

Analysis of Drug Related Impurities by HPLC in Ciprofloxacin Hydrochloride Raw Material

Short title: Analysis of Drug Related Impurities

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Abstract

In this study, we report the quality control results of drug related impurities analysis of seven raw materials of Ciprofloxacin Hydrochloride (CPF_HCl) marketed in Algeria. According to the European Pharmacopoeia (Eur Ph), the High-Performance Liquid Chromatography (HPLC) was used to analysis (B, C, D and E) impurities while Thin-Layer Chromatography (TLC) used for control of impurity A. The HPLC analysis showed that C1, C2, C3, C4 and C6 samples have an individual content of specified impurities (B, C, D, E), unspecified and the total of all present impurities conform to norms. C5 sample contains very high content (0.579 %) of impurity C which is a photodegradation product and the impurities total (0.625 %) exceeding limit while C7 sample has a slightly higher content (0.118 %) of unspecified impurity. The control solution of impurity A was not migrated in all developed TLC plates, so the system is not compliant, for this reason, an HPLC analysis protocol was developed. The results showed that impurity A content was conform in all samples except for the C6 sample which has an equal content to the limit. Therefore, we recommend revising the detecting technique of impurity A by TLC in the Eur Ph or replacing it by a more sensitive technique such as HPLC. **Keywords:** drug related impurities, specified, HPLC, TLC, Ciprofloxacin Hydrochloride.

1. Introduction

The identification and quantification of impurities in raw materials is very important to assure effective and safe treatment. So impurities control is a key component and a big challenge in pharmaceutical industry [1,2]. Impurities relate to starting materials, by-products, breakdown products or polymorphs. They can appear at the active pharmaceutical ingredients (APIs) production level as well as during or after the formulation process. Their concentrations may change upon storage of the product [2,3].

Chemically determination of related impurities in APIs is important because a long exposure at low concentrations, can have undesirable side effects or toxicity and/or may interfere with the drug's activity [3,4]. There are no toxicity studies for the majority of impurities, so impurities analysis is a critical step of quality control [5,6]. Therefore specific requirements for impurities are set by the regulatory authorities [6,7].

Ciprofloxacin Hydrochloride (CPF_HCl) is a synthetic antibiotic that is part of the list of essential drugs established by World Health Organization, manufactured by several generic laboratories in Algeria, their high rate of prescription by clinicians thanks to their numerous indications in the different infections (gynecological, urinary, digestive and respiratory,.... etc.). CPF_HCl has several associated impurities, they are well described and defined in the European Pharmacopoeia (Eur Ph) 8th edition. The specified impurities are: A, B, C, D and E, they are individually cited and limited by a specific acceptance criterion while the impurity F is not specified that is present but limited by an overall acceptance criterion [8]. According to Eur Ph, impurities B, C, D and E are searched by High Performance Liquid Chromatography (HPLC) while impurity A by Thin-Layer Chromatography (TLC) (Table I).

In this paper, we will analysis and evaluate the drug related impurities of seven samples of Ciprofloxacin Hydrochloride APIs marketed in Algeria using HPLC.

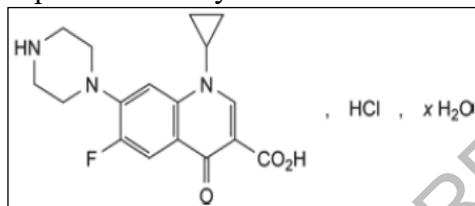
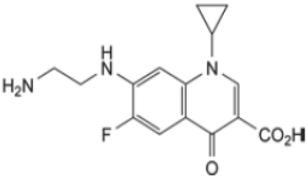
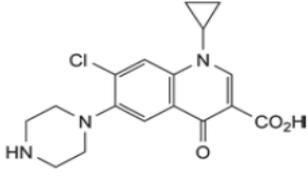
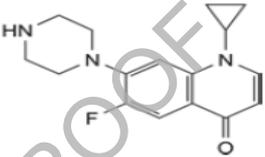
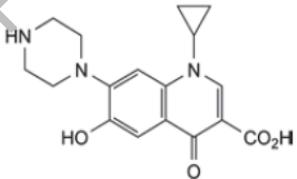


Figure 1. Chemical structure of CPF_HCl [8]

Table I. Related substances of CPF_HCl [7, 8].

