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Identification, Isolation and Characterization of New Process-related Impurities in Levofloxacin

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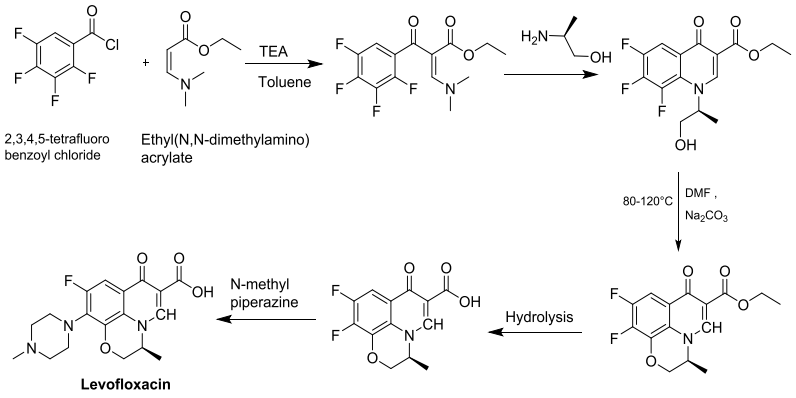
ABSTRACT

Three new process-related impurities were detected in levofloxacin sample by reversed-phase liquid chromatographic analysis method described in USP. Based on chromatographic elution order impurities were marked as Imp-1, Imp-2 and Imp-3. Impurities were isolated from Mother Liquor (ML) collected during various stages by using flash chromatographic method. These impurities were well characterized by Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) and Nuclear Magnetic Resonance (NMR). Mechanism of their formation is also discussed in this communication.

Keywords: Levofloxacin, Process-related impurities, Identification, HPLC

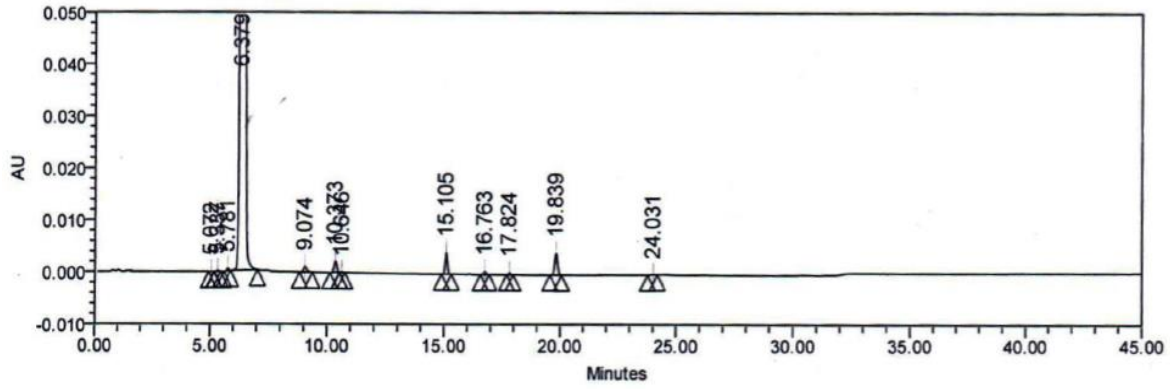
INTRODUCTION

Levofloxacin, (−)-(S)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7Hpyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid), an oral fluoroquinolones antibacterial agent, is the optical S-(-) isomer of ofloxacin. *In vitro* it is generally twice as potent as ofloxacin. Levofloxacin is active against most aerobic Gram-positive and Gram-negative organisms and demonstrates moderate activity against anaerobes (Davis et al.). Several methods have been reported for the synthesis of levofloxacin [1-4]. On the basis of literature survey, cost-effectiveness, overall good yield and less impurity formation we have designed the route of synthesis as shown in Scheme 1.



