

# Novel RP-HPLC-DAD and RP-UPLC Methods for Simultaneous Determination of Cefaclor and Methylparaben in their Dosage form and in its Impurity Cefaclor -Delta-3- Isomer



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## Abstract

Cefaclor (CFC), a new orally administered for the second generation of cephalosporin is the most effective for treatment of acute otitis media in children. So, the main task of this work is to develop and validate a novel RP-HPLC and RP-UPLC methods for simultaneous determination of cefaclor and methylparaben (MP) in their powder for oral suspension (POS) dosage form and in its impurity cefaclor-delta-3- isomer (CD<sub>3</sub>I). The chromatographic system is performed at ambient temperature using mobile phase composing of acetonitrile: methanol: 0.02M ammonium dihydrogen phosphate pH 4.7 ± 0.1 (25:10:65, v/v) on Agilent Eclipse XDB C18 column (250 mm X 4.6 mm, 5 µm particle size) at flow rate 1.0 mL/min, injection volume 20 µL for RP-HPLC and Waters CORTECS® C18 column (50 mm × 4.6 mm, 2.7 µm particle size) at flow rate 0.3 mL/min, injection volume 0.2 µL for RP-UPLC and UV detection at 265 nm. The method is linear for all analytes in the concentration range (70-700) µg/mL for CFC and (10-200) µg/mL for MP with correlation coefficients >0.999. The suggested method is validated according to ICH guidelines. Hence it is suitable for laboratory control of raw materials, bulk and finished products.

**Keywords:** Cefaclor; Methylparaben; Cefaclor -Delta-3- Isomer; RP- UPLC; RP- HPLC

## Introduction

Cefaclor (CFC) therapy is very effective for treating children aged 2-6 years affected by acute otitis media, with no significant difference related to treatment duration. Short-course treatments appear better in terms of compliance and tolerability [1]. Both cefaclor and amoxy-clav caused a significant improvement in all the signs and symptoms in the treatment of acute otitis media after 10-days treatment period. However, between-the-group comparisons showed that the lowering in most of the symptoms was significantly more in cefaclor drug than amoxicillin-clav [2]. CFC, (Figure 1a) is a semisynthetic product derived from a fermentation product follows the second generation of cephalosporin antibacterial group for oral administration. It is chemically named as (6R,7R)-7-[[[(2R)-2-Amino-2-phenylacetyl] amino]-3-chloro-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid monohydrate. It has a molecular formula of C<sub>15</sub>H<sub>14</sub>ClN<sub>3</sub>O<sub>4</sub>S, H<sub>2</sub>O and a molecular weight of 385.8. It has characters of appearance; White or slightly yellow powder and has solubility as slightly soluble in water, practically insoluble in methanol and in methylene chloride [3]. CFC is indicated for the treatment of the following types of infections caused by or likely to be caused by susceptible organisms: lower respiratory

infections, including pneumonia, bronchitis, and exacerbations of chronic bronchitis. Also, upper respiratory infections, including pharyngitis, tonsillitis and otitis media. Skin and skin structure infections. Urinary tract infections, including pyelonephritis and cystitis.

In more serious infections, otitis media, and infections caused by less susceptible organisms, the recommended dosage is 40 mg/kg/day in divided doses every 8 to 12 hours (maximum 2 g/day). For otitis media, 12 hourly administration appears equally effective [4]. MP (Figure 1b), is used as antimicrobial preservative. It was chemically named as (methyl 4-hydroxybenzoate), a molecular formula of (C<sub>8</sub>H<sub>8</sub>O<sub>3</sub>) and a molecular weight of 152.1. It has characters of appearance white or almost white, crystalline powder or colorless crystals and has characteristic of solubility as very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol [3]. Cefaclor -delta-3- isomer (CD3I), (Figure 1a) is used as antibacterial broad-spectrum drug. It was chemically named as (2R,6R,7R)- and (2S,6R,7R)-7-[[[(2R)-2-amino-2-phenylacetyl] amino]-3-chloro-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-3-ene-2-carboxylic acid (delta-3-cefaclor). It has a molecular formula of

$C_{15}H_{14}ClN_3O_4S$ , and a molecular weight of 367.8. It has characters of appearance: white powder and has solubility as slightly soluble in water [3].

