taken from American Pharmacopeia (USP). The certified OR reference standard and OR Impurity A (ORIA) reference standard consisting of the known impurities of the OR substance were obtained from the company Hunan Jiudian Pharma. Also, for the purposes of being used in the studies, the tablet product containing CF (750 mg)/OR (500 mg) as well as the placebo of this product were purchased from R&D Laboratories of Avis Pharmaceutical Industry and Trade Inc. Triethylamine, sodium dihydrogen phosphate dihydrate, 37% hydrochloric acid, 85% phosphoric acid in analytical purity and acetonitrile and methyl alcohol of the HPLC-grade purity were also supplied by Merck. The pure water used for the preparation of solutions and mobile phases was obtained from the Milipore Milli-Q Plus ultra-pure water equipment.

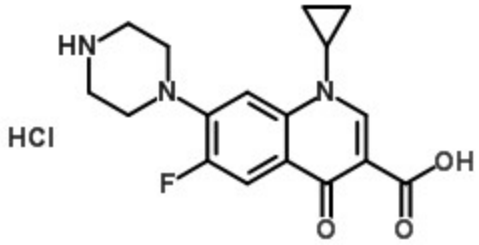

Fig. 1: Chemical structure of Ciprofloxacin HCl

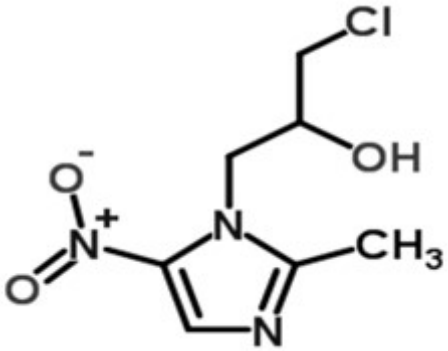

Fig. 2: Chemical structure of Ornidazole

Instrumentation

Shimadzu brand HPLC equipment with 20A Prominence UV-Visible detector composed of LC Solution Software, SPD-20A UV-Vis Detector, DGU-20A prominence degasser, LC-20AT Liquid Chromatography Pump, SIL-20AC Auto Sampler, CTO-10AS VP Column Furnace and 150 mm x 4.6 mm size, filler material with particle size of 5µm, G.L. Sciences brand Inertsil ODS-3 and Inertsil C8 HPLC columns were employed for the reverse-phase HPLC studies. Also, Mettler brand S20 Seven Easy pHmeter for the purposes of mobile phase pH adjustment, Shimadzu brand AUW220D analytical sensitive weigher for weighing, as supplementary in preparation of standard and sample solutions, Jeio Tech brand UCP-20 ultrasonic water bath was used.

Chromatographic conditions

For the study of impurity determination, it was employed in the gradient system as the mobile phase by mixing the buffer solution having a pH of 3.0 and acetonitrile at the below defined rates (table 1). While preparing the buffer solution, 2.54g sodium dihydrogen phosphate dihydrate was dissolved in 1liter water with the pH value adjusted to 3.0 using 85% phosphoric acid and 1mL of triethylamine was added for the purposes of avoiding tailing. The buffer and acetonitrile were mixed at the rate of 95:5 for the diluent. Standard dilutions were prepared using 0.01M HCl solution. It was taken as 1.0 mL/min mobile phase flow rate, 20 µL solution injection volume, 330 nm detector wavelength, 40°C column furnace temperature and 10°C the auto sampler temperature.

For the study of quantity determination; the buffer solution having a pH value of 3.0 with methanol and acetonitrile in the mobile phase was used by mixing at the rate of 750:125:125 for isocratic flow. The buffer solution was prepared by adjusting to pH 3.0 with phosphoric acid in 1 liter of aqueous solution containing 3.40g of sodium dihydrogen phosphate dihydrate and 1mL of triethylamine. Mobile phase flow rate was 1.5mL/min, with the injection volume of the solutions being 10µL, detector wavelength 330 nm, column furnace temperature 40°C and auto sampler temperature as 10°C.