Origin	Impurity	Structure	Analysis method
Synthesis by-product	Impurity A (Specified) Fluoroquinolonic acid: 7-Chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid.		TLC
Synthesis by-product	Impurity B (Specified) Defluorinated derivative: 1-Cyclopropyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.		HPLC

Photodegradation product	Impurity C (Specified) Ethylenediamine derivative: 7 - [(2-aminoethyl) amino] -1-cyclopropyl-6-fluoro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid.		HPLC
Synthesis by-product	Impurity D (Specified) 7-Chloro-1-cyclopropyl-4-oxo-6-(piperazin-1-yl) -1, 4-dihydroquinoline-3-carboxylic acid.		HPLC
Degradation product resulting from decarboxylation	Impurity E (Specified) Decarboxylic derivative: 1-Cyclopropyl-6-fluoro-7-(piperazin-1-yl) quinolin-4 (1H) -one.		HPLC
Hydroxylation product	Impurity F (Unspecified) 1-Cyclopropyl-6-hydroxy-4-oxo-7-(piperazin-1-yl) -1, 4-dihydroquinoline-3-carboxylic acid.		HPLC

2. Materials and Methods

Seven samples of CPF_HCl were collected from pharmaceutical producers installed in Algeria. They are labeled as follows: C1, C2, C3, C4, C5, C6 and C7 (**table II**).

Table II. Collection of CPF_HCl raw material from local producers

Sample	Local Producer	Batch number	Expiration Date	Manufacturer-Supplier
C1	Lab C1	A004801	04/2017	Unknown
C2	Lab C2	CIC 0074	01/2017	Baselux (Spain)
C3	Lab C3	CICA 4066	12/2019	Chemo (Swiss)
C4	Lab C4	10271610	07/2018	Dr Reddy's Laboratories (India)
C5	Lab C5	120801	08/2016	Pharmaceutical Co. LTD (China)
C6	Lab C6	KOFA0062	03/2017	Dr Reddy's Laboratories (India)

2.1. Research and quantification of impurities B, C, D and E by HPLC [8]

2.1.1. Standards, reagents and apparatus

The standard impurities "CPF_HCl for identification of SCR peaks (Containing impurities B, C, D and E)" was purchased from Eur Ph (Strasbourg, France). Acetonitrile (HPLC Grade), triethylamine and phosphoric acid produced by Sigma-Aldrich.

An HPLC-UV device (Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC systems) equipped with an automatic injector and UV detector.

2.1.2. Analysis protocol [8]

Mobile phase: 13 volumes of acetonitrile were mixed with 87 volumes of phosphoric acid at 2.45 g/L.

Test solution. 25 mg of CPF_HCl of each sample is dissolved in 50 mL of mobile phase.

Control solution (c). 1 mL of test solution was diluted in 500 mL of mobile phase.

Control solution (b). 2.5 mg of CPF_HCl for identification of SCR peaks is dissolved in 5 mL of mobile phase.

Chromatographic Conditions: temperature: 40 °C; flow: 1.5 mL/min, injection volume: 50 µL of control solution (b) and (c); detection: 278 nm; column C18:5 µm, 250× 4.6 mm.

2.2. Research of impurity A by TLC

2.2.1. Standards, reagents and apparatus

The standard "impurity A of Ciprofloxacin SCR" was purchased from Eur Ph. Acetonitrile (HPLC Grade), Ammonia, dichloromethane and methanol produced by Sigma-Aldrich. Silica gel plate F254 for TLC, chromatography tank and ultraviolet lamp at 254 nm (Cammag).

2.2.2. Procedure

Test solution. 50 mg of CPF_HCl is dissolved in 5 mL of water.

Control solution. 10 mg of impurity A standard is dissolved in mixture (0.1 mL of diluted ammonia and 90 mL of water), completed to 100 mL with water and 2 mL is diluted in 10 mL of water.

Mobile phase: acetonitrile, concentrated ammonia, methanol and methylene chloride (10:20:40:40 V/ V/ V/ V); the deposit volume: 5 µL.

Development: at the bottom of chromatography tank, a container of 50 ml concentrated ammonia is deposited. The vessel is closed and the plate is exposed to ammonia vapors for 15 min. The plate is developed on $\frac{3}{4}$; drying in air and examined under ultraviolet at 254 nm.

2.2.3. Limits

Sample is compliant, if impurity A spot isn't more intense than the main spot of control solution (0.2%)[10].

2.3. Research and quantification of impurity A by HPLC

2.3.1. Analysis protocol

Mobile phase: 50 volumes of acetonitrile were mixed with 50 volumes of phosphoric acid at 2.45 g / L.

Standard stock solution. 5 mg of standard is dissolved in 50 mL of mobile phase.

To determine the maximum absorption of impurity A, the standard solution is scanned in ultraviolet over a range of 200 to 400 nm.

Establishment of calibration curve. Five dilutions were prepared from the standard stock solution (0.1 mg/mL) (Table III).

Test solution. 50 mg of CPF_HCl is dissolved 5mL of water.

Table III. Dilution range of calibration curve.

1 st Dilution	2 nd Dilution	3 rd Dilution	4 th Dilution	5 th Dilution
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Stock solution (mL)	0,5	1	1,5	2	2,5
Solvent (mL)	9,5	9	8,5	8	7,5
Diluted solution (%)	0,05	0,10	0,15	0,20	0,25

Chromatographic conditions: temperature: 25.9 °C, flow: 1.5 ml/min, injection volume: 20 µL, detection at 260 nm, column C18: 5 µm, 150×4.6 mm.