Scheme 1: Route of synthesis of levofloxacin

During the synthesis and process optimization, process related impurities were critically monitored on USP described High Performance Liquid Chromatography (HPLC) methods, both procedure 1 and procedure 2 [5]. Three new impurities: Imp-1 (RT=10.37 min), Imp-2 (RT=15.10 min) and Imp-3 (RT=19.83 min) were detected on USP-procedure 2 method. These impurities levels were in the range of 0.1 to 0.3% (Figure 1).



Peak Results

	Name	RT	Area (µV*sec)	% Area	Height
1		5.072	308	0.00	55
2		5.337	1743	0.02	273
3		5.781	4008	0.04	611
4	Leofloxacin hemihydrate	6.379	11225141	99.07	1381659
5		9.074	11451	0.10	1150
6	unknown imp. 1	10.373	17687	0.16	2140
7		10.646	787	0.01	115
8	unknown imp. 2	15.105	26009	0.23	4121
9		16.763	4525	0.04	623
10		17.824	2162	0.02	387
11	unknown imp. 3	19.839	36546	0.32	4185
12		24.031	593	0.01	46
Sum			11330959.0	100.0	

Figure 1: HPLC chromatogram of levofloxacin sample showing three new impurities

As per guidelines recommended by International Conference on Harmonisation (ICH) on impurities in drug substances, any impurity which is present at level > 0.1% should be identified when maximum daily dose is ≤ 2 g/day [6]. Therefore an LC-MS method was developed to identify these unknown impurities. Further confirmation of the proposed impurities was done by NMR after their isolation by flash chromatography. Investigation was done to know the source of formation of these impurities. The present work describes the identification, isolation and characterization of three new unknown process related impurities formed in levofloxacin. Mechanism of formation of these impurities is also discussed in this paper.

EXPERIMENTAL

Materials and reagents

Samples of levofloxacin, intermediates and process related impurities were obtained from Center for Research & Development, Ipca Laboratories Ltd. (Mumbai, India). HPLC grade acetonitrile was purchased from Merck India Limited (Mumbai, India). De-ionized water was prepared using MilliQ plus purification system (Millipore, Bradford, USA). Deuterated dimethylsulfoxide and D_2O were purchased from Merck KGaA, Darmstadt, Germany.

HPLC analysis

Samples were analyzed on Waters Alliance 2690 HPLC system (Milford, MA, USA) equipped with Waters 2487 UV detector. Column YMC-ODS-A (4 mm × 15 cm; 3.0 µm packing L1) was used for chromatographic separation. The mobile phase consisting of Solution A (Acetonitrile: Buffer; 16: 84) and Solution B (Acetonitrile: Methanol: Buffer; 30: 20: 50) with timed gradient programme T (min)/A (%): 0/100, 5/100, 10/82, 15/40, 30/40, 30.1/100, 38/100 with flow rate of 1.0 ml/min was used. The injection volume was 10 µl and the detector wavelength was fixed at 280 nm. Buffer: Dissolve 3.08 g/l ammonium acetate and 8.43 g/l sodium perchlorate monohydrate in water and adjusted pH to 2.2 with phosphoric acid.

LC-MS instrumentation and methods

The liquid chromatography-heated electrospray ionization-tandem mass spectrometry (LCHESI- MS/MS) analysis was carried out on Q Exactive (Thermo Scientific, Waltham, Massachusetts, United States) Orbitrap Mass Spectrometer. The LC unit was consisted of an Ultimate 3000 quaternary gradient pump with a degasser and auto sampler. The chromatographic condition is almost same as mentioned above, only difference is in the buffer, here buffer is water with pH 2.2 adjusted by Trifluoroacetic Acid (TFA). The spray voltage was kept at 4.0 kV and capillary temperature at 320°C. Nitrogen was used as both sheath and auxiliary gas. Mass range was kept at m/z 100-1000. MS/MS studies were carried out by maintaining normalized collision energy at about 25% with the mass range m/z 150-1000.