Preparing the stock standard solutions

For commercial drugs containing CF and OR, in order to develop an alternative stability indicator impurity determination as well as developing new reverse-phase quantity determination methods, a series of standard solutions were prepared by diluting from the prepared stock standard solutions, from which calibration curves were formed. Later, for the tablet and test products containing CF and OR, applications were made and recoveries were controlled. The developed methods were validated by applying all validation parameters such as specificity, precision, linearity, accuracy, robustness and solution stability.

In the study of impurity determination; a standard solution having a concentration of 1200 µg/mL with diluent was prepared by taking a certain amount of the CF reference standard to prepare the stock CF standard solution. For the stock OR standard solution, a new solution with a concentration of 800 µg/mL was prepared by dilution with diluent from the OR reference standard. The new solutions were prepared from the CFIC reference standard and the ORIA reference standard with concentrations of 120 µg/mL and of 80 µg/mL with diluent for the stock CFIC standard solution and the stock ORIA standard solution, respectively.

For the study of quantity determination; the main stock solutions having concentrations of 750µg/mL from the

pure CF standard and 500µg/mL from the pure OR standard were prepared in a 0.01M HCl solution with a constant volume completed. A series of standard solutions were prepared from these stock solutions prepared at a concentration of 120-180µg/mL for CF and 80-120 µg/mL for OR, diluted with 0.01M HCl. These solutions were then used by filtration through a 0.2µm membrane filter.

Preparation of system suitability solutions
System suitability solutions were prepared for the purposes of testing HPLC system suitability before starting analyses in the developed methods. CF in the concentration of 600µg/mL, CFIC in the concentration of 1.2µg/mL and OR in the concentration of 400µg/mL were prepared by diluting from the stock standard solutions with diluent. Then, the prepared solutions were filtered through a 0.2 µ membrane filtration.

Preparation of spike standard solutions
The spike standard solutions containing 1.2µg/mL of CF and 0.8µg/mL of concentration were prepared by dilution of stock standard solutions with diluent and filtered through a 0.2µ membrane filter.

Preparation of the sample solutions
In the study of impurity determination; Twenty of the tablets containing 750mg of CF and 500mg of OR were weighed and crushed in a press until powder mixture is obtained. Sample solutions containing 600µg/mL CF and 400 µg/mL OR were prepared from this powder mixture using diluent. During the dissolution of the sample, an ultrasound water bath was employed. Solutions so prepared were filtered through a 0.2µ membrane filter.

For the study of quantity determination; 20 tablets each containing 750 mg CF and 500 mg OR were weighed and crushed in a press to obtain a homogenous powder mixture. Using such powder mixture, sample solutions were prepared to contain 150 µg/mL CF and 100 µg/mL OR in 0.01 M HCl medium. Such solutions prepared were strained through the 0.2 µ membrane filter.

Preparation of Placebo Solutions
The placebo sample equal to the contents of 60mg CF and 40mg OR was weighed in a balloon volumetric flask and resolved using diluent, complementing again using diluent up to the fixed volume of the balloon. During the dissolution of the placebo, an ultrasonic water bath was used again. These solutions were used by filtration through a 0.2µm membrane filter.

STATISTICAL ANALYSIS

Values such as mean, SD and RSD were calculated using Microsoft Excel program (Windows 10.0) with the data obtained from Shimadzu's LCsolution software LabSolutions Analysis Data System.

RESULTS

In order to develop an efficient method for determining the impurities related to both active substances, in the tablets containing the combination of CF and OR, the optimization of the method was performed first. As mobile phase; a mixture of buffer solution and acetonitrile was used and the ratios were optimized to be most appropriate, as previously described in the gradient elution program. Triethylamine was added into the phosphate buffer solution to increase the resolution, reduce the tailing factor of the peaks, and increase the theoretical number of plates. The pH value was also adjusted to 3.0. The best results for separating the of peaks were obtained in a GL Sciences Inertsil ODS-3 column with a size of 150 mm x 4.6mm and 5µm particle size filler. For the shortening of the analysis period, the mobile phase flow rate was adjusted to be 1.5 mL/min. In an effort to analyze both agents in a single wavelength, the wavelength of the detector was adjusted as 330 nm. In the process of developing a method for of the quantity determination; for commercial products containing CF and OR agents, some previously published studies have been examined (Simmy and Thoppil 2000; Aksoy *et al*., 2007, Lacroix *et al*., 1996, Vega *et al*., 1999, Dhandapani *et al*., 2010, Pande *et al*., 2009). As a result of the studies carried out, for the tablets containing CF and OR combination, an efficient method which can effectively separate both active substances were improved. For the purposes of method optimization, a buffer solution: methanol: acetonitrile mixture of 750: 125: 125 was used as the most suitable mobile phase. For increasing the resolution, reducing the tailing factor of the peaks and increasing the number of theoretical plates of the peaks, triethylamine was added to phosphate buffer solution and the pH value was adjusted to 3.0. The best result for the separation of the peaks was obtained in the GL Sciences Inertsil C_8 column with a size of 150 mm x 4.6 mm containing the filler of 5 µm particle size. For the purposes of reducing the analysis period, mobile phase flow rate was adjusted as 1.5 mL/min. The wavelength of the detector was adjusted as 330 nm to analyze both substances in a single wavelength.