HPLC method for determination of CFC is officially reported in both of British Pharmacopeia (BP) and United States Pharmacopeia (USP) [5]. It is still a limited number of analytical methods that are reported for the determination of CFC and MP individually or in combination with other drugs including high performance liquid chromatography (HPLC) [6-16], capillary electrophoresis and electrochemical methods [17-19], thin layer

chromatography (TLC) [20-22], UPLC and mass spectrometry [23], kinetics degradation and spectrophotometric [24-31], and comparative in vitro dissolution studies [32-34]. For the best of our knowledge, there is no RP-HPLC and RP-UPLC methods for simultaneous determination of cefaclor and methylparaben in their dosage form and in its impurity (cefaclor -delta-3- isomer). So, it is thought worthwhile to develop and validate a simple, precise and accurate RP-HPLC and RP-UPLC methods to resolve this binary mixture and determine cefaclor -delta-3-isomer in their dosage form.

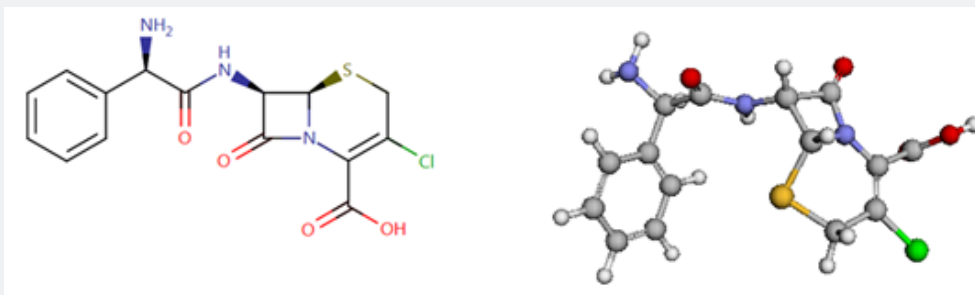


Figure 1a: Chemical structures of (a) Cefaclor & cefaclor delta-3- isomer.

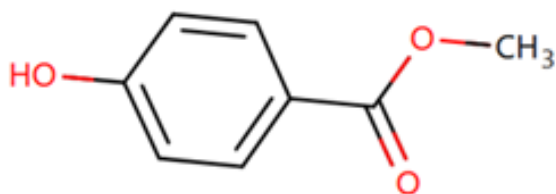


Figure 1b: Methylparaben.

## Experimental

### Materials and Reagent

CFC and MP are kindly supplied by Hikma pharmaceutical industries company, Beni-Suef, Egypt with potency >99.0%. Cefaclor-delta-3-isomer is purchased from store of USP with lot no. (R044L0). Alfatil® 125 mg/5mL "POS" (Batch No. 0004D4) is manufactured by Laboratories Ethypharm, France.

### Apparatus

Agilent 1260 infinity (Germany) HPLC system is supplied with preparative auto sampler (G1329B), Quaternary pump (G1311C), Thermostatic column compartment (G1316A), DAD detector VL (G1315D) and ChemStation (chem32) Software. Waters® UPLC System, a quaternary liquid chromatography provides plug with 2489 UV/Vis Detector (A17VTU408A), Sample manager FTN-R (D17VSM260N), Quaternary solvent manager-R (C17VQS618G) and Empower™ 3 Software. UV- 1800 double beam UV-Visible spectrophotometer (Shimadzu-Japan) with the highest resolution and spectral bandwidth of (1nm from 190-1100 nm range) is used for all absorbance measurements.

It is matched with 1 cm quartz cells. Perform data analysis by software (UV-Probe 2.3.3). METTLER TOLEDO density meter.

### Solvent Preparation

Use the mobile phase as solvent.

### Standard Solutions for Assay

**Stock standard solution (1000 µg/mL of CFC):** Carefully transfer 100 mg of CFC into 100 mL volumetric flask, add about 70 mL of the solvent. Sonicate for 10 min till complete dissolution then completes to mark with the same solvent and mix well.

**Stock standard solution (1000 µg/mL of MP):** Similarly weigh 100 mg of MP into 100 mL volumetric flask, add about 70 mL of the solvent Sonicate for 10 min till complete dissolution then completes to mark with the same solvent and mix well.