System compliance: linearity of calibration curve with a correlation coefficient greater than 0.990. The symmetry factor of impurity (A) peak must be between 0.8 and 1.5.

Identification of impurity A: by its retention time.

2.3.2. Results expression

The impurity A content of each sample is expressed by extrapolating of its area on the calibration curve: $y = aX + b$

y: impurity (A) area, **X:** impurity (A) concentration (%)

Calculus formula of impurity A content

$$\text{Impurity A content (\%)} = \frac{(\text{Peak Area} - b)}{a} \times \frac{50}{\text{Wieght (mg)}}$$

3. Results and discussion

3.1. Research and quantification of impurities B, C, D and E by HPLC

3.1.1. System compliance

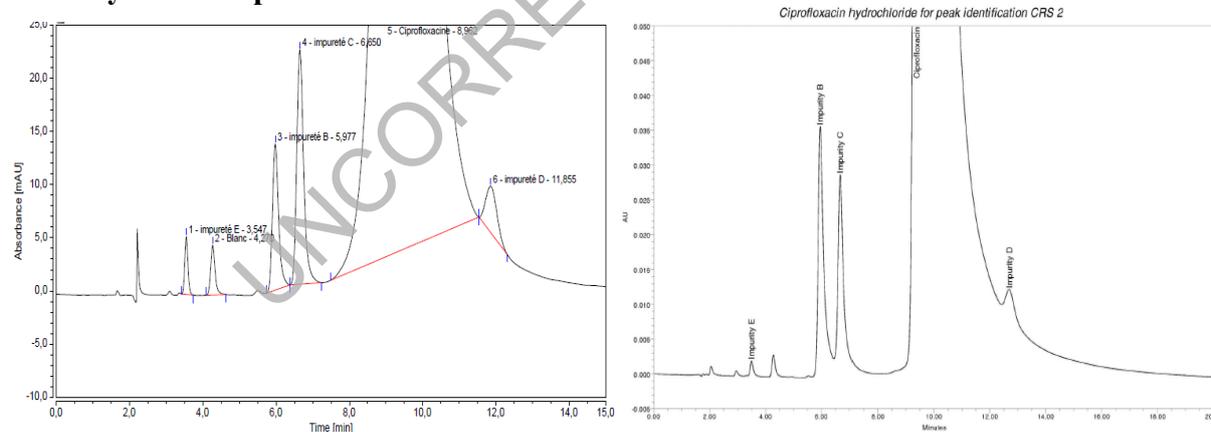


Figure 2. Chromatogram of control solution (b) **Figure 3.** Typical chromatogram. These two chromatograms were superimposable and comparable, which enabled us to identify the CPF_HCl main peak and impurity E, B, C and D peaks corresponding.

The retention time (RT) of CPF_HCl is 8.962, value close to that required by Eur Ph which must be at about 9 min. The RT obtained for each impurity (E, B, C and D) is respectively (3.547 min, 5.977 min, 6.650 min and 11.855 min). All these values are close to those given in the standard chromatogram or calculated from RRT (RT_Impurity E: 3.58 min, RT_Impurity B: 5.377 min, RT_Impurity C: 6.273 min, RT_Impurity D: 10.754 min).

The resolution between peaks of impurity B and C is 3, value complies with the required standard (at least 1,3). The symmetry factor of CPF_HCl peak is 1.4, conform to the Eur Ph standard (between 0.8 and 1.5). The symmetry factor of impurity (E, B, C and D) peaks is

(1.16, 1.31, 1.30, and 1.17). All these values are conforms. Therefore, the system compliance is validated.

