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Novel HPLC method for Levofloxacin and Its known impurities in Tablets dosage form

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Abstract:

Levofloxacin is a fluoroquinolone antibiotic that fights bacteria in the body. Levofloxacin is used to treat different types of bacterial infections. Simple HPLC method for Levofloxacin and six known impurities was developed and validated. Buffer: 8.5g ammonium acetate, 1.25g cupric sulphate and 1.g L-Isoleucine in 1000ml water. Mobile phase: Buffer and methanol 70:30 v/v; Diluent: Mobile phase; Column: Inertsil ODS-3V C18, 250*4.6mm, 5µ; Injection volume: 25µL; Column temperature 42°C; Flow rate: 0.7ml/min; Run time: 60min; Detector: 340nm. System suitability limits are NMT 10% RSD for 5 replicates and tailing factor NMT 1.8. Method validation was performed with precision, specificity, accuracy, linearity, limit of detection and quantification, ruggedness and robustness. Finalized method produced linear response in the specification limit was 0.998 to 1.000 correlation coefficient for levofloxacin and percentage recovery was found to be 98.00% to 102.0%.

Keywords: Levofloxacin, known impurities, HPLC method, United States of Pharmacopoeia.

Introduction:

Levofloxacin is a bacteriostatic L-isomer of ofloxacin and it is a third generation fluoroquinolone medicine. Chemically, levofloxacin is (-)-(S)-9-fluoro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid hemihydrate[1]. Levofloxacin is used to treat severe or life-threatening bacterial infections or bacterial infections that have failed to respond to other antibiotic classes[2-3]. Levofloxacin inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase[4-7]. Levofloxacin, like other fluoroquinolones, inhibits the A subunits of DNA gyrase, two subunits encoded by the gyrA gene. levofloxacin and its impurities chemical structures were represented in figure-1.

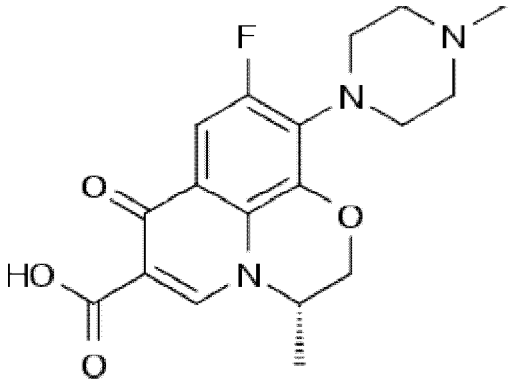

Figure-1: Levofloxacin and its impurities chemical structures

Literature confirmed the few reported methods to determine the levofloxacin and its combination with other drug products[8-14]. Goswami (2018), Sivasubramanian (2018) were reported combination method, Eldin (2018) reported levofloxacin and Daclastavir method. Objective of this method was to develop a simple and accurate method for six known impurities and Levofloxacin in tablets dosage form. USP method listed for four impurities but other two impurities are forming during product manufacturing and storage conditions.

Materials and Method:

Chemicals & Instrument:

Ammonium acetate, Cupric sulphate and L-Isoleucine AR grades; acetonitrile and methanol (HPLC grade); Water (Milli-Q grade); Waters alliance HPLC instrument with PDA detector; Inertsil ODS-3V C18, 250*4.6mm, 5µ.

Solutions preparation:

Buffer: 8.5g ammonium acetate, 1.25g cupric sulphate and 1.g L-Isoleucine in 1000ml water.
Mobile phase: Buffer and methanol 70:30 v/v
Diluent: Mobile phase
Std. soln.: 20 mg Levofloxacin std. in 100ml volumetric flask and dilution with diluent. 5ml of above solution dilute with 50ml diluent. 5ml of solution dilute 25ml with diluent.

Impurity stock: All known impurities each 2mg, D-isomer 5mg in 10ml and dilution with diluent. 2ml of solution dilute with 50ml diluent.
Identification sol.: 10mg Levofloxacin std. and 4ml of impurity stock solution in to 50ml and dilute with diluent.
Test sol.: 20 mg levofloxacin equivalent tablets fine powder in to 100ml and dilute with diluent. Sonicate 30min with intermediate shaking and filter with 0.45µ NYLON filter.

Chromatographic conditions:
Column: Inertsil ODS-3V C18, 250*4.6mm, 5µ; Injection volume: 25µL; Column temperature 42°C; Flow rate: 0.7ml/min; Run time: 60min; Detector: 340nm.
System suitability limits: NMT 10% RSD for 5 replicates and tailing factor NMT 1.8.

Results and Discussion:
Method optimization:
Method development was performed to separate all known impurities and Levofloxacin. Levofloxacin has official monograph in United States of Pharmacopoeia (USP), impurities method is by HPLC. USP Impurities method was specified four known impurities with acceptable limits. However, the main objective of this research was to develop a simple HPLC method for six known impurities. Initial method development was started as per the USP monograph.

Monograph method:
Mobile phase: 874g Cupric sulphate 918g L-isoleucine and 5.94g ammonium acetate in to 700ml water and 300ml methanol; Diluent: Acetonitrile and water 20:80 v/v; Column: L1 packing 250*4.6mm, 5µ; Injection volume: 25µL; Column temperature: 45°C; Flow rate: 0.8ml/min; Runtime: 2 times of levofloxacin RT; Wavelength: 360nm; System suitability: %RSD for Levofloxacin peak is NMT 2.0%; Tailing factor for standard peak NMT 1.8.
USP monograph impurities and objective method impurities were listed in below table-1.

Table-1: USP and objective method impurity profile comparison

S. No.	Impurity name	USP (NMT)	Objective method limit (NMT)	Remarks
1	10-fluoro levofloxacin	NA	0.1%	Objective impurity
2	Decarboxy levofloxacin	0.3%	-----	Controlled and listed as unspecified.
3	Levofloxacin impurity A (piperzine analog)	0.7%	0.2%	Stringent limit than USP.
4	Diamine derivative	0.3%	----	Controlled and listed as unspecified.

