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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC ASSAY METHOD FOR CEFIXIME TRIHYDRATE AND ORNIDAZOLE IN BULK DRUG AND COMBINED TABLET DOSAGE FORM

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Abstract: A simple, selective and stability indicating reverse phase high performance liquid chromatographic method was developed and validated for the determination of Cefixime trihydrate and Ornidazole in bulk drug and pharmaceutical dosage form. Separation and quantification were achieved on an ACE C_{18}, 5µm, 150 x 4.6 mm i. d. column. The mobile phase was Acetonitrile: 20mM KH_2PO_4 buffer (20:80) and buffer pH 5.5 adjusted using orthophosphoric acids after adding 0.1% triethylamine, at a flow rate of 0.8 ml/min and injection volume was 20µL.Detection was carried out at a wavelength of 310 nm. The method was validated for precision, accuracy and robustness. Cefixime trihydrate and Ornidazole were exposed to acidic, basic, oxidative, neutral, thermal and photolytic stress conditions and the stressed samples were analyzed by the proposed method. Good linear relationship in the concentration range of 10-60µg/ mL for Cefixime trihydrate with correlation coefficient of 0.998 and 25-150µg/ mL for Ornidazole with correlation coefficient of 0.997. Intra- and inter-day precision and accuracy were less than 2 % RSD for both analytes. The stressed sample chromatograms demonstrate the specificity of the proposed method for the determination of target analytes in presence of degradants.

Keywords: Cefixime trihydrate, Ornidazole, Stability indicating, RP-HPLC

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INTRODUCTION

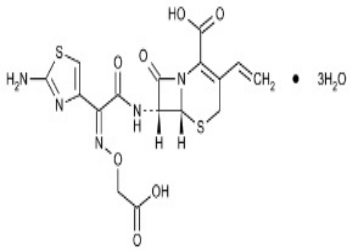
Cefixime trihydrate, (6R, 7R)-7-[[(Z)-2-(2-aminothiazol-4-yl)-2[(carboxymethoxy) imino] acetyl] amino]-3- ethenyl-8-oxo-5-thia-1-azabicyclo [4.2.0] Oct-2- ene-2- carboxylic acid. It is oral third generation cephalosporin and it is official in Indian Pharmacopoeia, British Pharmacopoeia, United State Pharmacopoeia, European Pharmacopoeia, and Japanese Pharmacopoeia. Cefixime trihydrate act by disrupt the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs). PBPs bind to the DAla-D-Ala at the end of muropeptides (peptidoglycan precursors) to crosslink the peptidoglycan. Beta-lactam antibiotics mimic this site and competitively inhibit PBP cross linking of peptidoglycan [1-3]. Ornidazole, (RS)-1-chloro-3-(2-methyl-5-nitroimidazol-lyl) propan-2-ol.It belongs to class of Nitroimidazoles and it is official in Indian pharmacopoeia. The Mechanism action of this drug is converted into reduction products that interact with DNA to cause destruction of helical DNA structure and strand leading to a protein synthesis inhibition [1-3]. Literature survey revealed HPLC [6-8] and UV [5] method for the analysis of CEFI and ORNI in combination, HPTLC [9] method for analysis of CEFI and ORNI in combination, stability study of CEFI [10] alone and stability study of ORNI [11] alone are done, HPLC method of CEFI and ORNI in combination with other drugs [12-20]. So far to our knowledge no stability indicating RP-HPLC assay method has been developed in combined dosage form. So the aim of this work is to develop simple stability indicating RP-HPLC assay method for determination of CEFI and ORNI in bulk drug and combined dosage form. Chemicals structures [4] of CEFI and ORNI are shown in Figure 1.