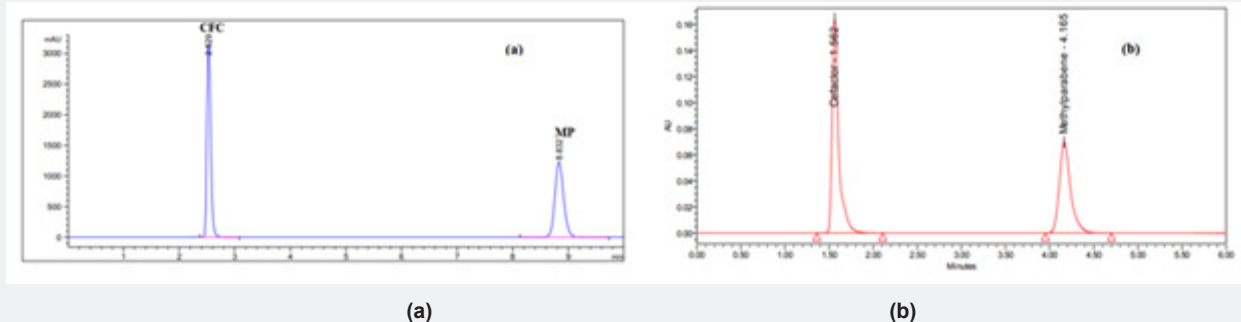
**Standard solution (625 µg/mL) of CFC and (150 µg/mL) of MP:** Accurately transfer 12.5 mL aliquot from CFC and 3mL of MP the previously prepared stock standard solutions into 20-mL volumetric flasks. Complete to mark with the same solvent. Mix

well and filter through 0.45 µm PTFE membrane filter and inject into the chromatographic system (Figure 2).

### Specific Gravity Determination

Take one bottle, add the quantity of water as mentioned in the product label. Close the bottle and shake well for 10 minutes.

Use suitable calibrated analytical balance, weigh an empty clean and dry pycnometer. Fill the pycnometer completely with water and weigh. Then repeat filling it with the reconstituted suspension and weigh and determine the specific gravity (density) according to the following equation:



**Figure 2:** Standard solution of 625 µg/mL of CFC and 150 µg/mL MP (a) HPLC chromatograms and (b) UPLC chromatograms using solvent as blank.

$$\text{Specific Gravity (Density)} = \frac{Wt_s - Wt_0}{WtW - Wt_0}$$

Where;

Wt<sub>0</sub>: Weight of empty pycnometer.

Wts: Weight of pycnometer filled with sample.

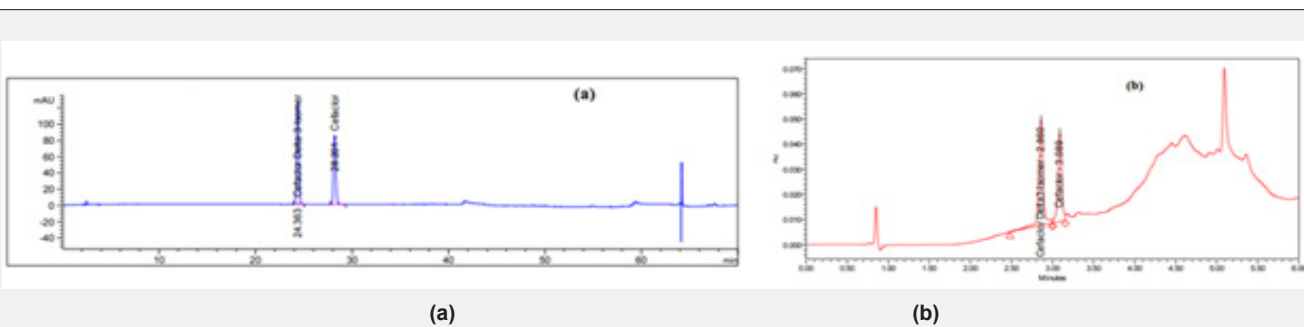
Wtw: Weight of pycnometer filled with water.

Or use the automated METTLER TOLEDO density meter.

### Pharmaceutical Preparation (Alfatil @125 mg /5 mL)

Constitute bottle of Alfatil @125 mg /5 mL POS as directed in the label and shake well for 10 minutes using multi shaker.