5	N-oxide impurity	0.7%	0.40%	Stringent limit than USP.
6	9-desfluoro levofloxacin	Other method	----	Controlled and listed as unspecified.
7	Dextrofloxacin / D-isomer		----	
8	9-piperazino isomer		----	
9	Ethyl ester impurity	NA	0.2%	Objective impurity

Method development was progressed to separate all the six impurities with different mobile phase conditions and columns. Finalized method was compared with USP monograph method and equivalency was performed. USP monograph method chromatogram was represented in below figure-2.

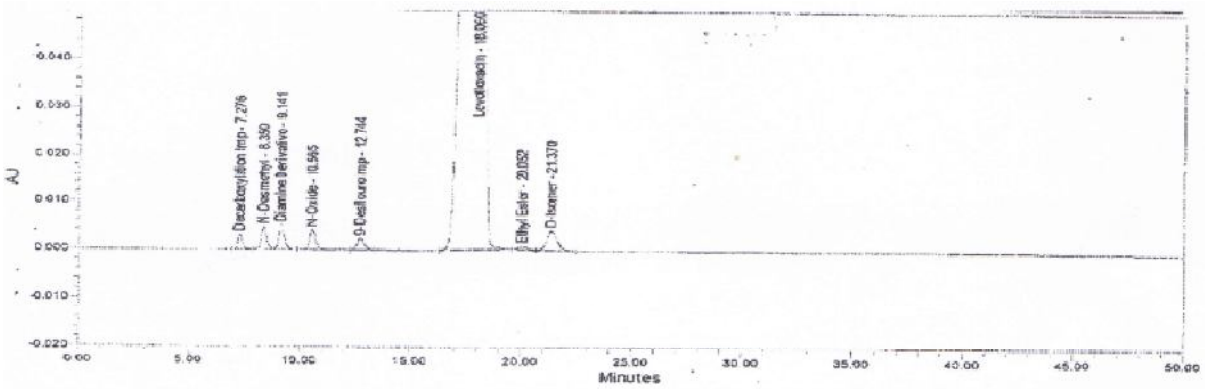

Figure-2: USP monograph method chromatogram

USP method observations:

Ethyl ester impurity was eluting nearest Levofloxacin peak (RRT 1.1) resolution was poor and recovery was found below 85%. 10-Fluoro Levofloxacin impurity peak eluted at same RRT of D-isomer impurity.

Developed method was separated Ethyl ester impurity and 10-fluoro levofloxacin impurity. Mobile phase was modified with phosphate buffer and column was changed and used Discovery HS F5, 150*4.6mm, 5µ column. Optimized method was validated as per the industry guidance and general practice.

Method validation:

System suitability:

Chromatographic conditions were applied to confirm the system suitability limits %RSD and tailing factor. %RSD was found 1.28% and tailing factor 1.1. Each impurities identification solution and spiked solutions were injected and confirmed the RRT values for each known impurity. RRF values were established with two different concentration levels. All impurities specification limits, RT,

RRT and RRF values were listed in below table-2. Diluted standard solution chromatogram was represented in figure-3. System suitability results were listed in table-3.

Table-2: Impurities RT, RRT and RRF values with specification limits.

S. No.	Impurity name	RT (min)	RRT	Limit (%)	RRF
1	Levofloxacin	23.06	NA	NA	1.0
2	Decarboxylation impurity	9.13	0.40	0.1	1.1
3	N-Desmethyl levofloxacin	11.26	0.49	0.2	1.03
4	Diamine derivative	12.53	0.54	0.1	0.77
5	N-Oxide impurity	14.31	0.62	0.3	0.9
6	9-desfluro impurity	16.80	0.73	0.3	0.62
7	Ethyl ester impurity	26.27	1.14	0.2	0.73
8	D-isomer impurity	28.89	1.25	0.8	0.97

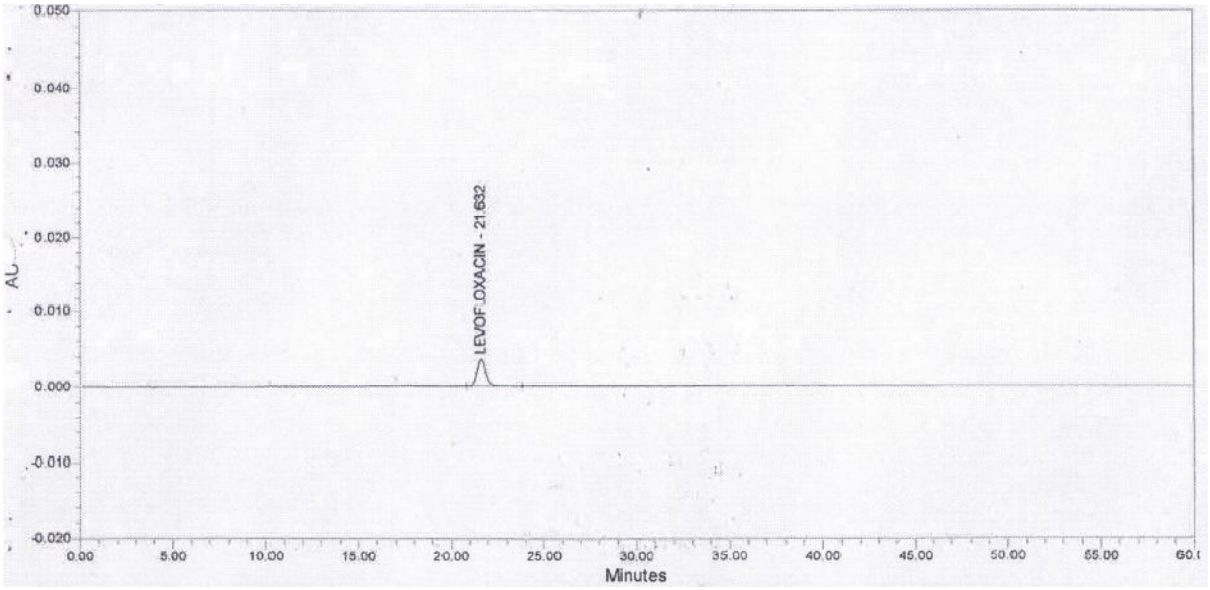

Figure-3: Diluted standard chromatogram

Table-3: Standard solution system suitability results

Sys. Suit.	1	2	3	4	5	6	Avg.	%RSD	Limit
Area	120808	120870	119963	119867	123979	122103	121265	1.28	10%
Tailing	1.1	1.2	1.1	1.2	1.2	1.1	1.15	NA	1.8