DISCUSSION

Selectivity
To prove the selectivity of the impurity determination method; CF and OR standard solutions, standard solutions related to the impurities, sample and placebo solutions were analyzed at the chromatographic conditions defined above. It was checked whether the peaks of CF, OR, and impurities overlap with the other peaks which could arise from placebo and solvent. In the results obtained according to table 2, where the system suitability parameters such as retention time, the number of theoretical plates, tailing factor, resolution and capacity

Table 1: Chromatographic conditions for the impurity determination (in gradient elusion)

Time (minute)	Mobile Phase A (%) Buffer Solution (pH: 3)	Mobile Phase B (%) Acetonitrile
0	95	5
2	95	5
10	80	20
20	80	20
25	95	5
32	95	5

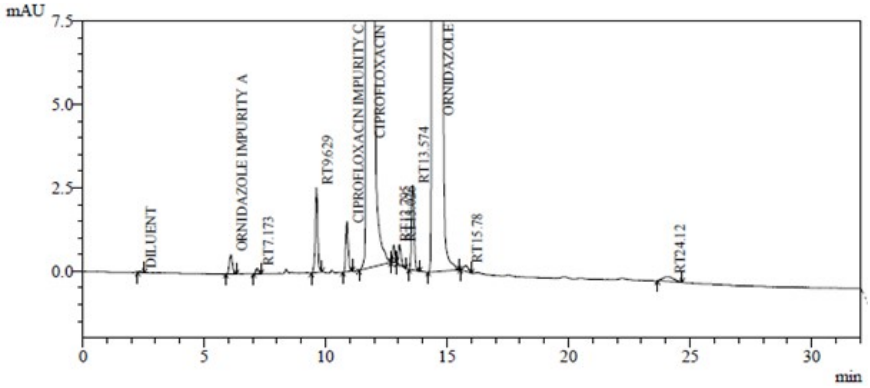

Fig. 3: Selectivity chromatogram for impurity determination

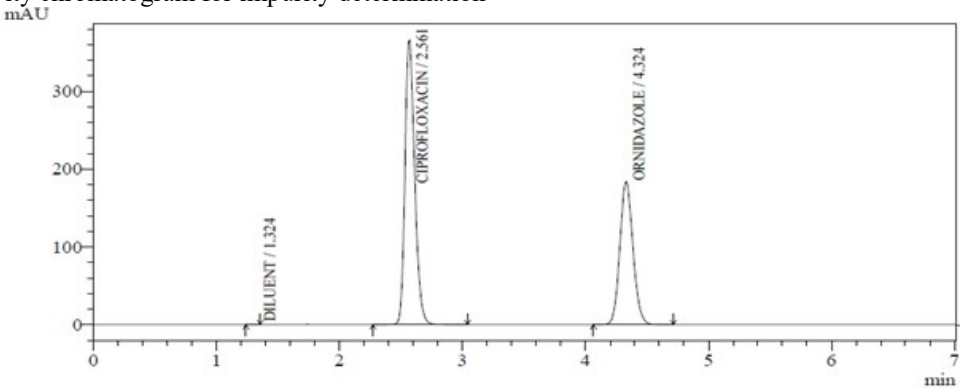

Fig. 4: Selectivity chromatogram for quantity determination

Table 2: Results of the selectivity studies for impurity determination

Peak name	Retention Time (RT)*	Theoretical Plate Number	Tailing Factor	Resolution	Relative Retention Time (RRT)	Capacity Factor
Solvent	2.36	2465	1.58	0.00	0.200	-
Placebo	-	-	-	-	-	-
ORIA	6.10	9815	1.04	17.16	0.518	1.59
RT 7.2	7.18	21716	1.21	4.90	0.610	2.05
RT 9.6	9.62	42132	1.02	12.79	0.818	3.08
CFIC*	10.87	48048	1.20	6.49	0.924	3.62
CF*	11.76	33042	1.56	3.88	1.000	3.99
RT 12.8	12.81	83354	1.07	4.79	1.089	4.44
RT 13.0	13.04	62715	1.46	1.21	1.109	4.54
RT 13.6	13.59	69088	1.13	2.62	1.155	4.77
ORIA*	14.59	37624	1.07	3.96	1.240	5.19
RT 15.8	15.78	25064	0.91	3.40	1.342	5.70
RT 24.1	24.12	11280	1.05	12.76	2.051	9.24

*RT: retention time (minute), CF: Ciprofloxacin reference standard, ORIA: Ordinazole impurity A reference standard, CFIC: Ciprofloxacin impurity C reference standard.

Table 3: Results of the selectivity studies for quantity determination