3.1.2. Samples Analysis

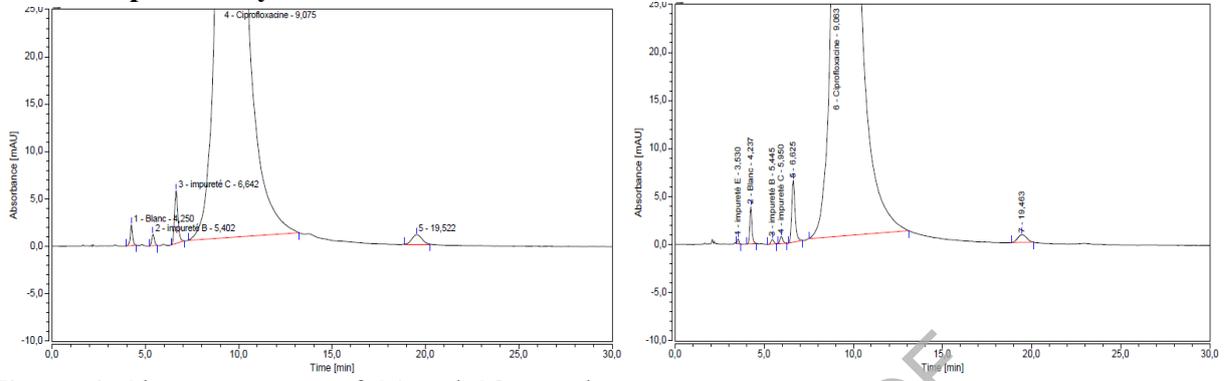


Figure 4. Chromatograms of C1 and C2 samples

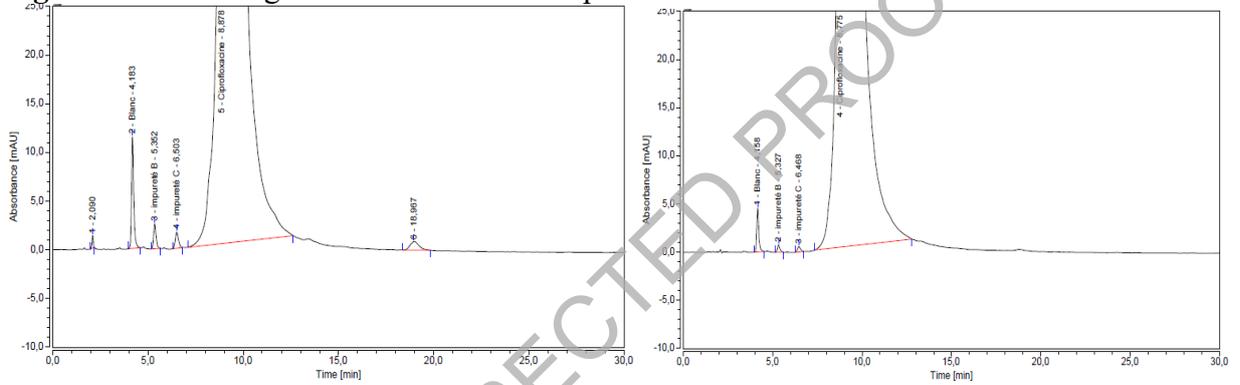


Figure 5. Chromatograms of C3 and C4 samples

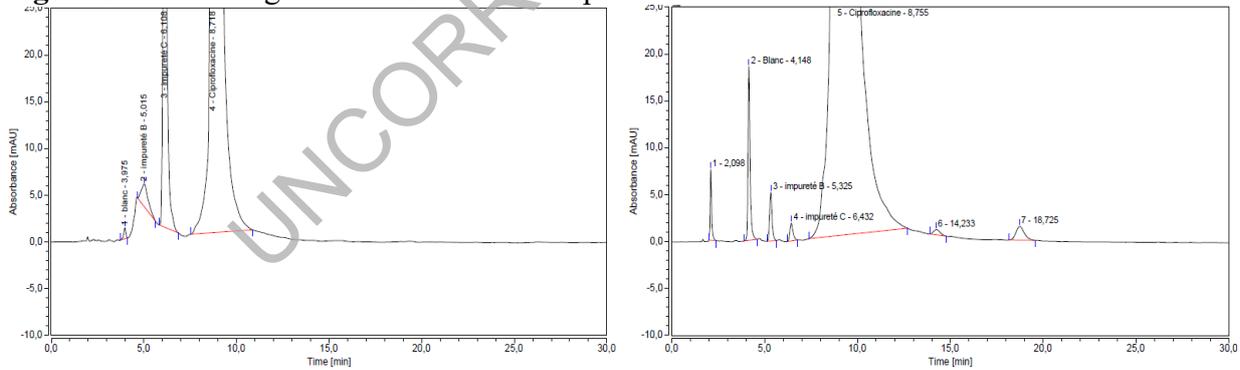


Figure 6. Chromatograms of C5 and C6 samples

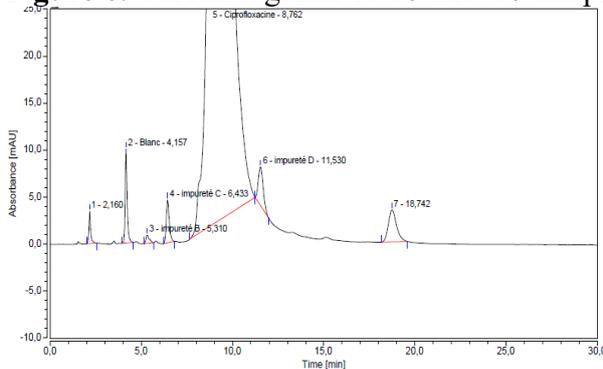


Figure 7. Chromatogram of C7 sample

Table IV. Individual content of (B, C, D, E, unspecified) impurity and the impurities total.