### Related Compounds



**Figure 3:** Standard solution of 50 µg/mL of each of CFC and CD3I (a) HPLC chromatograms and (b) UPLC.

System suitability solution: dissolve a quantity of USP cefaclor, delta-3 isomer RS in the standard solution to obtain a solution having a concentration of about 0.05 mg per mL and filter through 0.45 µm PTFE membrane filter and inject into the chromatographic system (Figure 3). Chromatograms using solvent as blank. Test solutions: constitute a bottle of Alfatil @125 mg /5 mL POS as directed in the label and shake well for 10 minutes using multi shaker. Transfer an accurately weighed portion of sample, freshly mixed and free from air bubbles,

Use the previously determined specific gravity to calculate the required weight per grams for each test as the following equation:

$$\text{Required weight for each test} = \text{Specific gravity} \times \text{volume (5 mL)}$$

Transfer an accurately weighed portion of the resulting suspension, freshly mixed and free from air bubbles, dilute quantitatively with solvent in 200-mL volumetric flask to obtain a final solution (equivalent to about 125 mg of CFC and 30 mg MP). Sonicate if necessary, to ensure complete dissolution of the CFC. Filter through 0.45 µm PTFE membrane filter and inject into the chromatographic system.

equivalent to about 50 mg of cefaclor (about 1.9066 gm), to 10-mL volumetric flasks, dilute each with solvent to volume, and mix. Sonicate briefly if necessary, avoid heating and filter through 0.45 µm PTFE membrane filter and inject into the chromatographic system (Figure 4).

### Mobile Phase Preparation

**a) Solution A:** Dissolve 2.3 g of ammonium dihydrogen phosphate in 1000 mL of water and adjust with phosphoric acid to a pH of 4.7±0.1.

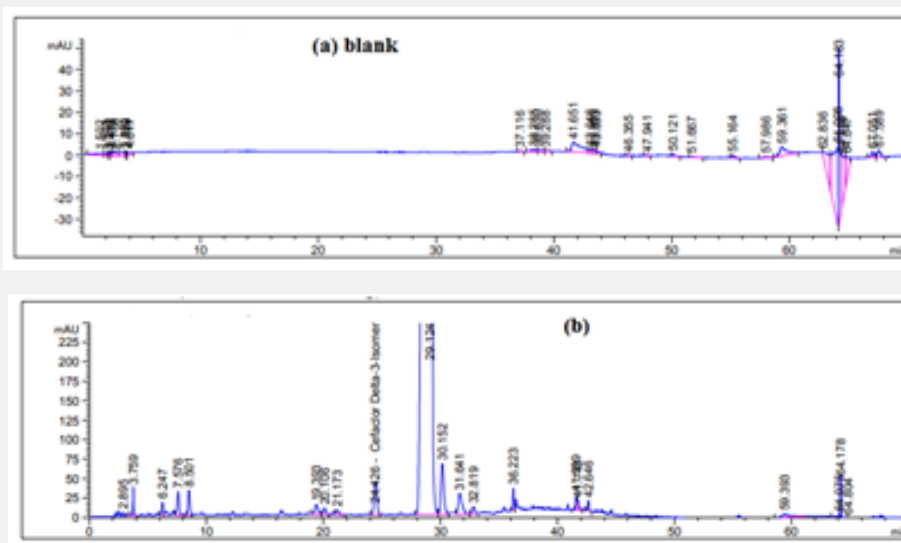


Figure 4: HPLC chromatograms of (a) blank (b) Related compound of Alfatil 125 mg POS chromatograms using solvent as blank.

Table 1: The HPLC chromatograph for related compound is programmed as follows.

Initial	Flow	Inj. Vol	Solution A	Solution B	Elution
			(%)	(%)	
0	1	20µL	90	10	equilibration
28	1	20µL	70	30	linear gradient
42	1	20µL	70	30	linear gradient
55	1	20µL	0	100	isocratic
60	1	20µL	90	10	reset composition
70	1	20µL	90	10	re-equilibration

Table 2: The UPLC chromatograph for related compound is programmed as follows.