NMR spectroscopy

The ^{1}H, ^{13}C and DEPT measurements were recorded on a Bruker AVANCE 400 NMR spectrometer (Fallanden, Switzerland) instrument at 300 K. The exchangeable protons were identified by D_2O exchange experiment. The phase sensitive double quantum filtered correlation spectroscopy (DQF-COSY), Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) was also performed using the same instrument. The ^{1}H and ^{13}C chemical shift values were reported on the δ scale in ppm relative to dimethyl sulfoxide (2.49 ppm) and (39.5 ppm) respectively.

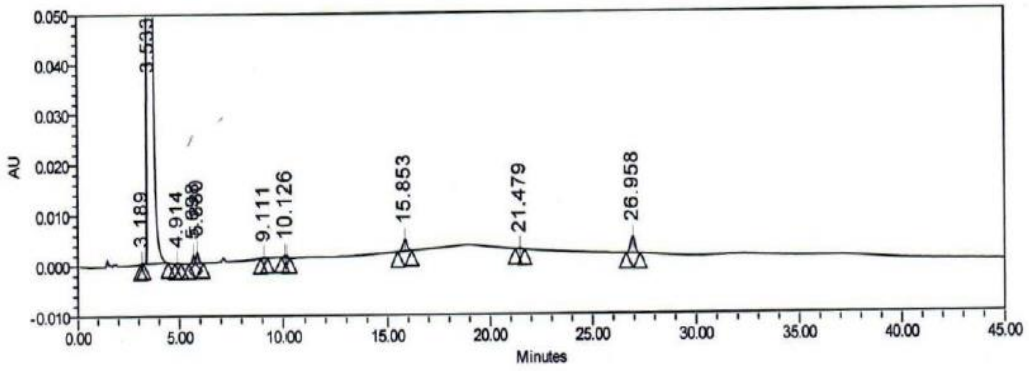
Flash chromatography

Flash chromatography instrument COMBIFLASH RF 200i (Teledyne ISCO, NE, USA) at wavelength 280 nm (UV absorbance). Column used for the separation was RediSep Rf Normal Phase Flash Columns, 40-60 μ. Solvent mixture used for the separation was MDC/MeOH (95:5) and flow rate was kept at 30 ml/min.

RESULTS AND DISCUSSION

Identification of unknown impurities

HPLC analysis of Levofloxacin API using chromatographic method showed three unknown impurities in the level of 0.1-0.3%. Impurities were marked as Imp-1 (RT=10.37 min), Imp-2 (RT=15.10 min) and Imp-3 (RT= 19.83 min) along with levofloxacin peak (RT=6.37 min) as depicted in typical chromatogram (Figure 1). HPLC analysis of same sample on LC-MS compatible method shown these impurities at retention time 5.88 (Imp-1), 15.85 (Imp-2), 26.95 (Imp-3) respectively along with Levofloxacin peak at RT=3.53 (Figure 2).



Peak Results

	RT	Area (μV*sec)	% Area	Height	USP Plate Count	USP Tailing	USP Resolution	
1	3.189	1274	0.01	291	10941	0.86		
2	3.533	14291083	99.15	1565365	3291	1.55	1.85	
3	4.914	1502	0.01	216	11613	1.20	6.33	
4	5.698	13135	0.09	1686	8497			
5	5.880	18962	0.13	2265	10246			← imp. 1
6	9.11	1530	0.01	147	16024	0.89	11.92	
7	10.126	5225	0.04	480	18141	0.93	3.43	
8	15.853	32866	0.23	2310	28272	0.94	16.73	← imp. 2
9	21.479	2369	0.02	220	91635	1.27	16.71	
10	26.958	45742	0.32	3293	84902	0.99	16.47	← imp. 3
Sum		14413686.6	100.0					