Peak name	Retention Time (RT)	Theoretical Plate Number	Tailing Factor	Resolution	Relative Retention Time (RRT)*	Capacity Factor
Solvent	1.30	4179	1.58	-	0.52	-
Placebo	-	-	-	-	-	-
CF	2.56	4102	1.36	10.20	1.00	0.94
OR	4.32	6651	1.17	9.48	1.69	2.27

*Relative retention times (RRT) were defined according to CF reference standard.

Table 4: Results of the accuracy studies for impurity determination

Sample Number	(%) Results							
	CFIC*		ORIA*		UBI*		TI*	
	Intra-day	Between-days	Intra-day	Between-days	Intra-day	Between-days	Intra-day	Between-days
S-1	0.058	0.024	0.025	0.064	0.075	0.075	0.130	0.112
S-2	0.055	0.025	0.025	0.063	0.075	0.074	0.131	0.112
S-3	0.055	0.025	0.026	0.063	0.075	0.074	0.130	0.112
S-4	0.056	0.025	0.027	0.069	0.073	0.077	0.129	0.118
S-5	0.056	0.025	0.026	0.068	0.073	0.077	0.128	0.117
S-6	0.056	0.022	0.026	0.063	0.073	0.067	0.130	0.119
Mean	0.056	0.024	0.026	0.065	0.074	0.074	0.130	0.115
SD*	0.001	0.001	0.001	0.003	0.001	0.004	0.001	0.003
RSD %*	2.0	5.0	2.9	4.2	1.5	5.0	0.8	2.9

*SD: Standard Deviation, RSD %: Relative Standard Deviation, CFIC: Ciprofloxacin Impurity C Reference Standard, ORIA: Ornidazole Impurity A Reference Standard, UBI: Highest Single Unknown Impurity, TI: Total Impurities

Table 5: Results of the accuracy studies for quantity determination

Sample Number	Ciprofloxacin (Results %)		Ornidazole (Results %)	
	Intra-day	Between-days	Intra-day	Between-days
S-1	101.1	100.3	97.4	99.0
S-2	100.8	101.3	97.4	99.8
S-3	100.7	102.1	98.7	99.8
S-4	99.8	101.4	97.9	100.0
S-5	100.6	100.4	98.6	99.0
S-6	99.8	101.9	97.9	98.9
Mean	100.5	101.2	98.0	99.4
SD	0.537	0.746	0.563	0.517
RSD (%)	0.5	0.7	0.6	0.5

Table 6: Results of the linearity studies for impurity determination

Parameters	CF	CFIC	OR	ORIA
Regression Equation	$y = 3.84.10^7 x + 2599.30$	$y = 3.04.10^7 x + 32.30$	$y = 4.52.10^7 x + 576.40$	$y = 5.25.10^7 x + 7.58$
Linearity (µg/mL)	0.05-1.6	0.05-2.2	0.04-1.6	0.04-1.6
Correlation Coefficient	0.9998	0.9999	0.9999	0.9999