Initial	Flow	Inj.Vol	Solution A	Solution B	Elution
			(%)	(%)	
0	0.3	0.2µL	90	10	equilibration
1.8	0.3	0.2µL	70	30	linear gradient
2.7	0.3	0.2µL	70	30	linear gradient
3.6	0.3	0.2µL	0	100	isocratic
4.4	0.3	0.2µL	90	10	reset composition
6	0.3	0.2µL	90	10	re-equilibration

b) **Solution B:** Prepare a mixture of methanol and acetonitrile (30:70), degassing for not more than 2 minutes (Tables 1 & 2).

### Construction of Calibration curves

Separately transfer different aliquots from the stock standard solutions into separate series of 10 mL volumetric flasks covering the concentration ranges (70-700) µg/mL for CFC and (10-200) µg/mL for MP. Triplicate 20 µL injections are injected for each concentration applying the recommended chromatographic conditions. The chromatograms are recorded, and the peak areas of CFC and MP are estimated, the calibration curves relating the obtained integrated peak areas to the corresponding

concentrations are constructed and the regression equations are computed.

## Results and Discussion

### Methods Development and Optimization

Many systems of different compositions and ratios are tried including organic: methanol (100%); methanol: purified water (50:50, v/v) and acetonitrile: purified water (50:50, v/v). Also (0.2 to 2.0 mL/min) flow rates are tried on HPLC and UPLC system, to gain the optimum wavelength for the solution, 20 µg/mL of CFC and 25 µg/mL of MP are measured at 200 to 400 nm (Figure 5). Thus, 265 nm was selected as the most suitable wavelength. Preliminary studies involved trying C18 reversed

phase columns. The best developing system using mobile phase composing of acetonitrile: methanol: 0.02M ammonium dihydrogen phosphate pH 4.7 ± 0.1 (25:10:65, v/v) on Agilent Eclipse XDB C18 column (250 mm X 4.6 mm, 5 µm particle size) at flow rate 1.0 mL/min, injection volume 20 µL for RP-HPLC and Waters CORTECS® C18 column (50 mm × 4.6 mm, 2.7 µm particle size) at flow rate 0.3 mL/min, injection volume 0.2 µL for RP-UPLC and UV detection at 265 nm. Another advantage for UPLC system is the relatively short run time (6min) of the analysis, under isocratic conditions. This selected developing system allows good separation with good R<sub>t</sub> values without tailing of the separated bands and good theoretical plates.

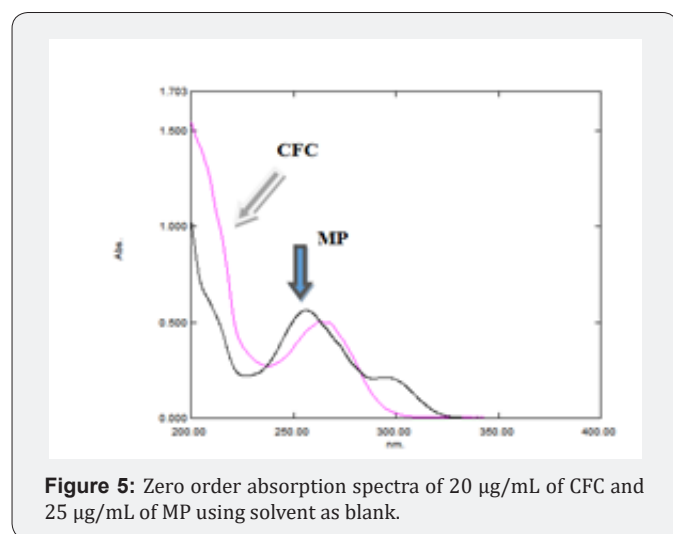


Figure 5: Zero order absorption spectra of 20 µg/mL of CFC and 25 µg/mL of MP using solvent as blank.

Accuracy and Recovery

Table 4: Data of accuracy and recovery for CFC and MP by the proposed HPLC and UPLC methods.

CFC Standard Solution (µg/ mL)	MP Standard Solution (µg/ mL)	HPLC Recovery%		UPLC Recovery%	
		CFC	MP	CFC	MP
70		98	100	98.7	99.5
		99	98	98.3	99.3
		99	100.7	99.4	100.3
100		101	98	99	100.2
		100	99	99.4	100.4
		100.3	99	99.2	100.1
170		100.5	100.6	100.2	99.8
		99.7	100.8	100.5	99.3
		99.8	99.8	100.33	99.8
Accuracy (Mean) ± RSD		99.70±0.91	99.54±1.10	99.45±0.76	99.86±0.42

The recovery is calculated for triplicate of three concentrations (70, 100 and 170 µg/mL) comparing the individual peak response with that of the reference solution. As mentioned in (Table 4).