MATERIALS AND METHOD

Reagents and Chemicals

Cefixime trihydrate (CEFI) and Ornidazole (ORNI) obtain from Mann Pharmaceutical Pvt. Ltd Mehsana. Acetonitrile and methanol were (HPLC Grade, Merck Specialties Private Limited, Mumbai, India).Water (HPLC Grade, Rankem, RFCL Limited, New Delhi, India). Potassium Dihydrogen Ortho Phosphate (HPLC Grade), Triethylamine and Ortho Phosphoric Acid obtain from Finar Chemicals Ltd, Ahmedabad. Sodium hydroxide, Hydrochloric acid and Hydrogen peroxide (30%) obtain from Finar Chemicals Ltd, Ahmedabad. Marketed tablet formulation Ornicef* (200mg CEFI & 500mg ORNI) was taken from local market.

a)



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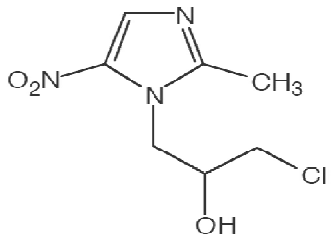

Figure 1: It shows Chemical structures of a) Cefixime trihydrate b) Ornidazole.

Instrumentation and chromatographic conditions

The HPLC system was (LC 2010CHT, Shimadzu) with PDA detector. Data acquisition was performed on LC Solution software. The detector was set at 310 nm. The HPLC separation and quantitation were achieved on ACE C_{18}, 5µm, 150 x 4.6 mm i.d. column. All determinations were performed at 25 ºC. The mobile phase was (20:80) Acetonitrile: 20mM KH_2PO_4 buffer and buffer pH 5.5 adjusted using orthophosphoric acid after adding 0.1% triethylamine, which was run Low pressure gradient. Flow rate was 0.8 ml/min. and injection volume was 20µL.

Preparation of standard solutions

A combined standard solution of CEFI and ORNI was prepared by dissolving 10 mg of CEFI and 25 mg of ORNI in 10 ml volumetric flask and dissolved in Methanol. Appropriate dilutions were made in Acetonitrile: water (20:80v/v) to obtain standard stock solution (1000µg/mL CEFI and 2500µg/mL ORNI).From this solution make working standard solution (100µg/mL CEFI and 250µg/mL ORNI).

Preparation of sample solutions

The content of ten tablets of Ornicef® was accurately weighed, crushed and well mixed. A portion of the powder equivalent to 10mg CEFI and 25mg ORNI was accurately transferred to a

10 mL volumetric flask and dissolved in 10 ml methanol in an ultrasonic bath for 2 min and then filtered through 0.45 µm membrane filters (Millipore, Milford, MA, USA). Appropriate dilutions were made in Acetonitrile: water (20:80v/v) to obtain sample stock solution (1000µg/mL CEFI and 2500µg/mL ORNI).From this solution make working sample solution (100µg/mL CEFI and 250µg/mL ORNI).

Forced degradation conditions

Acid degradation: About 10 mg of standard CEFI and 25 mg of standard ORNI were weighed accurately and transferred in to 10 mL volumetric flasks and dissolve with 2 mL methanol. Add 8 mL of 1N HCl in flask for acid hydrolysis. Solutions were kept for 1.5 hr at 60°C.

Alkaline degradation: About 10 mg of standard CEFI and 25 mg of standard ORNI were weighed accurately and transferred in to 10 mL volumetric flasks and dissolve with 2 mL methanol. Add 8 mL of 0.001N NaOH in flask for alkaline hydrolysis. Solutions were kept for 10min at room temperature.

Oxidative degradation: About 10 mg of standard CEFI and 25 mg of standard ORNI were weighed accurately and transferred in to 10 mL volumetric flask and dissolve with 2 mL methanol. Add 8 mL of 3%H2O2 in flask for oxidation. Solutions were kept for 1.5hr at room temperature.

Neutral degradation: About 10 mg of standard CEFI and 25 mg of standard ORNI were weighed accurately and transferred in to 10 mL volumetric flask and dissolve with 2 mL methanol. Add 8 mL of water in flask for neutral hydrolysis. Solution was refluxed for 24 hours at 60^0C.

Photolytic degradation: About tablet powder equivalent to 10 mg and 25 mg accurately weighed powder amount of CEFI and ORNI were exposed to UV 254nm in photolytic chamber for 10days. After this exposure, the drug powder was mixed and transferred in to 10 mL volumetric flask, dissolved in methanol.