Ciprofloxacin Hydrochloride Sample	Impurity	Impurity Area (mAU.min)	Control (c) Area (mAU.min)	Control (c) Weight (mg)	Dilution Factor Control (c)	Control (c) Concentration (µg/mL)	Theoric Concentration of Control (c) (µg/mL)	Theoric Concentration of Control (c) (%)	Correction Factor	Real Concentration of Control (c) (%)	Individual Content of Impurity (%)	Impurities Total (%)	Norms (%)
C1	Imp B	0.202	3.375	50.5	0.00002	1.01	1	0.2	0.7	0.202	0.008	0.087	
	Imp C	1.189	3.375	50.5	0.00002	1.01	1	0.2	0.6	0.202	0.043		
	Imp D	ND	3.375	50.5	0.00002	1.01	1	0.2	1.4	0.202	ND		
	Imp E	ND	3.375	50.5	0.00002	1.01	1	0.2	6.7	0.202	ND		
	Imp Unspf 1	0.606	3.375	50.5	0.00002	1.01	1	0.2	1	0.202	0.036		
C2	Imp B	0.086	3.514	50.7	0.00002	1.014	1	0.2	0.7	0.203	0.003	0.136	
	Imp C	0.137	3.514	50.7	0.00002	1.014	1	0.2	0.6	0.203	0.005		
	Imp D	ND	3.514	50.7	0.00002	1.014	1	0.2	1.4	0.203	ND		
	Imp E	0.052	3.514	50.7	0.00002	1.014	1	0.2	6.7	0.203	0.020		
	Imp Unspf 1	1.404	3.514	50.7	0.00002	1.014	1	0.2	1	0.203	0.081		
Imp Unspf 2	0.465	3.514	50.7	0.00002	1.014	1	0.2	1	0.203	0.027			
C3	Imp B	0.411	3.190	50.2	0.00002	1.004	1	0.2	0.7	0.201	0.018	0.070	
	Imp C	0.424	3.190	50.2	0.00002	1.004	1	0.2	0.6	0.201	0.016		
	Imp D	ND	3.190	50.2	0.00002	1.004	1	0.2	1.4	0.201	ND		
	Imp E	ND	3.190	50.2	0.00002	1.004	1	0.2	6.7	0.201	ND		
	Imp Unspf 1	0.093	3.190	50.2	0.00002	1.004	1	0.2	1	0.201	0.006		
Imp Unspf 2	0.473	3.190	50.2	0.00002	1.004	1	0.2	1	0.201	0.030			
C4	Imp B	0.126	3.559	50.2	0.00002	1.004	1	0.2	0.7	0.201	0.005	0.010	Imp B ≤ 0.2 Imp C ≤ 0.2 Imp D ≤ 0.2 Imp E ≤ 0.3 Imp Unspf ≤ 0.1 Imp Total ≤ 0.5 Exculsion Limit = 0.05
	Imp C	0.153	3.559	50.2	0.00002	1.004	1	0.2	0.6	0.201	0.005		
	Imp D	ND	3.559	50.2	0.00002	1.004	1	0.2	1.4	0.201	ND		
	Imp E	ND	3.559	50.2	0.00002	1.004	1	0.2	6.7	0.201	ND		
C5	Imp B	1.038	3.190	50.2	0.00002	1.004	1	0.2	0.7	0.201	0.046	0.625	
	Imp C	15.342	3.190	50.2	0.00002	1.004	1	0.2	0.6	0.201	0.579		
	Imp D	ND	3.190	50.2	0.00002	1.004	1	0.2	1.4	0.201	ND		
	Imp E	ND	3.190	50.2	0.00002	1.004	1	0.2	6.7	0.201	ND		
C6	Imp B	0.775	3.284	50.0	0.00002	1	1	0.2	0.7	0.200	0.033	0.147	
	Imp C	0.366	3.284	50.0	0.00002	1	1	0.2	0.6	0.200	0.013		
	Imp D	ND	3.284	50.0	0.00002	1	1	0.2	1.4	0.200	ND		
	Imp E	ND	3.284	50.0	0.00002	1	1	0.2	6.7	0.200	ND		
	Imp Unspf 1	0.664	3.284	50.0	0.00002	1	1	0.2	1	0.200	0.040		
	Imp Unspf 2	0.221	3.284	50.0	0.00002	1	1	0.2	1	0.200	0.013		
Imp Unspf 3	0.763	3.284	50.0	0.00002	1	1	0.2	1	0.200	0.046			
C7	Imp B	0.189	3.071	50.5	0.00002	1.01	1	0.2	0.7	0.202	0.009	0.302	
	Imp C	0.861	3.071	50.5	0.00002	1.01	1	0.2	0.6	0.202	0.034		
	Imp D	1.287	3.071	50.5	0.00002	1.01	1	0.2	1.4	0.202	0.119		
	Imp E	ND	3.071	50.5	0.00002	1.01	1	0.2	6.7	0.202	ND		
	Imp Unspf 1	0.349	3.071	50.5	0.00002	1.01	1	0.2	1	0.202	0.023		
	Imp Unspf 2	1.790	3.071	50.5	0.00002	1.01	1	0.2	1	0.202	0.118		