Precision

**Repeatability:** repeatability is proceeded using 6 replicates of the standard solution of the compound being studied (625 µg/mL) for CFC and (150 µg/mL) for MP. The system is precise

Method Validation

The proposed method is validated, in accordance with ICH guidelines [35].

Linearity and Range

The linearity of the suggested methods is obtained in the concentration range (70 – 700 µg/mL) for CFC and (10 – 200 µg/mL) for MP. From the graphical presentation, the relation of peak response (A) plotted at X axis and concentration (C) plotted at Y axis is evaluated on the base of least-square linear regression equation (A= slope C + Y intercept). The LOD and LOQ of the method are calculated using the expression (3.3σ/ slope) and (10σ/slope) respectively. The obtained coefficient of regression is >0.999. Linearity results are shown in (Table 3).

Table 3: Regression and validation parameters of the proposed HPLC and UPLC methods for determination of CFC and MP.

Parameter	HPLC		UPLC	
	CFC	MP	CFC	MP
Linear				
Range (µg/mL)	70-700	10-200	70-700	10-200
Slope	2.2033	5.434	2.1976	10.6332
Intercept	3.6943	10.601	1.6449	4.0712
Correlation coefficient	0.9998	0.9998	0.9999	0.9999
Repeatability	0.12	0.21	0.08	0.33
LOD a (µg/mL)	4.31	1.6	3.8	1.2
LOQ a (µg/mL)	13.06	4.8	9.7	3.8

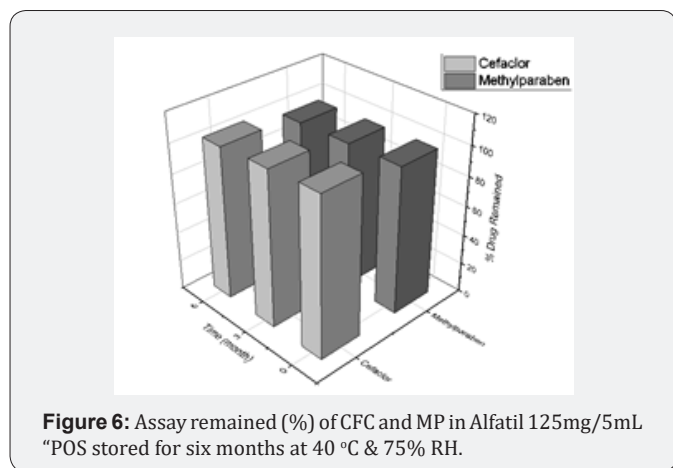
as the relative standard deviation RSD ≤ 2%. High precision of the method is obtained as shown in (Table 3). Intermediate precision (ruggedness): Intermediate precision expresses the within-laboratories variations: different days, different analysts, different equipment's, etc. Good results are obtained and presented in (Table 5).

**Robustness:** for measuring the capability of the method to remain unaffected by small, but deliberate, variations in method's parameters such as: influence of variations of flow

rate change ( $\pm 0.1$  mL/min), wave length change ( $265 \pm 2.0$  nm), column temperature change ( $25 \pm 5$  °C), and pH of the mobile phase ( $\pm 0.2$ ). Good results are gained as presented in (Table 5).

**Stability of the analytical solution:** After preparing the standard solution, part of it was stored in fridge and another one is stored at room temperature. After about 24 hours, these solutions are tested against freshly prepared standard; relative standard deviation should be less than 2%. These results are displayed in (Table 5).

**Assay of Dosage Form**



**Figure 6:** Assay remained (%) of CFC and MP in Alfatil 125mg/5mL "POS stored for six months at 40 °C & 75% RH.

The suggested method is performed by assaying six samples from the finished product with the same concentration (Alfatil@125 mg /5 mL POS) to determine the assay of CFC and MP (Figure 6). The obtained results are displayed in (Table 6).