Thermal degradation: About tablet powder equivalent 10 mg and 25 mg accurately weighed powder amount of CEFI and ORNI were exposed at 60^0C for 7days. After this exposure, the drug powder was mixed and transferred in to 10 mL volumetric flask, dissolved in methanol.

Finally,1 mL of the individual stress solutions taken to 10ml volumetric flask neutralized and diluted up to mark with ACN: WATER (20:80) to obtain Cefixime trihydrate (100 µg/mL) & Ornidazole (250 µg/mL) before injection.

Validation procedure

The method was validated in accordance with the ICH requirements [21], which involved accuracy, precision, linearity, specificity, robustness, limit of detection and limit of quantitation.

System suitability

The column efficiency, peak symmetry and Chromatographic peak resolution were calculated for the standard solutions (Table 2). The values obtained demonstrated the suitability of the system for the analysis of this drug combination. Here tailing factor for peaks of CEFI and ORNI was less than 2% and resolution was satisfactory. The peaks obtained for CEFI and ORNI were sharp and have clear base line separation. The repeatability of these parameters was checked by injecting six solutions of CEFI and ORNI.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The stress studies were performed for CEFI and ORNI bulk drugs to provide indication of the stability indicating property of the method and specificity of the proposed method. Intentional degradation was attempted to stress condition of acidic, basic and neutral hydrolysis, oxidation, dry heat (thermal) and UV light to evaluate the ability of the proposed method to separate CEFI and ORNI from its degradation products. Peak purity test was carried out for the CEFI and ORNI peak by using PDA detector in all stress samples.

Robustness

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by changing four small changes.

1) Column Temperature (25±2^{0}C)

2) Mobile phase flow rate (0.8±0.1mL/min)

3) Organic phase modifier (20mM KH_2PO_4 + Acetonitrile, 82+18 and 78+22 v/v)

4) pH (±0.2 units)

After each change the target concentration of test sample solution was injected and calculated % RSD with system suitability parameters was checked.

Linearity and Range

The linearity response was determined by analyzing six independent levels of calibration curve in the range of 10-60 µg/mL of ORNI and 25 - 150 µg/mL of ORNI.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were calculated using following formula:

$$LOD = 3.3 \times \sigma / S \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$LOQ = 10 \times \sigma / S \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where, σ = standard deviation of response

S = slope of the calibration curve

Precision

a) Repeatability

Repeatability was determined by analysing CEFI and ORNI test solution having the concentration 20 µg/mL and 50µg/mL respectively for 6 times. Calculate % RSD for CEFI and ORNI.

b) Intermediate precision

Intermediate precision was determined by analysing of CEFI (20, 30 and 50µg/mL) and ORNI (50, 75 and 100 µg/mL) test solutions for three times on the different day. Calculate % RSD for CEFI and ORNI.

Accuracy

Accuracy was determined by calculating recovery of CEFI and ORNI by the standard addition method. Known amount of standard solution of CEFI (16, 20 and 24 µg/mL) and ORNI (40, 50 and 60 µg/mL) were added to prequantified solution of CEFI (20 µg/mL) and ORNI (50 µg/mL). Each solution was injected in triplicate and recovery was calculated from regression equation of calibration curve by measuring peak areas.

RESULT AND DISCUSSION

Method Development

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs

that are to be detected. Cefixime trihydrate has λmax 287 nm and Ornidazole has λmax 312 nm. Both drugs are present in market formulation in 2:5 ratios. Chromatogram at different wavelength was taken, in that at 310 nm, both drugs give good peak height and shape. Also at 310 nm, all the degradation products were detected clearly. So, 310 nm was selected for simultaneous estimation of Cefixime trihydrate and Ornidazole in tablet dosage forms. Here ACE C_{18}, 5µm, 150 x 4.6 mm i.d. column used. The mobile phase was Acetonitrile: KH_2PO_4 buffer (20:80) and buffer pH 5.5 adjusted using orthophosphoric acid after adding 0.1% triethylamine, at a flow rate of 0.8 ml/min. Finally the Retention time of CEFI at 3.05min and ORNI at 10.75min with good resolution and theoretical plates observed.