Table 7: Working range and correction factors for impurity determination

Peaks	(µg/mL)			Correction Factor
	LOD	LOQ	Range	
CF	0.01	0.05	0.05-1.6	-
CFIC	0.01	0.05	0.05-2.2	0.79
OR	0.01	0.04	0.04-1.6	-
ORIA	0.01	0.04	0.04-1.6	1.16

Table 8: Results of the linearity studies for quantity determination

Parameters	CF	OR
Regression Equation	$y = 1.36.10^7 x - 6432.35$	$y = 1.42.10^7 x - 6152.99$
Linearity (µg/mL)	120-180	80-120
Correlation Coefficient	0.9995	0.9994

Table 9: Results of the accuracy studies for impurity determination

CFIC Recovery Results					
Added Concentration (µg/mL)	Measurement Concentration (µg/mL)	Recovery (%)	Mean Recovery (%)	SD	RSD (%)
0.045	0.046	102.22	101.48	1.283	1.26
0.045	0.046	102.22			
0.045	0.045	100.01			
0.797	0.815	102.26	102.30	0.441	0.43
0.797	0.819	102.76			
0.797	0.812	101.88			
1.593	1.627	102.13	101.95	0.166	0.16
1.593	1.623	101.88			
1.593	1.622	101.82			
ORIA Recovery Results					
Added Concentration (µg/mL)	Measurement Concentration (µg/mL)	Recovery (%)	Mean Recovery (%)	SD	RSD (%)
0.052	0.051	98.08	98.72	1.110	1.12
0.052	0.051	98.08			
0.052	0.052	100.02			
1.024	0.956	93.36	93.07	0.352	0.38
1.024	0.954	93.16			
1.024	0.949	92.68			
2.008	1.883	93.77	93.59	0.160	0.17
2.008	1.877	93.48			
2.008	1.878	93.53			

Table 10: Results of the accuracy studies for quantity determination

Ciprofloxacin Recovery Results					
Added Concentration (µg/mL)	Measurement Concentration (µg/mL)	Recovery (%)	Mean Recovery (%)	SD	RSD (%)
120.39	121.02	100.52	100.54	0.055	0.06
120.56	121.16	100.49			
120.26	120.98	100.60			
150.37	150.68	100.21	100.01	0.396	0.40
150.16	150.56	100.26			
150.54	149.86	99.55			
180.06	179.5	99.69	99.52	0.307	0.31
180.19	179.66	99.70			
180.40	178.89	99.16			
Ornidazole Recovery Results					
Added Concentration (µg/mL)	Measurement Concentration (µg/mL)	Recovery (%)	Mean Recovery (%)	SD	RSD (%)
80.35	79.49	98.93	99.05	0.132	0.13
80.10	79.45	99.19			
80.20	79.43	99.04			
100.40	98.76	98.37	98.60	0.21	0.21
100.01	98.78	98.78			
100.15	98.81	98.66			
120.10	117.67	97.98	98.03	0.131	0.13
120.05	117.86	98.17			
120.20	117.70	97.92			

Table 11: Results of the robustness studies for impurity determination

Operation No	Peak Name	Tailing Factor*	Capacity Factor*	Theoretical Number of Plates*	RSD (%)*	Resolution**
1	CF	1.12	3.8	64774	0.32	4.1
	OR	1.06	4.9	35996	0.23	
2	CF	1.08	3.8	63478	0.35	4.1
	OR	1.06	4.8	34581	0.34	
3	CF	1.15	3.5	65011	0.38	4.0
	OR	1.05	4.7	34744	0.34	
4	CF	1.09	3.6	64534	0.46	4.1
	OR	1.06	4.6	35132	0.41	

*The data related to standard solution, **The resolution between CFIC and CF peaks related to system suitability solution, Operation No-1: 40°C –2°C column oven temperature change, Operation No-2: 40°C +2°C column oven temperature change, Operation No-3: 1.5 mL/min –0.1 mL/min mobile phase flow rate change, Operation No-4: 1.5 mL/min +0.1 mL/min mobile phase flow rate change