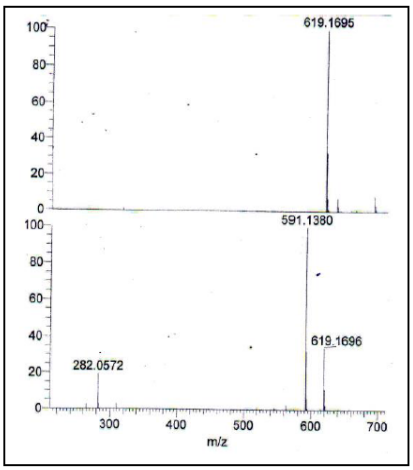
Figure 2: HPLC chromatogram of levofloxacin in LC-MS compatible method

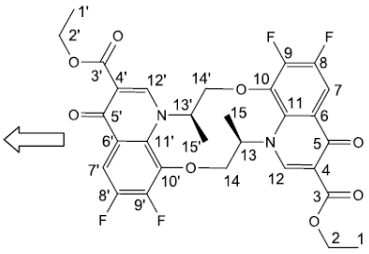
LC-MS analysis of the sample revealed that the levofloxacin peak (RT=3.53), Imp-1 (RT=5.80), Imp-2 (15.85) and Imp-3 (26.95) has a protonated molecular weight of 362.1505, 619.1695, 316.1289 and 355.1466 respectively. MS/MS analysis of Imp-1 gave two major fragments at m/z 591.1380 and 282.0572 (Figure 3a). MS/MS analysis of Imp-2 gave one major fragment at m/z 298.1183 (Figure 3b). While that of Imp-3 shown major fragments at m/z 327.1150 and 309.1045 (Figure 3c). On the basis of mass spectral data the structures of all three impurities were proposed as shown in Figures 3a-3c. The structures of these impurities were verified by NMR analysis after their isolation by flash chromatography.

Isolation of impurities by flash chromatography

Impurities were isolated from Mother Liquor (ML) collected during various stages by using flash chromatographic method as described above. Higher concentration of Imp-3 and Imp-1 were present in stage (IV) ML, while Imp-2 was isolated from the ML obtained at final stage of Levo. Purification of intermediate (IV) was carried out in two steps. First is acetone slurry treatment given to crude intermediate (IV) and ML obtained after filtration of slurry was subjected to flash chromatography to get Imp-3.

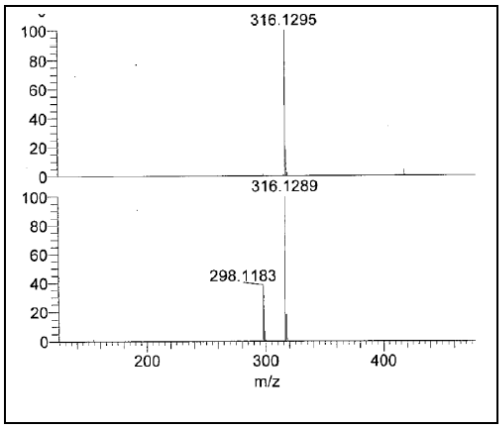
Second step is crystallization of above obtained intermediate (IV) in DMF followed by filtration. Obtained ML was used for the isolation of Imp-1. Similarly at last stage crystallization of levofloxacin was carried out in ethanol and the ML obtained after filtration was subjected to flash chromatography for the isolation of Imp-2. Impurities isolated were found to have HPLC purity > 95%. These impurities were further used for structure elucidation without any further purification.

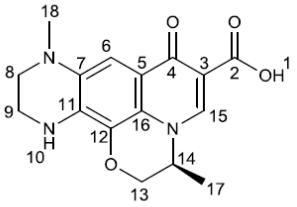




Imp.-1

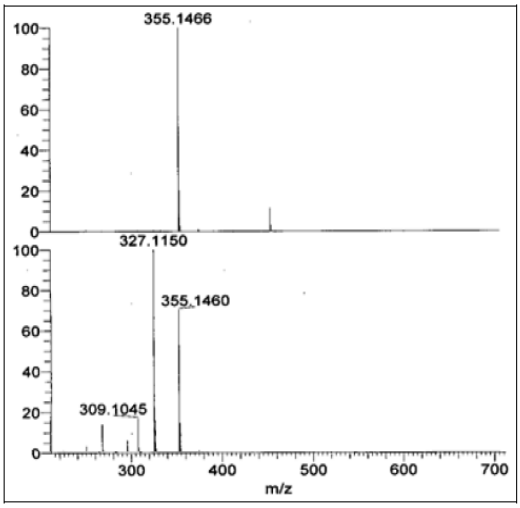
Figure 3a: MS/MS spectra and proposed structure of imp-1