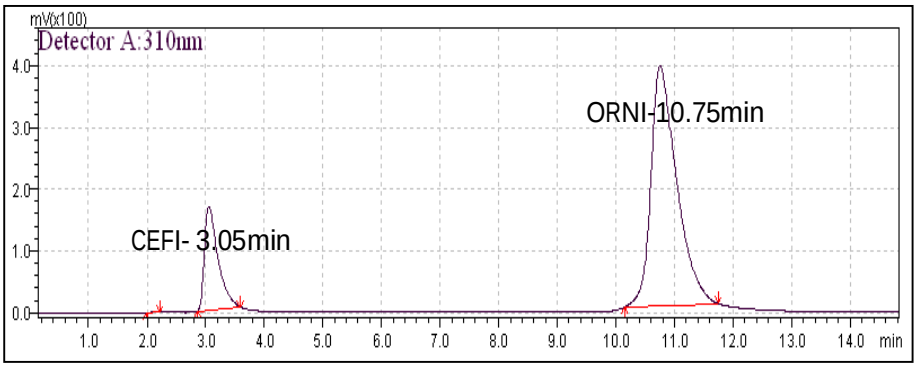

Figure 2: It shows Chromatogram of CEFI (100µg/mL) and ORNI (250µg/mL) in Standard mixture

Forced degradation studies

Forced degradation studies were established by subjecting samples of CEFI and ORNI standard solutions to degradation in HCl, NaOH and H_2O_2 and also in neutral, photolytic and Thermal condition degradation samples were analyzed using the proposed method. Minor degradations of CEFI (~13.7%) and ORNI (~7.36%) were observed under acidic conditions at 60°C for 1.5hr. Small degradation product peaks were observed as shown in Figure 3. All acidic degradation products were chromatographically resolved from target analytes.

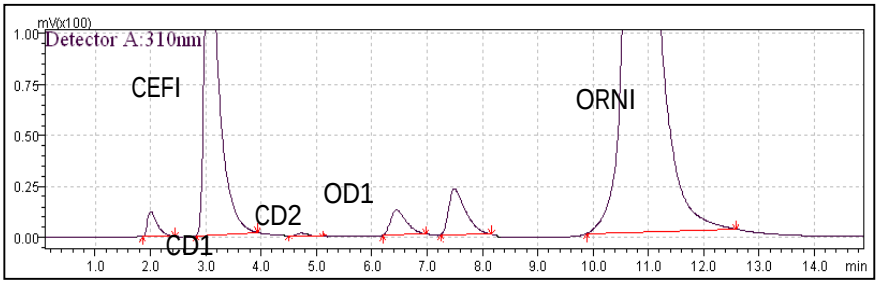

Figure 3: It shows Chromatogram of CEFI and ORNI degradation in 1N HCl (60°C).

ORNI peaks showed approximately 17.1% degradation under alkaline condition at room temperature for 10min. The degradation product is shown in Figure 4. The main alkaline degradation products was eluted at 7.5 min. CEFI also liable to degradation in alkaline condition but in this mild condition it was not degraded bcoz according to ICH guideline 10-30% degradation of each drug was optimized for SIAM.