Precision:

Method precision and system precision was performed for levofloxacin 750mg tablets as per the test procedure mentioned in materials and method. Six preparations were performed and intermediate precision was performed on different HPLC with different column and analyst. Precision results were tabulated for all spiked known impurities and %RSD was calculated. Precision and intermediate precision results were tabulated in table-4. As such test sample and known impurities spiked sample chromatograms were represented in figure-4 and 5.

Table-4: Precision and intermediate precision results

Impurity name	**Precision samples**						**Avg.**	**%RSD**
	1	**2**	**3**	**4**	**5**	**6**		
Decarboxylation impurity	0.099	0.106	0.101	0.104	0.101	0.102	0.102	2.43
N-Desmethyl levofloxacin	0.212	0.222	0.216	0.220	0.209	0.216	0.216	2.24
Diamine derivative	0.115	0.119	0.110	0.116	0.108	0.112	0.113	3.60
N-Oxide impurity	0.313	0.331	0.320	0.325	0.317	0.320	0.321	1.96
9-desfluro impurity	0.328	0.346	0.335	0.345	0.332	0.337	0.337	2.12
Ethyl ester impurity	0.187	0.201	0.188	0.195	0.190	0.189	0.192	2.79
D-isomer impurity	0.783	0.844	0.807	0.829	0.787	0.809	0.810	2.91
Total impurities	2.33	2.47	2.37	2.43	2.34	2.38	2.89	2.16
Impurity name	**Intermediate Precision samples**						**Avg.**	**%RSD**
	1	**2**	**3**	**4**	**5**	**6**		
Decarboxylation impurity	0.111	0.113	0.111	0.111	0.112	0.11	0.112	0.88
N-Desmethyl levofloxacin	0.231	0.227	0.226	0.226	0.224	0.224	0.226	1.14
Diamine derivative	0.118	0.116	0.118	0.118	0.118	0.118	0.118	0.69
N-Oxide impurity	0.323	0.322	0.321	0.337	0.319	0.323	0.324	1.99
9-desfluro impurity	0.324	0.303	0.304	0.320	0.314	0.323	0.315	2.97
Ethyl ester impurity	0.177	0.176	0.182	0.179	0.178	0.177	0.178	1.20
D-isomer impurity	0.820	0.821	0.813	0.821	0.820	0.822	0.820	0.40
Total impurities	2.387	2.361	2.358	2.395	2.368	2.383	2.375	0.64