Imp: impurity, Unspf: unspecified, ND: not detected

According to the Eur Ph standards, the individual content of impurity B, C and D must be less than or equal to 0.2%, impurity E, less than or equal to 0.3% and unspecified impurity less than or equal to 0.1%. Any other impurity with an individual content less than or equal to 0.05% (exclusion limit) shall not be taken into consideration. The impurities total content shall not exceed 0.5%.

C1, C2, C3, C4 and C6 samples have an individual content of specified impurity (B, C, D and E) or unspecified and the impurities total in the required standards.

C5 sample contains a very high content (0.579%) of impurity C compared to the limit, and a total (0.625%) exceeding the norm. This explains that sample has degraded in impurity C

which is a photodegradation product despite having been well preserved. This result is consistent since the sample was analyzed on date close to its expiry date (August 2016), or it degraded during handling.

C7 sample has an individual content of unspecified impurity (known structure such as impurity F or unknown structure) equal to 0.118%, slightly higher than the general acceptance criterion and a total in the norm.

3.2. Research of impurity A by TLC

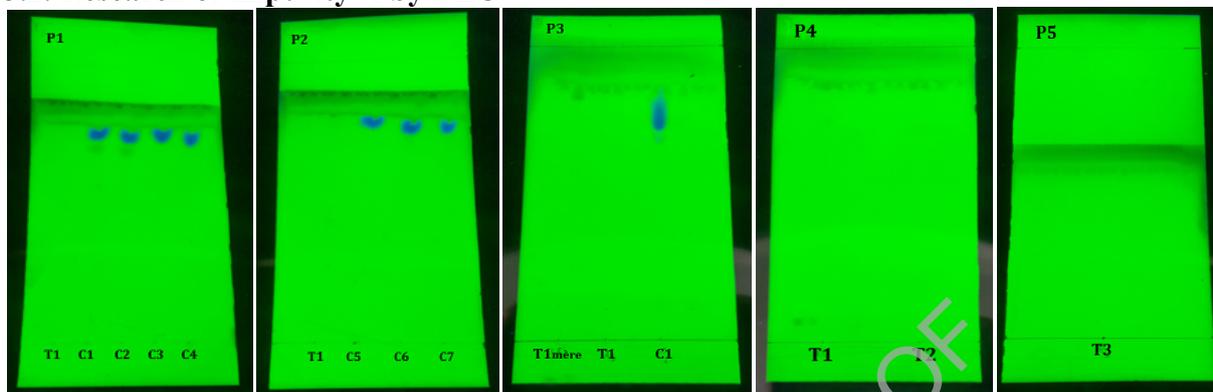


Figure 8. TLC plates revelation under the UV lamp

T1: 1st control solution (0.02 mg/mL) prepared from the first vial

T1 stock: 1st stock control solution (0.1 mg/mL) prepared from the first vial.

T2: 2nd control solution (0.02 mg/mL) prepared from the first vial.

T3: 3rd control solution (0.02 mg/mL) prepared from a second standard vial.

The first plate revealed four main spots corresponding to test solution of C1, C2, C3 and C4 samples and no spot of the control solution appeared. The second plate revealed three main spots corresponding to test solution of C5, C6 and C7 samples and no spot of control solution appeared. Due to the absence of control migration, a third plate is prepared in which the stock control solution (0.1 mg/mL) is deposited but still has not been migrated.

The TLC was re-tested several times while using:

- New reagents to prepare the mobile phase (plate 3);
- New plates silica gel F254 for TLC (plate 3);
- Second control solution prepared from the first vial (plate 4);
- Third control solution prepared from a second vial of impurity standard (plate 5).

The control was not migrated in all developed TLC plates, so the system is not compliant. For this reason, an HPLC analysis protocol for impurity A was developed.

3.3. Research and quantification of impurity A by HPLC

3.3.1. Maximum absorption of the standard solution

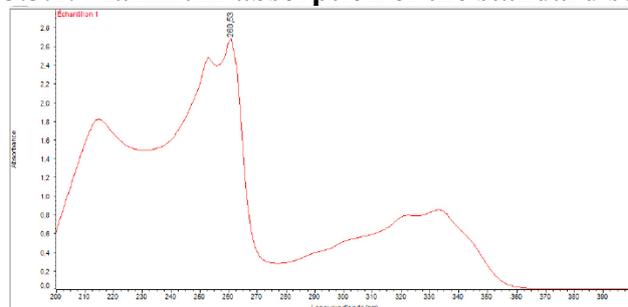


Figure 9. Absorption spectrum of impurity A in ultraviolet

3.3.2. System compliance

The chromatograms obtained with the various standard solutions of the calibration range are shown below.