Table 12: Results of the robustness studies for quantity determination

Operation No	Peak Name	Tailing Factor	Theoretical Number of Plates	RSD (%)	Resolution*
1	CF	1.29	3107	1.02	10.9
	OR	1.22	3942	3.12	
2	CF	1.28	3085	0.98	10.9
	OR	1.22	3872	3.08	
3	CF	1.33	3251	0.97	11.0
	OR	1.23	4693	3.05	
4	CF	1.32	3162	1.01	11.1
	OR	1.22	4548	3.08	

*The resolution between CF and OR peaks, Operation No-1: 40°C –2°C olumn oven temperature change, Operation No-2: 40°C +2°C olumn oven temperature change, Operation No-3: 1.5 mL/min –0.1 mL/min mobile phase flow rate change, Operation No-4: 1.5 mL/min +0.1 mL/min mobile phase flow rate change

Table 13: Solution stability study results for the impurity determination

Time- Injection Number	CFIC (%)	ORIA (%)	UBI (%)*	TI (%)*
Start – Inj 1	0.054	0.025	0.075	0.160
Start - Inj 2	0.055	0.025	0.075	0.161
16. hour - Inj 1	0.055	0.025	0.073	0.161
16. hour - Inj 2	0.055	0.025	0.073	0.161
36. hour - Inj 1	0.054	0.025	0.074	0.160
36. hour - Inj 2	0.053	0.025	0.074	0.160
Mean	0.054	0.025	0.074	0.161
SD	0.001	0.000	0.001	0.001
RSD (%)	1.5	0.0	1.2	0.3

*UBI: Highest Single Unknown Impurity, TI: Total Impurities

Table 14: Solution stability study results for the quantity determination

Time- Injection Number	Areas of Ciprofloxacin (mAU)		Areas of Ornidazole (mAU)	
	Standard Solution	Sample Solution	Standard Solution	Sample Solution
Start – Inj 1	1959.64	1925.34	1369.77	1330.32
Start - Inj 2	1941.16	1943.66	1359.87	1341.23
12. hour - Inj 1	1949.37	1920.08	1361.79	1326.47
12. hour - Inj 2	1953.47	1933.71	1365.30	1334.69
24. hour - Inj 1	1939.99	1918.17	1355.22	1325.31
24. hour - Inj 2	1942.82	1922.67	1357.63	1329.52
Mean	1947.70	1927.30	1361.60	1331.30
SD	7.806	9.690	5.290	5.890
RSD (%)	0.4	0.5	0.4	0.4

factor are given together and in the chromatogram in fig. 3, any other peaks which interfere with the CF, OR and impurity peaks were not observed. Relative Retention Times (RRT's) were also given for the comparison of the retention times of the other impurity peaks with respect to this main peak, with referring to the main peak retention times. Occasionally, retention times for system or analyst-derived peaks could change, but RRT's do not change.

To prove the selectivity of the quantity determination method; CF and OR standard solutions, sample solutions and placebo solutions were analyzed under the above defined chromatographic conditions. Whether or not CF and OR peaks overlapped with the other peaks which could arise from placebo and solvent was controlled. In the findings combining such system suitability parameters as retention time, the number of theoretical plates, tailing factor, resolution, and capacity factors (table 3) and with regard to the retention times presented in the chromatogram, other than CF peak at 2.56 minutes and OR peak at 4.32 minutes, no overlapping peak was observed (fig. 4).

Precision

For the intra-day or inter-day precision analyzes acquired both from impurity and quantity determination studies; average, standard deviation (SD) and relative standard deviation (RSD %) data between 6 consecutive analyzes outcomes were evaluated. The standard solutions prepared were injected to the auto-sampler intra-day and inter-day (on different days) serially 6 times. In the sample solutions so prepared, CFIC, ORIA, the highest single unknown impurity value (UBI) and total impurities (TI) were examined. In addition, 6 sample solutions prepared in parallel were analyzed intra-day and inter-day. The RSD values found according to all repeatability analysis results so obtained were found to be below 10% with regard to impurity analyzes and below 2% with regard to quantity determination (see, respectively table 4 and table 5).

Linearity

According to the results of linearity analysis of impurity determination; standard solutions were prepared from stock standard solutions at 5 different concentrations. When these solutions were analyzed, it was observed that there was a proportional increase between the concentrations and the peak areas in the calibration curves and it was found that all the correlation coefficients exceed 0.9998 (table 6).