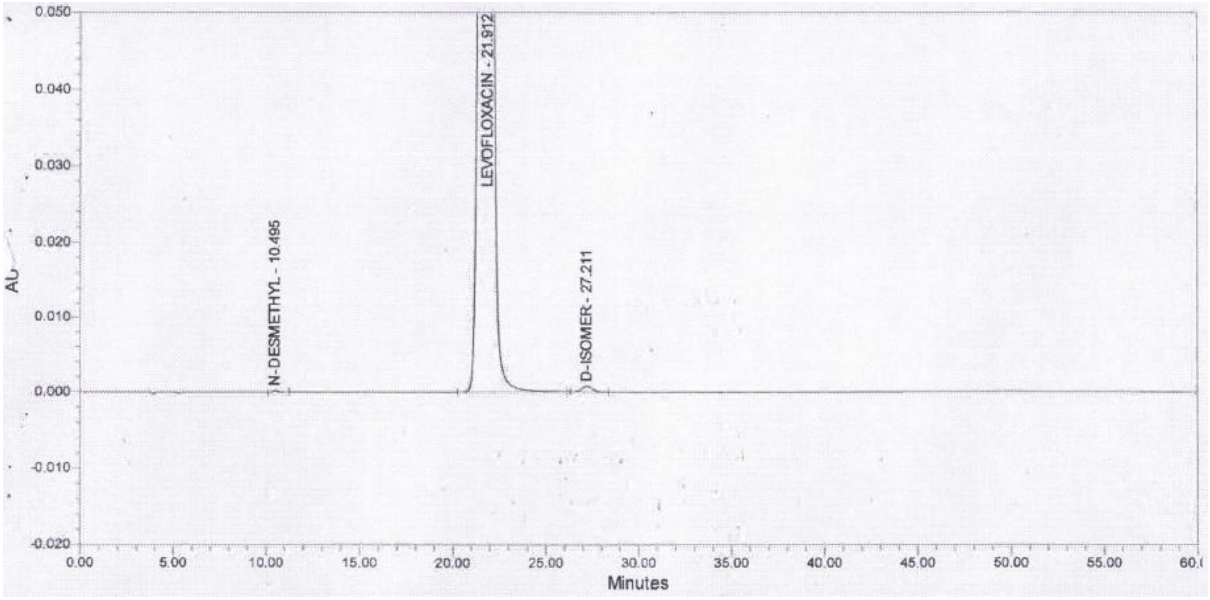

Figure-4: As such sample chromatogram

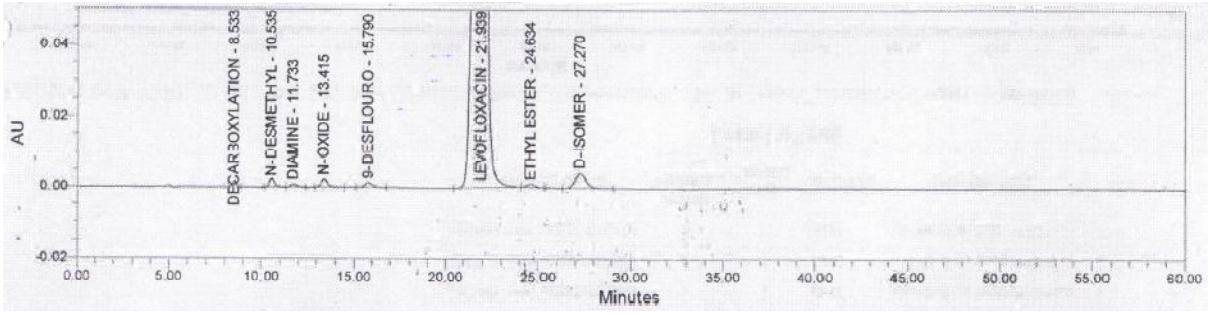

Figure-5: Impurities spiked sample chromatogram

Specificity:

Specificity was performed to evaluate the degradation behaviour of the formulation drug product. Acidic, basic, peroxide, thermal UV light, water and humidity stress conditions were applied. Peak purity plot and % degradation were calculated and reported in table-5. Degradation studies chromatogram peak purity plots were represented in figure-6 to 13.

Table-5: Degradation conditions and results.

S. No.	Stress condition	% Net degradation	Peak purity			Mass balance
			Angle	Threshold	Flag	
1	As such	0.284	0.121	0.290	NO	NA
2	Acid (5N HCl/24hr 70°C)	0.27	0.694	1.242	NO	103.74
3	Base (5N NaOH /24hr 70°C)	0.322	0.657	1.257	NO	104.21
4	Peroxide %H_2O_2	4.235	0.799	1.674	NO	95.47
5	Thermal 105°C/ 7days	0.581	0.096	0.296	NO	103.36
6	Water 24hr / 70°C	0.322	0.279	0.523	NO	102.60
7	Humidity 90% RH 25°C / 7days	0.368	0.525	1.109	NO	96.50
8	UV/ Sunlight 200w hr/sq/ 1.2mil lux hr	0.279	0.149	0.288	NO	104.67