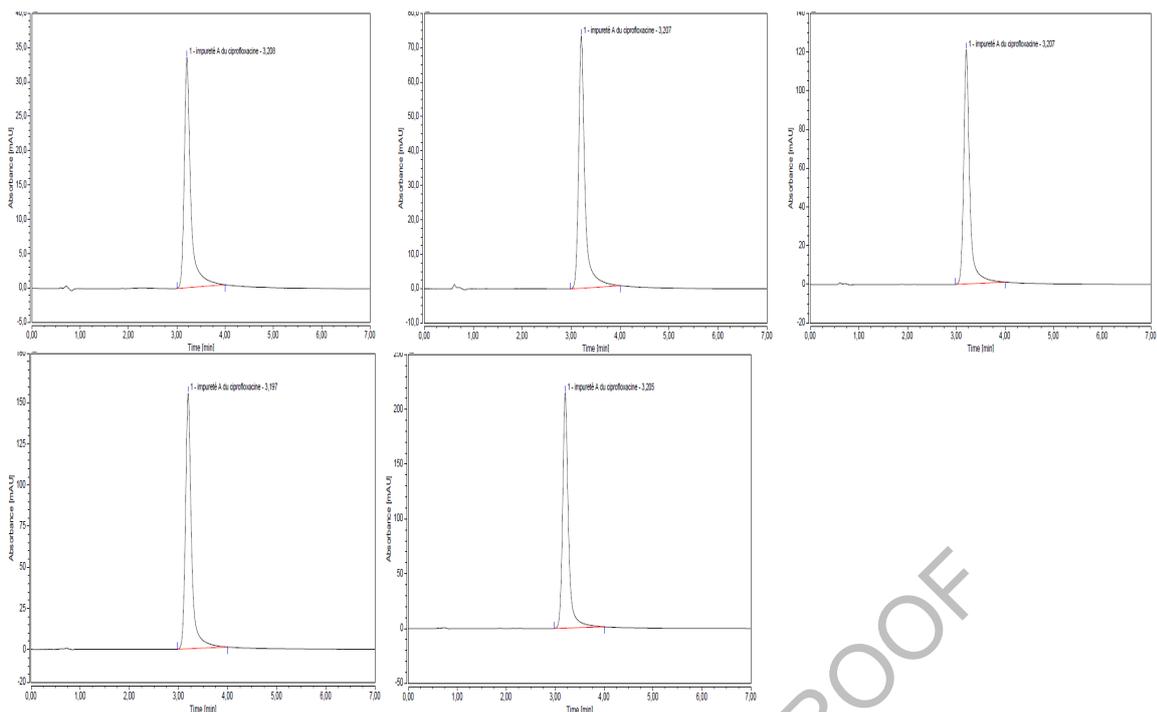


Figure 10. Chromatograms of standard solution at 0.05%, 0.1%, 0.15%, 0.2% and 0.25% respectively

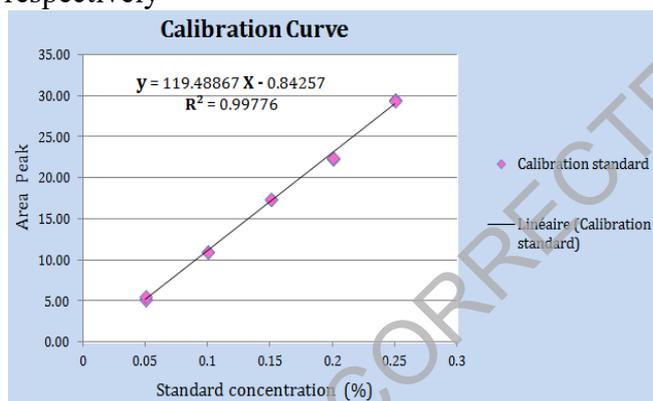


Figure 11. Calibration curve of standard Solution

The maximum absorption of impurity A is 260 nm and its retention time is 3,208 min. The symmetry factor of the impurity A peak is 1.40, conform to the norm. Correlation coefficient of calibration curve is 0.997, which shows that the curve linearity is validated, so the system is compliant.