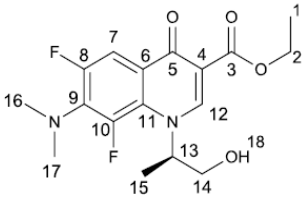




Imp.-2

Figure 3b: MS/MS spectra and proposed structure of imp-2





Imp.-3

Figure 3c: MS/MS spectra and proposed structure of imp-3

Structural elucidation of impurities

In order to determine the molecular formula of impurities, these figures of measured accurate masses were plugged into elemental composition calculator.

Imp-1 with accurate mass m/z 619.1695, gave molecular formula $C_{30}H_{27}F_4N_2O_8^+$ when subjected to elemental composition calculator. Two major fragment in MS2 were obtained at m/z 591.1380 (-28Da) attributed to the loss of $–C_2H_4$ group while fragment at m/z 282.0572 with the loss of 337 Da indicated some similarity with example-1, compound (8) [4] and Levofloxacin related compound B [5]. Presence of ethyl side chain is further confirmed by ^{1}H where methyl & methylene protons were observed at 1.39 and 4.36 ppm respectively. Presence of ddd peak between 4.55 to 4.79 ppm with high coupling constant suggests cyclized methylene ring with restricted degree of rotation for sp^3 hybridized methylene group. Doublet of doublet peak at 8.03 ppm indicated aromatic proton in vicinity of two fluorine atom.

This further authenticated by typical ^{13}C-^{19}F splitting pattern in ^{13}C-NMR data. Downfield singlet at 8.54 ppm along with other information confirmed the presence core quinolin-4(1H)-one structure. On integrating methyl group as 3H, ^{1}H-NMR spectrum showed total of 13H. This was not in accordance with MS data, where molecular formula revealed 26H. Hence it was confirmed that the present impurity is dimer moiety. Combining together NMR and mass spectral data, methyl proton was integrated as 6H. Based on collective information this impurity was confirmed as diethyl (9S,19S)-5,6,15,16-tetrafluoro-9,19-dimethyl-3,13-dioxo-8,9,18,19-tetrahydro-3H,13H-[1,7]dioxa[4,10]diazacyclododecino[2,3,4-ij:8,9,10-i'j']diquinoline-2,12-dicarboxylate. Complete ^{1}H/^{13}C spectral assignment is shown in Table 1.

Accurate mass peak of Imp-2 was at m/z 316.1295, which gave closest possible molecular formula $C_{16}H_{18}N_3O_4$. MS2 fragment at m/z 298.1183 (-18 Da) attributed to loss of water molecule from carboxylic acid terminal. ^{1}H-NMR spectrum Imp-2 showed two methyl group, one doublet at 1.42 ppm and another singlet at 2.92 ppm. Two aromatic protons, one singlet at 7.04 and another at 8.66 ppm along 7 with D_2O exchangeable protons at 6.51 ppm and 16.49 ppm were observed.