**System Suitability**

System suitability testing is an integral part of much analytical procedure. The tests are based on the concept that the equipment's, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability is checked by calculating the tailing factor (T), column efficiency (N), resolution (Rs) factors. All calculated parameters are found to be within the acceptable limits indicating good selectivity of the method and assuring system performance (Table 7).

**Specificity**

Method selectivity is established for the finished product being studied and placebos matrix containing all excipients of the dosage form. The results referred that no interference is detected at the retention times of CFC and MP in placebo solution. Also, the standard addition technique is applied by adding known concentrations of pure form for both drugs to dosage form and the recovery of the added pure forms are calculated to check the specificity and selectivity of the method. The obtained results are shown in (Table 6).

**Table 5:** Ruggedness, robustness and stability of analytical solution of the proposed methods.

Parameter	HPLC		UPLC		Limit %
	CFC	MP	CFC	MP	
Day to Day	0.8	0.7	0.4	0.6	RSD $\leq$ 2.0%
Analyst to Analyst	0.9	1.2	0.6	0.8	
Column to Column	0.5	0.8	0.6	0.9	
Flow rate change ( $\pm 0.1$ mL/min)	0.7	0.9	0.5	0.7	
pH changes of mobile phase ( $\pm 0.2$ )	0.8	0.9	0.9	0.8	
Wave length change ( $230 \pm 2.0$ nm)	1	1.3	0.9	1	
Column temperature change (30,25 °C)	1.2	1.4	1	0.8	
Fresh Sample	0.2	0.3	0.2	0.2	
Stored Sample in fridge	0.4	0.5	0.5	0.5	
Stored Sample in room temperature	0.8	0.8	0.7	0.9	

**Table 6:** Assay results for the determination of CFC and MP in their dosage form by the proposed HPLC and UPLC methods and application of standard addition technique.

CFC Standard Solution add ( $\mu\text{g/ mL}$ )	MP Standard Solution add ( $\mu\text{g/ mL}$ )	HPLC Recovery%		UPLC Recovery%	
		CFC	MP	CFC	MP
10	10	99	98	99.5	99.3
		98	99	99.6	99.5
		98.7	99	99.3	98.6
20	20	100	99	98	99.8
		99	98	99	99.4
		100	99	99.5	99.3

30	30	100	98	100.3	100.3
		99.5	98	100.1	100.8
		99.1	98.6	100.7	100.1
Accuracy (Mean) ± RSD		99.26±0.69	98.51±0.50	99.56±0.79	99.68±0.65

**Table 7:** System suitability testing parameters of the developed methods.

Item	HPLC		UPLC		Reference values
	CFC	MP	CFC	MP	
Tailing factor	1.2	1.3	0.88	0.77	T ≤ 2
Injection precision	0.15	0.33	0.08	0.26	RSD ≤ 1%
Number of theoretical plates (N)	3300	4125	7568	5693	N > 2000
Resolution	-	4.8	5.6	6.2	Rs > 2
Retention time (R <sub>t</sub> )	0.12	0.18	0.13	0.07	RSD ≤ 1%
Selectivity	-	3.2	3.7	4	k' > 2

### Drug Remained under Accelerated Condition

Accelerated stability study intended to increase the rate of physical changes as (appearance, pH, water content...etc.) or chemical degradation (assay, dissolution, related substances and so on) by using immoderate conditions of elevated temperature and humidity. So, stability study is performed under storage conditions (40 ± 2°C / 75 ± 5 % RH (relative humidity) for six months. Products are recommended to analysis at three points (e.g. 0, 3 and 6 months). The obtained results shown in (Table 8).

**Table 8:** Accelerated stability study results for the determination of CFC and MP in their dosage form by the proposed HPLC method.

Pharmaceutical formulation Alfatil 125 POS	HPLC			Limit%
	Accelerated Stability Studies			
	0	3	6	
Cefaclor	107	105	103	90-120%
Methylparaben	99	98	97	

### Conclusion

A novel, accurate and sensitive RP-HPLC-DAD and RP-UPLC methods were validated and developed according to the requirements of ICH guidelines for the simultaneous determination of cefaclor and methylparaben in their dosage form and in its impurity (cefaclor-delta-3- isomer). Based on the above results, the analytical method is valid, achieved reduced time for analysis of assay & related compound and can be used for regular routine quality control analysis and stability study.

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