3.3.3. Samples analysis

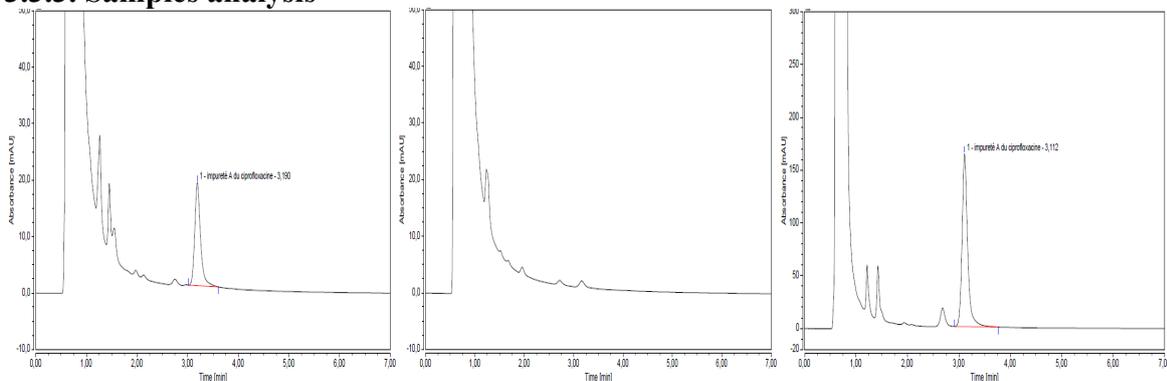


Figure 12. Chromatogram of C1, C2 and C3 samples

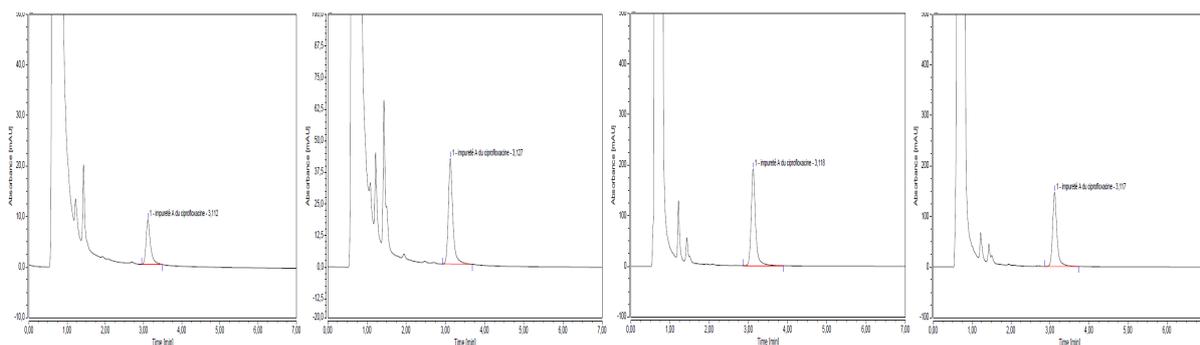


Figure 13. Chromatogram of C4, C5, C6 and C7 samples

Table V. Individual content of impurity A

Ciprofloxacin Hydrochloride Sample	Injection Number	Weight (mg)	Peak Area	a	b	Impurity A Content (%)	Average (%)	Norm (%)
C1	1	50.15	2.412	119.4887	0.84257	0.01	0.01	Impurity A < 0.2
	2	50.15	2.440	119.4887	0.84257	0.01		
C2	1	50.20	ND	119.4887	0.84257	ND	ND	
	2	50.20	ND	119.4887	0.84257	ND		
C3	1	50.00	20.457	119.4887	0.84257	0.16	0.16	
	2	50.00	20.471	119.4887	0.84257	0.16		
C4	1	50.10	1.536	119.4887	0.84257	0.01	0.01	
	2	50.10	1.529	119.4887	0.84257	0.01		
C5	1	50.25	5.496	119.4887	0.84257	0.04	0.04	
	2	50.25	5.460	119.4887	0.84257	0.04		
C6	1	50.20	24.431	119.4887	0.84257	0.20	0.20	
	2	50.20	24.516	119.4887	0.84257	0.20		
C7	1	50.20	18.704	119.4887	0.84257	0.15	0.15	
	2	50.20	18.772	119.4887	0.84257	0.15		

According to Eur Ph, the individual content of impurity A must be less than 0.2%.

Impurity A was not detected in C2 sample, while C1, C3, C4, C5 and C7 samples had a content conform but the C6 sample had a content equal to the limit.

4. Conclusion

The specified and unspecified impurities (A, B, C, D, E) were precisely determined in seven samples of CPF_HCl by HPLC. The C1, C2, C3, C4 and C6 samples have an individual content of specified impurities (B, C, D, E), unspecified and the total of all present impurities conform to norms. C5 sample contains a very high content of impurity C which is a photodegradation product and the impurities total exceeding limit while C7 sample has a slightly higher content of unspecified impurity. The impurity A content is conform in all samples except for the C6 sample which has an equal content to the limit. According to the detecting technique of impurity A by TLC in the Eur Ph, the control solution was not migrated, so we recommend revising this method or replacing it by a more sensitive technique such as HPLC.

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