Table 1: ^{1}H, ^{13}C NMR data of levofloxacin impurities, Imp-1, Imp-2 and Imp-3

Imp-2					Imp-3					Imp-1				
Position	**Integration**	**δ (ppm)**	**Multiplicity, *J* (Hz)**	**^{13}C (δ in ppm), multiplicity, *J* (Hz)**	**Position**	**Integration**	**δ (ppm)**	**Multiplicity, *J* (Hz)**	**^{13}C (δ in ppm), multiplicity, *J* (Hz)**	**Position**	**Integration**	**δ (ppm)**	**Multiplicity, *J* (Hz)**	**^{13}C (δ in ppm), multiplicity, *J* (Hz)**
1	1H	16.49	s	-	1	3H	1.40-1.44	t, (7.1)	14.4	1,1’	6H	1.39-1.43	t (7.1)	14.4
2	-	-	-	167.8	2	2H	4.32-4.40	m	61.0	2, 2’	4H	4.36-4.41	q (7.1)	61.3
3	-	-	-	105.9	3	-	-	-	165.1	3, 3’	-	-	-	164.8
4	-	-	-	175.5	4	-	-	-	108.1	4, 4’	-	-	-	111.3
5	-	-	-	114.7	5	-	-	-	171.7	5, 5’	-	-	-	171.6
6	1H	7.04	s	97.1	6	-	-	-	127.2-127.3 (dd, 5.1, 2.2)	6, 6’	-	-	-	126.7 (d, 5.9)
7	-	-	-	127.5	7	1H	7.05-7.09	dd, (12.7, 1.5)	107.3-107.5 (dd, 22.7, 2.2)	7, 7’	2H	8.03-8.08	dd (9.8, 0.7)	110.1-110.3 (d, 18.3)
8	2H	3.49	s	47.9	8	-	-	-	153.0-155.5 (dd, 248.8, 7.3)	8, 8’	-	-	-	147.2-149.7 (dd, 254.7, 34.4)
9	2H	3.20-3.25	m	40.3	9	-	-	-	134.2-134.5 (t, 14.6)	9, 9’	-	-	-	147.1-149.6 (dd, 254.7, 37.3)
10	1H	6.51	s	-	10	-	-	-	145.0-147.6 (dd, 248.1, 6.6)	10, 10’	-	-	-	137.5-137.6 (d, 12.4)
11	-	-	-	130.0	11	-	-	-	123.3-123.4 (d, 13.9)	11, 11’	-	-	-	131.0 (t, 2.9)
12	-	-	-	136.1	12	1H	8.63	s	147.5	12, 12’	2H	8.54	s	145.7
13	2H	4.26-4.48	dd, (77.5, 11.3)	68.1	13	1H	5.30-5.36	m	60.3-60.5 (d, 20.5)	13, 13’	2H	5.92-5.98	m	57.4
14	1H	4.78-4.83	m	55.2	14	2H	3.87-4.40	m	64.3	14, 14’	4H	4.55-4.79	ddd, (95.6, 9.8, 6.4)	77.1
15	1H	8.66	s	142.3	15	3H	1.68-1.70	d, (7.1)	16.9	15, 15’	6H	1.73-1.75	d (7.1)	19.2
16	-	-	-	121.7	16	3H	3.05-3.06	t, (4.4)	43.2-43.3 (t, 4.4)					
17	3H	1.42-1.44	d (6.6)	18.4	17	3H	3.05-3.06	t, (4.4)	43.2-43.3 (t, 4.4)					