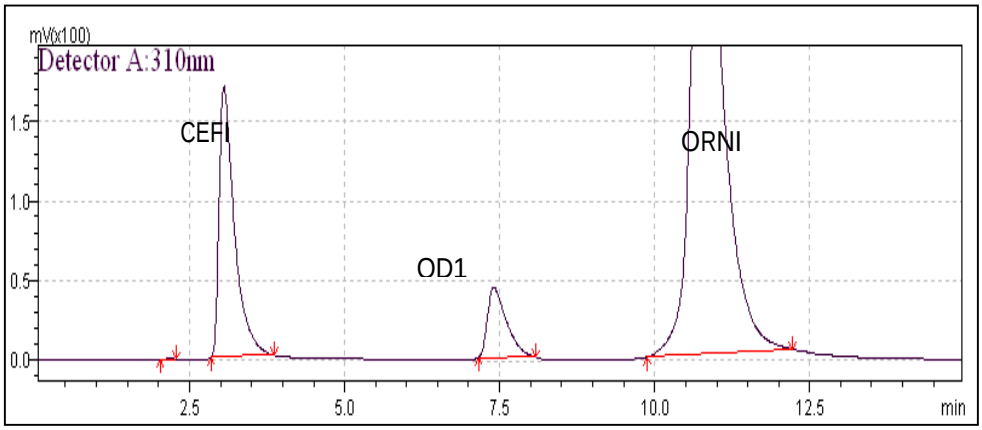

Figure 4: It shows Chromatogram of CEFI and ORNI degradation in 0.001N NaOH at room temperature.

CEFI peaks showed approximately 13.7% degradation under Oxidative condition (3% H_2O_2) at room temperature for 1.5hr. The degradation product is shown in Figure 5. The main oxidative degradation product was eluted at 2.6 min. ORNI are stable in this mild condition it was not degraded bcoz according to ICH guideline 10-30% degradation of each drug was optimized for SIAM.

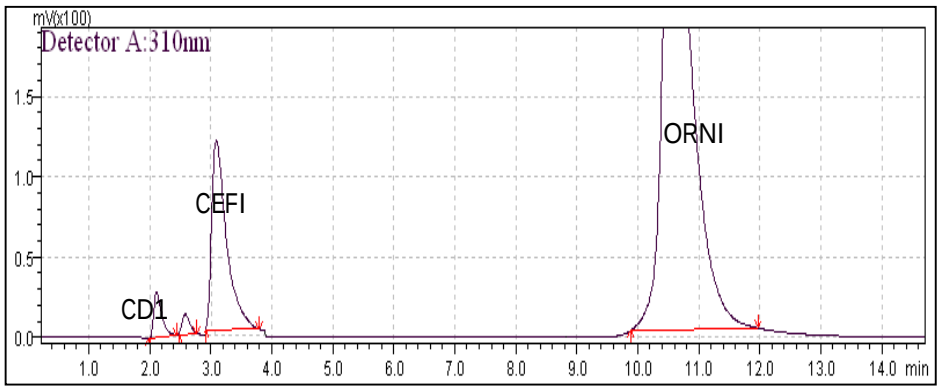

Figure 5: It shows Chromatogram of CEFI and ORNI degradation in 3%H_2O_2 at room temperature.

CEFI peaks showed approximately ~7.15% degradation under Neutral condition at 60°C for 24hr. The degradation product is shown in Figure 6. The neutral degradation product was eluted at 2.5 min. ORNI was stable in this neutral condition.

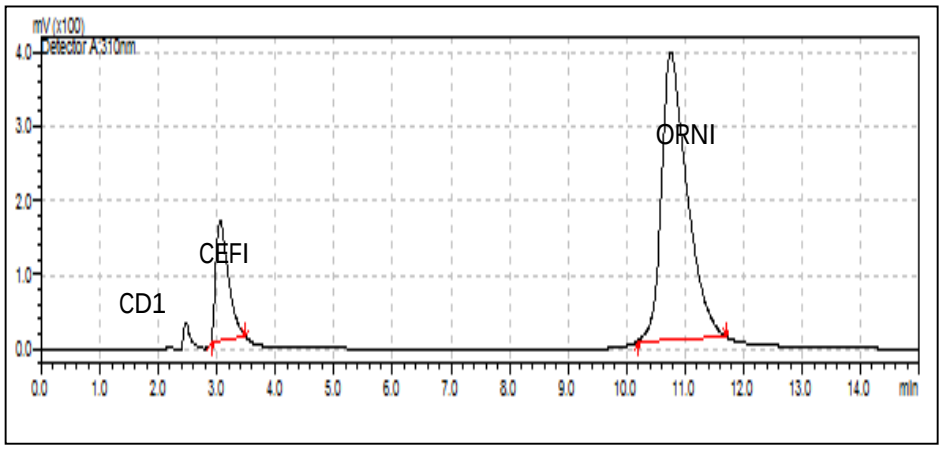

Figure 6: It shows Chromatogram of CEFI and ORNI degradation In neutral at (60°C).