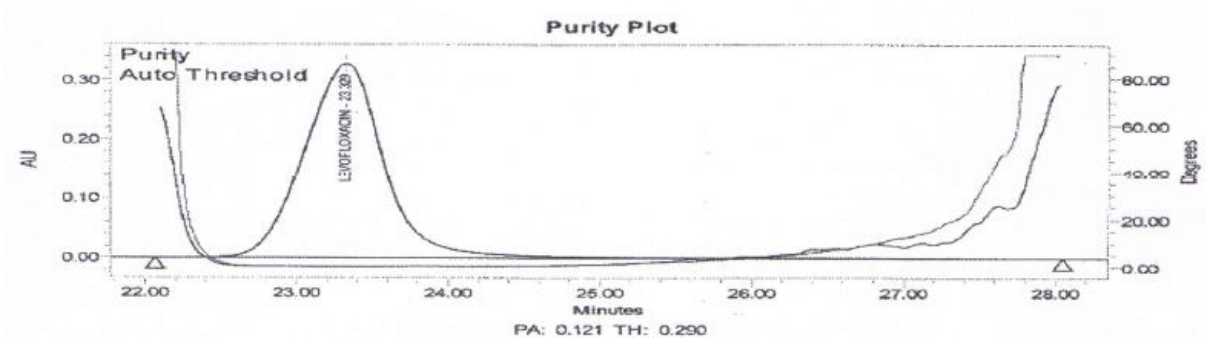

Figure-6: As such sample peak purity plot

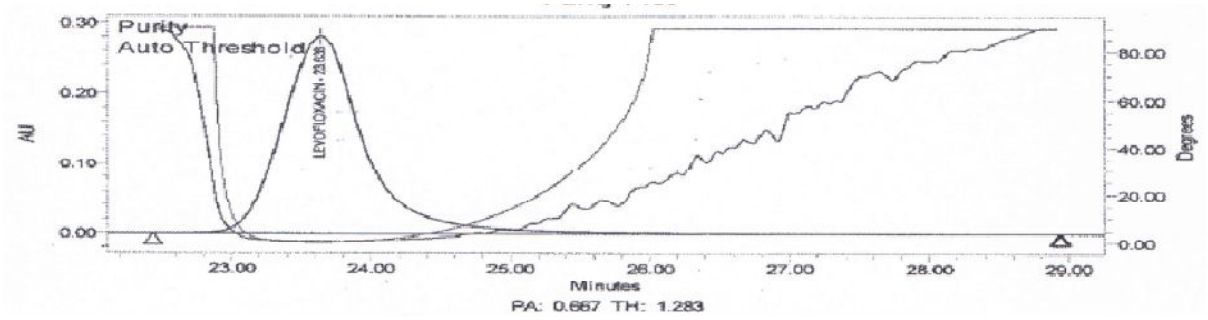

Figure-7: Acid degradation sample peak purity plot

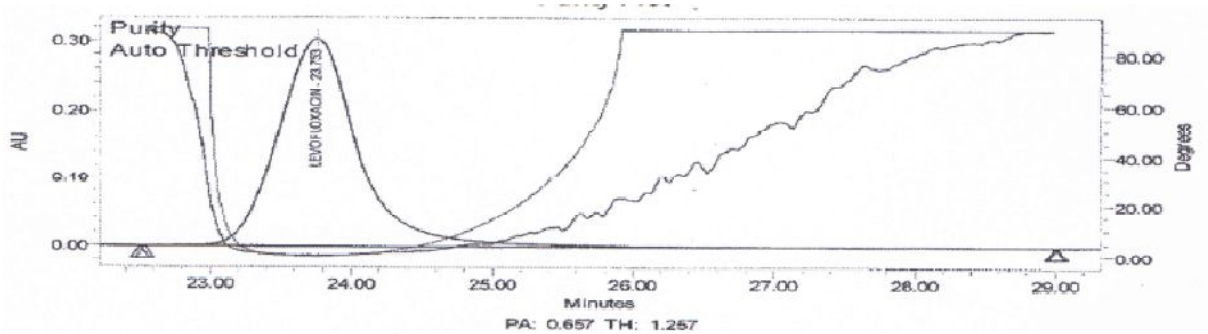

Figure-8: Base degradation sample peak purity plot

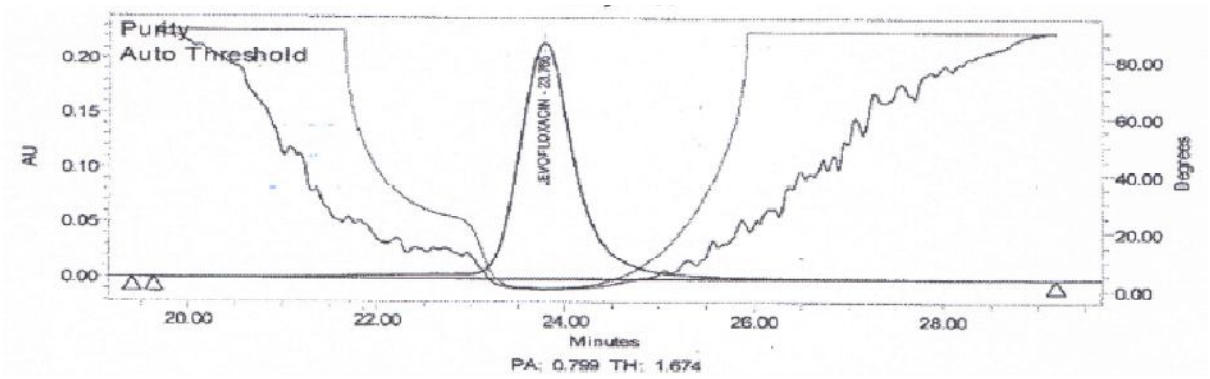

Figure-9: Peroxide degradation sample peak purity plot

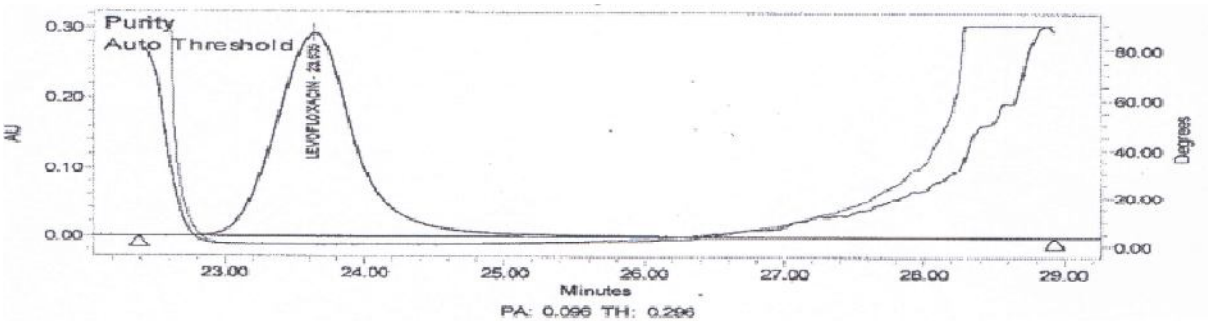

Figure-10: Thermal degradation sample peak purity plot

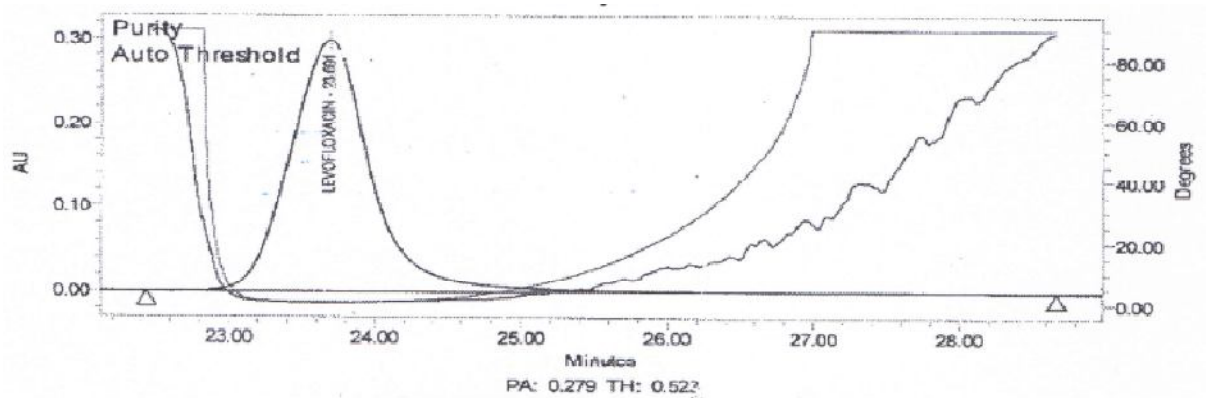

Figure-11: Water degradation sample peak purity plot

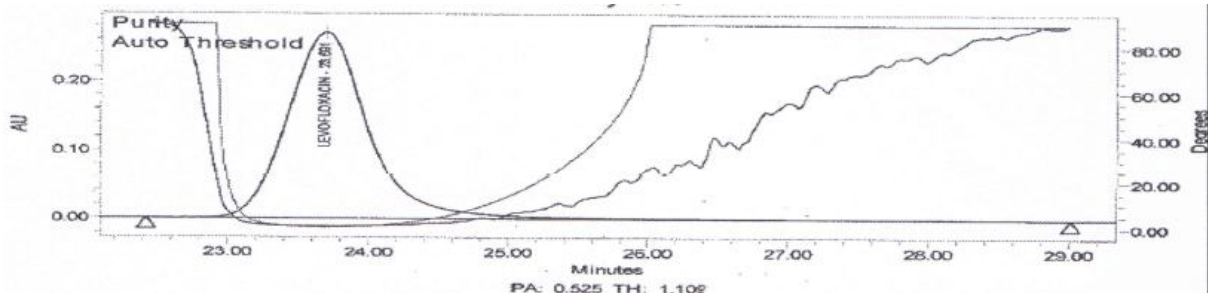