The working intervals and correction factors related to known impurities (CFIC and ORIA) were determined considering the slope values obtained from the calibration curves found as a result of the linearity study. LOD and LOQ values were obtained from the signal (S)/noise (N) proportions of peaks. The concentration at which the S/N ratio was 3/1 or 2/1 as LOD value and the concentration at which the S/N ratio was 10/1 were determined as LOQ value. The stock standard solutions were diluted at suitable rates using diluent. The LOD values for all CF, CFIC, OR and ORIA were 0.01µg/mL and LOQ values were obtained 0.05µg/mL for CF and CFIC and 0.04µg/mL for OR and ORIA (table 7).

According to the results of linearity analysis of quantity determination; the main stock solutions of 750µg/mL from the pure CF standard and 500µg/mL from the OR standard were prepared in 0.01 M HCl solvent. Later, 5 different study standard solutions were prepared from such stock solutions namely the concentrations of 120, 135, 150, 165 and 180µg/mL for CF and 80, 90, 100, 110 and 120µg/mL for OR. These solutions were analyzed and their correlation coefficients were found to be over 0.9994 (table 8).

Accuracy

Impurity determination recovery study; spike sample solutions containing 3 different known concentrations (LOQ, 100 and 200 %) of CF, CFIC, OR and ORIA were prepared and these solutions were analyzed repeatedly 3 times to yield recovery (accuracy) values. The recovery values were between 92 and 103% for both active substances while the RSD values obtained after measurements were found to be below 1.3% (table 9).

Quantity determination recovery study; spike sample solutions containing 3 known different concentrations of (80, 100 and 120%) CF and (120, 150 and 180%) OR were prepared and these solutions were analyzed repeatedly 3 times to find out the recovery (accuracy) values. The recovery values were between 97 and 101% for both active substances while the RSD values obtained after measurements were found to be below 0.5% (table 10).

Robustness

The effects of these changes on system suitability and analysis results were evaluated under definite chromatographic conditions by making changes ±2° C in column temperature and ±0.1 ml / min in mobile phase flow rate for both impurity and quantity determination analysis studies. According to the analysis results obtained, system suitability was provided (table 11 and table 12).

Solution stability

In the impurity determination, the solution stability parameter; the standard and the sample solutions prepared were analyzed in certain intervals by keeping them at a temperature of +5°C±3°C in dark for 36 hours. At the end of this waiting period, when the results of CFIC, ORIA, UBI and TI analysis were compared with those of the initial analysis, the change between the results and RSD

values were found to be below 10%. The standard and sample solutions were observed to be stable at +5°C±3°C, in dark for 36 hours (table 13).

In the study of quantity determination, for the purposes of evaluating solution stability parameter; the standard and sample solutions prepared were kept at room temperature and in dark for 24 hours, and analyzed in certain intervals. At the end of the waiting period, when the analysis results were compared with the initial analysis results, the change between the results was found to be below 2% and the standard and sample solutions were stable at room temperature, in the dark and for 24 hours (table 14).

CONCLUSION

In this study, both first-time impurity determinations as well as a practical and feasible new alternative, reverse-phase HPLC quantity determination methods for CF and OR active ingredients co-existing in pharmaceutical tablet products have been developed and successfully applied.

Primarily, a new method has been provided in the literature by determining the impurities involved in first of these methods developed for tablet products containing both active ingredients simultaneously. At the same time, this new method also allows analysis in reverse-phase HPLC system, UV-Visible detector, single wavelength, low LOD and LOQ levels. Later, a new reverse-phase liquid chromatography quantitation method has also been developed which may also be an alternative from various aspects to the other liquid chromatographic methods in the literature; ease of use in the application and economy, lower peak tailing factors, more uniform peak shapes, satisfaction of peak widths and sharpness, quite higher recovery values and quite lower RSD%.

These two methods developed at the same time were validated by testing the selectivity, precision, accuracy, LOD, LOQ, linearity, stability and solution stability parameters in the direction of the ICH guide. Thus, validation studies have proven that the methods are feasible and reliable.

In addition, the main advantage of both of these methods is that they are highly suitable for routine analysis of impurities and simultaneous quantitative determinations of this binary combination product, which is located in solid dosage forms for use in quality control laboratories.

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