CEFI peaks showed approximately 2.85% degradation under photolytic degradation at UV 254 nm for 10days. The degradation product is shown in Figure 7. The major degradation product was eluted at 2.6 min. ORNI are stable.

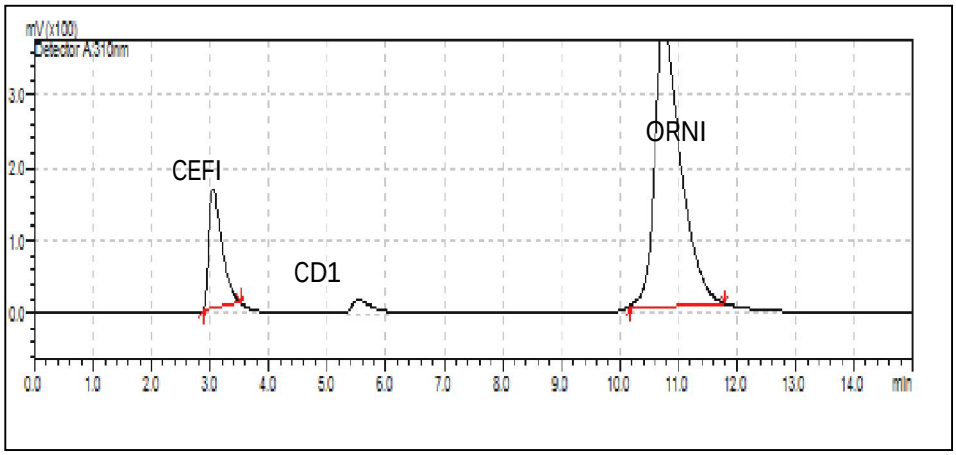

Figure 7: It shows Chromatogram of CEFI and ORNI degradation In photolytic at UV 254nm.

METHOD VALIDATION

The developed method was validated according to the ICH guidelines [21], for the following parameters: system suitability, specificity, linearity, precision, accuracy and LOD/LOQ.

System suitability

System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were the chromatographic peak resolution, theoretical plate number and tailing factor. The repeatability of these parameters was checked by injecting six solution of CEFI and ORNI are shown in Table 1.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The stress studies were performed for CEFI and ORNI bulk drugs to provide indication of the stability indicating property of the method and specificity of the proposed method. Intentional degradation was attempted to stress condition of acidic, basic and neutral hydrolysis, oxidation, dry heat (thermal) and UV light to evaluate the ability of the proposed method to separate CEFI and ORNI from its degradation products. Peak purity test was carried out for the CEFI and ORNI peak by using PDA detector in all stress samples. Peak purity has shown in Table 2.

Linearity and Range

The linearity of CEFI was found to be in the range of 10-60 µg/mL with correlation coefficient 0.998 and the linearity of ORNI was found to be in the range of 25-125 µg/mL with correlation coefficient 0.997 as shown in Table 3 & 4.

Precision

The % RSD for repeatability of CEF and ORNI were found to be 0.7 and 0.53,

Respectively which has shown in Table 5.The % RSD for Intermediate Precision was found to be in the range of 0.54-0.92 for CEF and 0.41-0.84 for ORNI, respectively, which indicates the method is precise. Data are shown in Table 6.

Accuracy

The accuracy study was carried out by the standard addition method. The percent Recovery was found in the range of 98.75 -101.0 % and 99.40-100.34 % for CEFI and ORNI, respectively and this indicates accuracy of the method. Data are shown in Table 7.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The Limit of detection (LOD) was found to be 0.047 and 0.209 µg/mL; while the Limit of quantification (LOQ) was found to be 0.14 and 0.63 µg/mL for CEFI and ORNI, respectively.LOD and LOQ data are shown in Table 8.