Figure-12: Humidity degradation sample peak purity plot

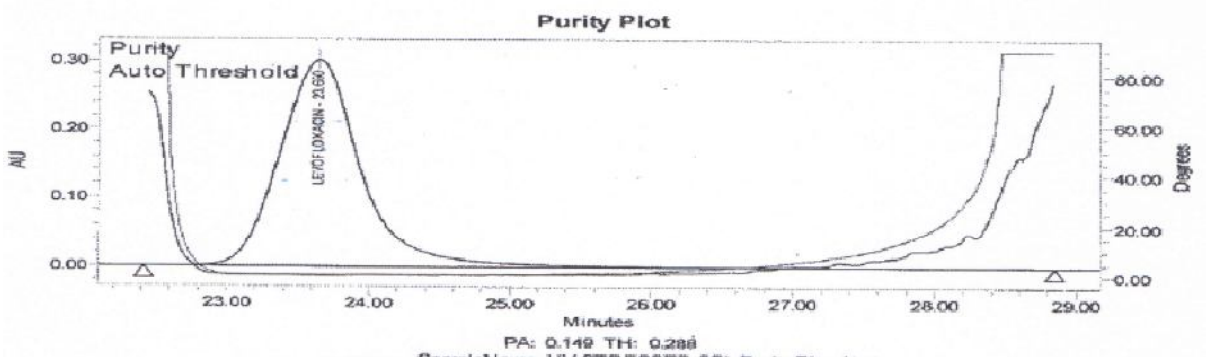

Figure-13: UV light degradation sample peak purity plot

Limit of detection (LOD) and Limit of quantification (LOQ):

LOD and LOQ were established with signal to noise (S/N ratio) method. S/N ratio values for levofloxacin and its impurities were found from 2.0 to 3.4 LOD concentration and 9.0 to 11.4 LOQ concentration levels. LOQ concentration precision was performed and confirmed the %RSD. LOD and LOQ concentration chromatograms were represented in figure-14 and 15. LOD and LOQ concentration results were tabulated in table-6.