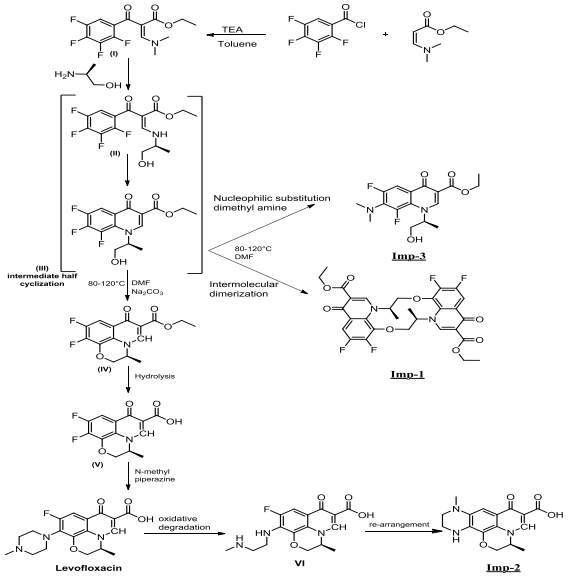


Figure 4: Possible pathway for the formation of Imp-1, Imp-2 and Imp-3

This confirmed the partial structural skeleton of molecule is similar to parent molecule except peak at 6.51 ppm. Absence of typical fluorine coupling impact on aromatic peak at 7.04 ppm in 1H and aromatic carbon peaks in the ^{13}C spectrum indicated that the fluorine atom is severed from moiety. Based on NMR and MS data it is now suggestive that fluorine is replaced by fused piperazine ring where one of the nitrogen substituted with singlet methyl at 2.92 ppm while second nitrogen contained exchangeable hydrogen appearing at 6.51 ppm. Taken together, the structure of Imp-2 is confirmed to be (S)-3,9-dimethyl-7-oxo- 2,3,9,10,11,12-hexahydro-7H-[1,4]oxazino[2,3,4-ij]pyrazino[2,3-g]quinoline-6-carboxylic acid. Complete $^{1}H/^{13}C$ spectral assignment is shown in Table 1.

Based on Imp-3 accurate mass (m/z 355.1460) the obtained molecular formula by elemental composition calculator was $C_{17}H_{21}F_2N_2O_4$. MS2 fragment at m/z 327.1150 (-28 Da) and m/z 309.1045 (-18 Da) attributed to loss of $–C_2H_4$ and H_2O group from possible ethyl ester side chain. Presence of ethyl side chain is further confirmed by ^{1}H where methyl and methylene protons were observed at 1.40 and 4.32 ppm respectively. Presence of doublet of doublet peak at 7.05 ppm suggests aromatic proton in vicinity of two fluorine atom. This further authenticated with ^{13}C-NMR. Downfield singlet at 8.62 ppm along with other information confirmed the presence core quinolin-4(1H)-one structure. Additionally triplet peak at 3.05 ppm corresponding to 6H, correlating to triplet at 43.2 ppm in ^{13}C-NMR confirms presence of dimethyle amine group present in vicinity to fluorine atom. Combining together it was confirmed that the one of the fluorine atom of aromatic ring was substituted by N,N-dimethyl amine which has hampered the scope of desired cyclization process. Therefore, this impurity was confirmed as ethyl (S)-7-(dimethylamino)-6,8-difluoro-1-(1-hydroxypropan-2-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate. Complete $^{1}H/^{13}C$ spectral assignment is shown in Table 1.

Formation of impurities

During synthesis of half cyclized intermediate (III), unstable intermediate (II) is formed along with release of N, N-dimethyl amine before attainment of intended cyclized intermediate (IV).

At stage (III) minor quantity of fluorine atom at meta position to heterocyclic nitrogen is substituted by *N*, *N*-dimethyl amine to give Imp-3. Later, when temperature is elevated to achieve (III), as per reaction mechanism hydrogen atom of hydroxyl functional group and fluorine fuse together to give cyclized product (IV) with elimination of -HF. However, concurrently two molecule of (II) undergo 9 elimination-dimerization processes to give Imp-1. Proceeding reaction for (V), N-methyl piperazine substitutes fluorine atom to give Levo. At this stage it very likely that Levo may undergo oxidative degradation, stimulating ring opening and de-alkylation of N-methylpiperazine moiety [7,8]. Further this open ring (VI) re-cyclized to give Imp-2. The collective outcome of MS and NMR confirmed the structure of unknown impurities and the reaction scheme with pathway of formation of impurities is predicted as shown in Figure 4.

CONCLUSION

Three new process related impurities have been identified in levofloxacin sample when analyzed by UPS mentioned procedure 2. Isolation of unknown impurities has been carried out by using flash chromatography. The structural characterization of the isolated impurity was established by using LC-MS/MS and NMR technique.

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