Robustness

The typical variations studied under this parameter were flow rate, mobile phase composition, Temperature and pH. Overall %RSD was found to be in range of 0.21-0.48 and 0.18-0.21 for CEFI and ORNI respectively, which was below 2% which indicates that the proposed method is robust. Robustness data were shown in Table 9.

Analysis of marketed formulation (Ornicef) by proposed method

Applicability of the proposed method was tested by analyzing the commercially available marketed formulation (**Ornicef).** The percentage of CEFI and ORNI were obtained from the calibration curve. Results as % Assay were shown in Table 10

CONCLUSIONS

In this work, specific, Precise, accurate and stability-indicating HPLC method for the determination of CEFI and ORNI in the presence of degradation products was developed and validated. The stability of CEFI and ORNI under various stress conditions were investigated using a forced degradation study. All of the degradation products were well resolved from the target analytes demonstrates the stability-indicating power of the method. The information presented in this study could be used for quality control studies of pharmaceutical dosage form of these combinations.

Table 1: It shows System Suitability Parameters

Parameter	CEFI	ORNI
Retention. Time	3.052 ±0.1	10.752 ±0.15
Tailing Factor	1.827 ±0.03	1.496 ±0.01
Resolution	2.22 ±0.53	12.57 ±0.32
Theoretical Plate	2112.012 ±17.03	3565.123 ±21.54

Table 2: It shows Summary of Peak purity

Stress Condition	Cefixime			Ornidazole		
	Impurity	Peak Purity	Single point threshold	Impurity	Peak Purity	Single point threshold
Standard	Not detected	1.0	1.0	Not detected	1.0	1.0
Test	Not detected	1.0	1.0	Not detected	1.0	1.0
Acid	Not detected	0.999972	0.999937	Not detected	0.999982	0.999944
Alkaline	Not detected	0.999976	0.999952	Not detected	0.999993	0.999965
Neutral	Not detected	0.999987	0.999969	Not detected	0.999981	0.999971
Oxidative	Not detected	0.999983	0.999972	Not detected	0.999992	0.999953
Thermal	Not detected	0.999967	0.999952	Not detected	0.999985	0.999964
Photolytic	Not detected	0.999985	0.999975	Not detected	0.999977	0.999938

Table 3: It shows Linearity Data of CEFI and ORNI

SR. No.	CEFI			ORNI		
	Conc. (µg/mL)	Peak Area Mean* ± S.D (n=3)	% RSD	Conc. (µg/mL)	Peak Area Mean* ± S.D (n=3)	% RSD
1	10	271141±379.5	0.14	25	1185690±3082.7	0.26
2	20	540062±1944.2	0.36	50	2371380±6877	0.29
3	30	793529±3015.4	0.38	75	3257071±11725.4	0.36
4	40	1021652±5006.09	0.49	100	4742762±31776.5	0.67
5	50	1335252±7610.9	0.57	125	5928452±40906.3	0.69
6	60	1606750±12211.3	0.76	150	7114142±58335.9	0.82

*Average of three determinations

Table 4: It shows Linearity results of CEFI and ORNI

Parameters	CEFI	ORNI
Linearity Range (µg/mL)	10-60	25-150
Regration line equation	y = 26548x – 1109	y = 47770x – 80000
Correlation coefficient	0.998	0.997

Table 5: It shows Repeatability Data for CEFI and ORNI

Sr.No.	CEFI (20 µg/mL)			ORNI (50 µg/mL)		
	Peak Area	**Mean* ± S.D**	**% RSD**	**Peak Area**	**Mean* ± S.D**	**% RSD**
1	540062	533605±3735.2	0.7	2271381	2297082±12176.5	0.53
2	532456			2314226		
3	536921			2296270		
4	530047			2282420		
5	530912			2295240		
6	521236			2322960		