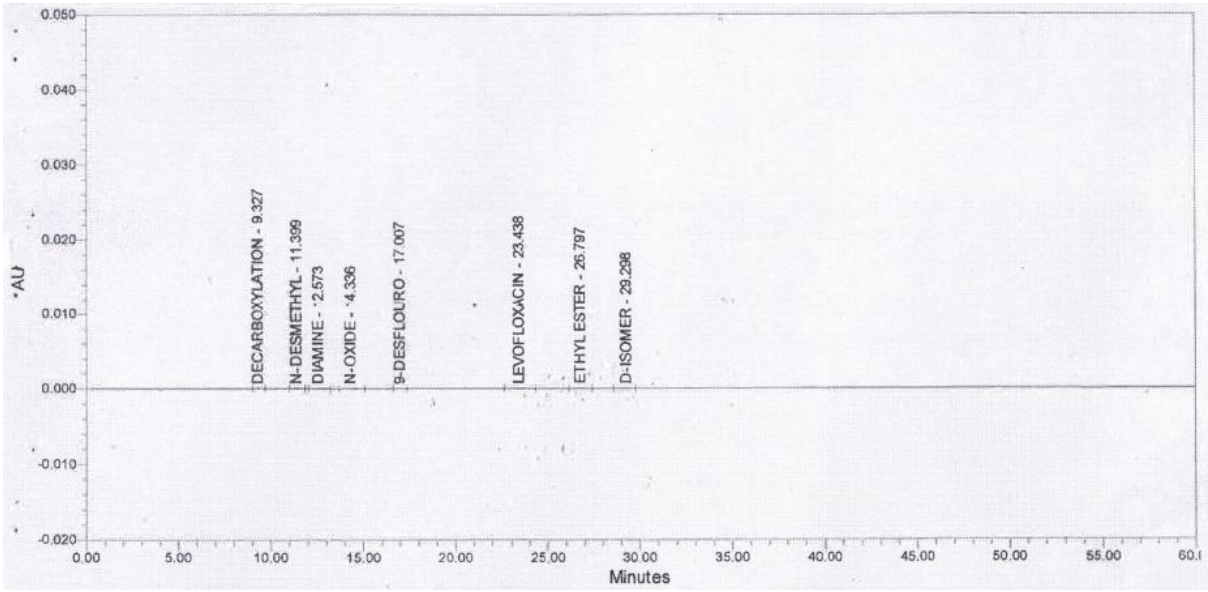

Figure-14: LOD solution chromatogram

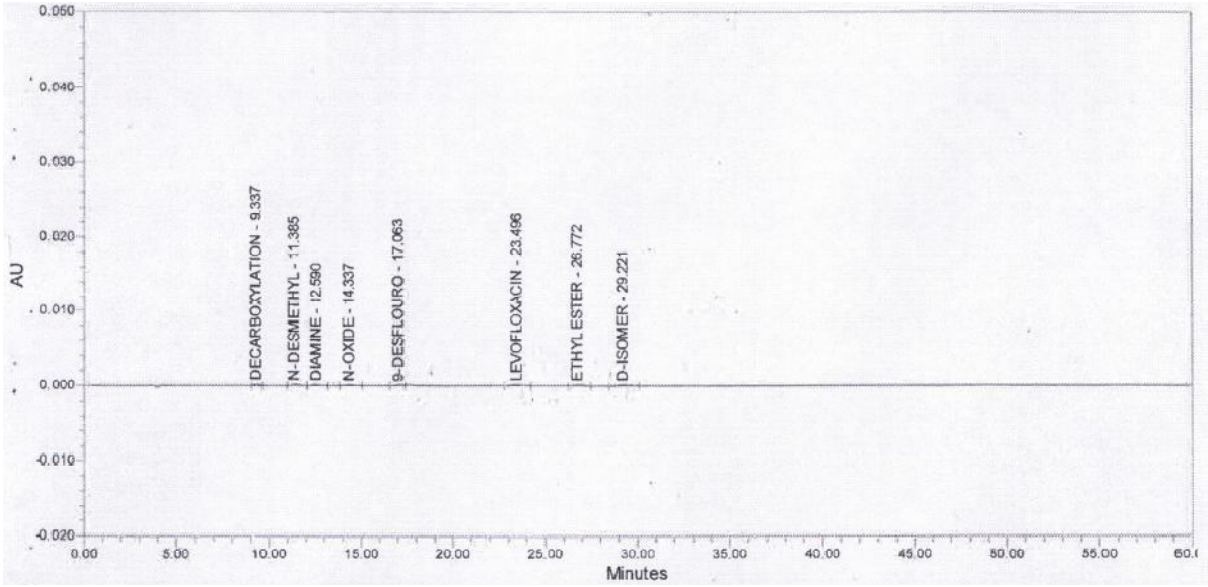

Figure-15: LOQ solution chromatogram

Table-6: LOD and LOQ results

Compound name	LOQ			LOD		
	ppm	% level	S/N ratio	ppm	% level	S/N ratio
Levofloxacin	0.08	0.04	10.7	0.040	0.02	3.0
Decarboxylation imp	0.04	0.02	10.0	0.020	0.01	29
N-desmethyl imp	0.05	0.025	9.5	0.025	0.01	2.8
Diamine imp	0.05	0.025	10.6	0.025	0.01	2.5
N-oxide imp	0.05	0.025	9.1	0.025	0.01	2.1
9-desfluro imp	0.08	0.04	9.7	0.040	0.02	2.0
Ethyl ester imp	0.09	0.045	10.5	0.045	0.02	2.3
D-isomer imp	0.06	0.03	11.0	0.030	0.02	2.0

Linearity:

Linearity was conducted from LOQ concentration to 150% of the specification limit. Linearity concentration levels are LOQ, 30%, 50%, 80%, 100%, 130% and 150%. Linearity results were calculated for correlation coefficient (area Vs conc.), slope, intercept, bias for 100% response. Linearity results were tabulated in table-7.

Table-7: Linearity Results

Conc. (%)	levofloxacin		Decarbox. imp		N-desmethyl imp		Diamine imp		N-oxide imp	
	Conc. (ppm)	Peak area	Conc. (ppm)	Peak area	Conc. (ppm)	Peak area	Conc. (ppm)	Peak area	Conc. (ppm)	Peak area
LOQ	0.08	8802	0.04	3315	0.05	4460	0.05	2868	0.05	3248
50	0.78	47287	0.11	7119	0.22	13595	0.11	4801	0.0	16060
75	1.18	68771	0.16	10668	0.33	20340	0.16	7149	0.46	24018
100	1.96	118800	0.22	14384	0.44	27114	0.22	1048	0.61	33735
125	2.75	166624	0.27	17989	0.54	33541	0.27	12443	0.76	40798
150	3.14	188121	0.33	21661	0.65	40705	0.33	14752	0.91	49621
Corr. Coe.(r)		1.000	0.999		1.000		0.997		0.999	
Slope (m)		59450	63725		60475		44540		53740	
Intercept (b)		1732.7	476.9		837.7		248.49		239.7	
Bias for 100% response		1.5	3.3		3.1		2.4		0.7	

		LOQ	50%	75%	100%	125%	150%	C.C	Slope	Inter.	Bias
9-desfluro	Conc.	0.08	0.33	0.50	0.67	0.83	1.00	0.998	33949	733.37	3.1
	Area	3004	12625	17254	23423	30141	33652				
Ethyl ester	Conc.	0.09	0.22	0.33	0.43	0.54	0.65	0.999	43102	199.4	1.1
	Area	3618	9600	13335	18315	22755	28382				
D-isomer	Conc.	0.06	0.79	1.19	1.58	1.98	2.37	1.000	60705	795.1	0.8
	Area	3678	47963	69799	94008	118951	144582				

Accuracy:

Accuracy of the method was validated with three different concentration levels 50%, 100% and 150%. 100% level was performed with six replicate preparations and other 50% and 150% levels were three replicates. Recovery was calculated from added concentration and recovered concentrations. Accuracy results were tabulated in table-8

Table-8: Accuracy Results

Accuracy level	Decarboxylation imp.			N-desmethyl imp			Diamine imp		
	Conc. µg/ml		%	Conc. µg/ml		%	Conc. µg/ml		%
	Added	Found	Recovery	Added	Found	Recovery	Added	Found	Recovery
50%	0.110	0.102	93.0	0.218	0.216	99.3	0.109	0.112	102.5
		0.104	94.8		0.214	98.3		0.118	108.0
		0.104	94.8		0.218	100.2		0.124	113.4
100%	0.219	0.198	90.3	0.45	0.424	97.4	0.219	0.230	105.3
		0.212	96.7		0.444	102.0		0.238	108.9
		0.202	92.1		0.432	99.3		0.220	100.7
		0.208	94.8		0.440	101.1		0.232	106.2
		0.202	92.1		0.418	96.1		0.216	98.9
		0.204	93.0		0.432	99.		0.224	102.5
150%	0.329	0.34	104.3	0.653	0.662	101.4	0.328	0.334	101.9
		0.339	103.0		0.656	100.5		0.334	101.9
		0.348	105.7		0.668	102.3		0.344	104.9
	N-oxide imp			9-desfluro imp			Ethyl ester imp		
50%	0.304	0.334	109.7	0.333	0.352	105.8	0.195	0.176	90.7
		0.338	111.0		0.348	104.6		0.174	89.7
		0.340	111.7		0.352	105.8		0.178	91.8
100%	0.609	0.626	102.8	0.666	0.656	98.6	0.390	0.374	96.4
		0.662	108.7		0.692	104.0		0.402	103.6
		0.640	105.1		0.670	100.7		0.376	96.9
		0.650	106.7		0.690	103.7		0.390	100.5
		0.634	104.1		0.664	99.8		0.380	97.9
		0.640	105.1		0.674	101.3		0.378	97.4
150%	0.913	0.958	104.9	0.998	1.014	101.6	0.585	0.576	99.0
		0.954	104.5		1.002	100.4		0.574	98.6
		0.976	106.9		1.016	101.8		0.586	100.7
D-isomer imp									
50%	0.791	0.804	101.7	100%			1.581	1.566	99.0
		0.786	99.4					1.688	106.8
		0.814	103.0					1.614	102.1
150%	2.372	2.468	104.1					1.658	104.9
		2.434	102.6					1.574	99.6
		2.498	105.3					1.618	102.3

Ruggedness:

Ruggedness of the chromatographic conditions was evaluated with intermediate precision such as different analyst, different HPLC system and column. Intermediate precision results found satisfactory and results were listed in precision. Solution stability (mobile phase, standard and test solutions) were evaluated for day-0, day-1 and day-2. Ruggedness results were tabulated in table-9 and 10.

Table-9: Ruggedness results (solution stability)

Stability condition		**Bench top**			**Refrigerator**		
Time in days		**Day-0**	**Day-1**	**Day-2**	**Day-0**	**Day-1**	**Day-2**
Std. similarity factor		NA	0.97	0.98	NA	0.98	0.97
Total impurities	Test-1	2.053	1.978	1.992	2.053	1.996	1.951
	Test-2	2.165	2.150	1.963	2.165	2.113	2.073
Decarboxylation imp	Test-1	0.099	0.099	0.099	0.099	0.099	0.099
	Test-2	0.106	0.104	0.106	0.106	0.106	0.105
N-desmethyl imp	Test-1	0.214	0.207	0.204	0.214	0.207	0.201
	Test-2	0.221	0.220	0.216	0.221	0.222	0.219
Diamine imp	Test-1	0.115	0.108	0.112	0.115	0.119	0.114
	Test-2	0.119	0.114	0.112	0.119	0.116	0.112
N-oxide imp	Test-1	0.313	0.308	0.299	0.313	0.305	0.303
	Test-2	0.331	0.324	0.316	0.331	0.325	0.314
9-desfluro imp	Test-1	0.328	0.309	0.303	0.328	0.314	0.299
	Test-2	0.346	0.335	0.319	0.346	0.334	0.314
Ethyl ester imp	Test-1	0.187	0.171	0.159	0.187	0.172	0.161
	Test-2	0.201	0.179	0.169	0.201	0.178	0.168
D-isomer imp	Test-1	0.797	0.776	0.816	0.797	0.780	0.775
	Test-2	0.841	0.874	0816	0.841	0.833	0.842

Table-10: Ruggedness results (mobile phase stability)

M.P. Bench top stability	Total impurities		Decarboxylation imp		N-desmethyl imp		Diamine imp	
	Test-1	Test-2	Test-1	Test-2	Test-1	Test-2	Test-1	Test-2
Day-0	2.053	2.165	0.099	0.106	0.214	0.221	0.115	0.119
Day-1	2.057	2.036	0.102	0.216	0.216	0.215	0.116	0.110
Day-2	2.041	2.029	0.104	0.213	0.213	0.212	0.110	0.115
	N-oxide imp		9-desfluro imp		Ethyl ester imp		D-isomer imp	
Day-0	0.313	0.313	0.328	0.346	0.187	0.201	0.797	0.841
Day-1	0.317	0.314	0.33	0.325	0.172	0.173	0.803	0.799
Day-2	0.313	0.312	0.319	0.313	0.168	0.167	0.813	0.804

Robustness:

Robustness was performed to confirm the changes in the test procedure such as mobile phase solvent ratio, pH, flow rate and column oven temperature. Sample preparation filter validation was performed with PVDF and NYLON filters. Robustness filter validation results were satisfactory and tabulated in table-11. Method changes results were represented in table-12.

Table-11: Robustness results (Filter validation)

Filter solution	Total impurities		Decarboxylation imp		N-desmethyl imp		Diamine imp	
	Test-1	Test-2	Test-1	Test-2	Test-1	Test-2	Test-1	Test-2
Centrifuged	2.072	2.051	0.109	0.111	0.225	0.220	0.122	0.119
PVDF	2.067	2.044	0.115	0.110	0.226	0.224	0.115	0.111
NYLON	2.083	2.017	0.114	0.112	0.235	0.218	0.122	0.112
	N-oxide imp		9-desfluro imp		Ethyl ester imp		D-isomer imp	
Centrifuged	0.322	0.313	0.292	0.290	0.177	0.173	0.282	0.824
PVDF	0.320	0.323	0.285	0.282	0.173	0.178	0.834	0.816
NYLON	0.322	0.310	0.290	0.281	0.178	0.171	0.822	0.813

Table-12: Robustness results (Method changes)

Levofloxacin system suitability	Flow rate (ml/min)			Column oven temperature (°C)			M.P organic solvent ratio		
	0.6	**0.7**	**0.8**	**37**	**42**	**47**	**90%**	**100%**	**110%**
Tailing factor (NMT 1.8)	1.2	1.1	1.2	1.2	1.1	1.2	1.2	1.1	1.2
%RSD (NMT 10%)	0.6	0.2	0.8	0.6	0.2	1.4	1.7	0.2	07

Conclusion:

Levofloxacin and its impurities were separated and quantified with simple and rugged HPLC method. Method equivalency was performed against the USP pharmacopeia method. Equivalency results were satisfactory and method validation was performed to confirm the method extendedness. Validation results were satisfactory precision %RSD, degradation results, correlation coefficient, % recovery and system suitability. Eventually, results confirmed that this method can be used to check the regular manufacturing activity.

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