*Average of six determinations

Table 6: It shows Intermediate Precision data for CEFI and ORNI

Drug	Conc (µg/mL)	Inter-day Precision	
		Mean* ± S.D	**% RSD**
CEF	20	525618 ±2838.34	0.54
	30	789489 ±6236.96	0.79
	40	1051430 ±9673.15	0.92
ORNI	50	2290665±9552.07	0.41
	75	3491762±22172.69	0.63
	100	4700821±39486.89	0.84

*Average of three determinations

Table 7: It shows Results of Accuracy study for CEF and ORNI

Amount sample taken (µg/mL)		Amount std* added (µg/mL)		Amount found* (µg/mL)		% Recovery ± %RSD	
CEFI	ORNI	CEFI	ORNI	CEFI	ORNI	CEFI	ORNI
20	50	16	40	15.83	39.76	98.93 ± 0.14	99.40 ± 0.27
20	50	20	50	19.75	50.17	98.75 ± 0.32	100.34 ± 0.41
20	50	24	60	24.24	59.41	101.0 ± 0.47	99.0 ± 0.57

*Average of three determinations

Table 8: It shows LOD and LOQ Data

Parameters	CEFI (µg/mL)	ORNI (µg/mL)
LOD	0.047	0.209
LOQ	0.14	0.63

Table 9: It shows Robustness data of CEFI and ORNI

Condition		CEFI		ORNI	
		% Assay	**% RSD**	**% Assay**	**% RSD**
As Such		99.95	0.37	99.91	0.29
Temp. (25°C)	23 °C	99.97	0.25	100.1	0.18
	27 °C	99.59		99.76	
Flow rate	0.7 mL/min	100.2	0.41	99.94	0.26
(0.8 mL/min)	0.9 mL/min	99.72		99.84	
Mobile phase ACN :Buffer (20:80 v/v)	18:82	100.12	0.54	100.05	0.31
	22:78	99.43		99.9	
pH	5.3	99.96	0.24	100.1	0.19
	5.7	99.85		99.89	

Table 10: It shows Analysis of marketed formulation of CEFI and ORNI

Formulation	Label claim (mg/tablet)		% Assay(Mean* ± S.D, n=6)	
	CEFI	**ORNI**	**CEFI**	**ORNI**
Ornicef	200	500	99.95	99.91

*Average of six determinations

Table 11: It shows Summary of validation parameters for Cefixime trihydrate and Ornidazole

Sr. No.	Parameters		CEFI	ORNI
1	Specificity		Specific because all degradation product well separated from main analyte peak and peak purity near to 0.999	
2	Linearity range		10-60 µg/mL	25-150 µg/mL
3	Regression line equation		Y=26548x-1109	Y=47770x+80000
4	Correlation coefficient		0.998	0.997
5	Precision (%RSD)	Repeatability	0.7	0.53
		Intermediate	0.54-0.92	0.41-0.84
6	Accuracy (% recovery)		98.93-101.0	99.0-100.34
7	Limit of Detection (µg/mL)		0.047	0.209
8	Limit of Quantitation (µg/mL)		0.14	0.63
9	Robustness (% RSD of Assay)		0.24-0.54	0.18-0.31

Table 12: Summary of stress degradation study

Sr.No.	Stress type	Stress Condition	Relative retention time	No of peaks		%Degradation			
				CEFI	ORNI	Individual		Combination	
						CEFI	ORNI	CEFI	ORNI
1	Acid hydrolysis	1 N HCl at 60°C for 1.5 hr	CD1 0.43 CD2 0.60 OD1 0.697	2	1	14.70	7.89	13.7	7.36
2	Alkali hydrolysis	0.001 N NaOH at room temperature for 10 min	OD2 0.70	-	1	-	18.4	-	17.1
3	Oxidative	3% H2O2 at room temperature for 1.5 hr	CD3 0.232	1	-	14.64	-	13.47	-
4	Neutral Hydrolysis	24 hr at 60°C	CD4 0.225	1	-	7.05	-	7.15	-
5	Thermal	At 60°C for 7 days	-	-	-	-	-	-	-
6	Photolytic	UV 254nm for 10 days	CD5 0.5161	1	-	2.98	-	2.85	-
				1	-	-	-	2.84*	

* % Degradation in formulation

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