Research Paper

A Novel LC-MS/MS Method Development and Validation for Ultra-trace Level Determination of Three Genotoxic Efavirenz Impurities

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A novel, simple, sensitive, specific and rapid reverse-phase liquid chromatography-electrospray ionization-mass spectrometry method was established and validated for the ultra-trace analysis of efavirenz-related compound C, 8-hydroxy efavirenz and its impurity 1, which are potential genotoxic impurities associated with efavirenz. These were separated on high performance liquid chromatography and detected with tandem mass spectrometry in the positive ionization mode with MRM transitions, m/z 314.15>244.10, 212.10>57.10, 305.70>69.05 and 364.15>291.25. A gradient method was developed to separate the impurities using 0.01 M ammonium acetate buffer and methanol at 1 ml/min flow-rate. Samples were analysed using a Hypersil C-18 column (5 µm, 250×4.6 mm). The calibration curves have shown good linearity in the concentration range of 2.5-78 ppb and a correlation coefficient of 0.99. Mean intra and inter day precision was 2.6-3.2 ppm and 2.5-2.6 ppm, respectively. For the 3 genotoxic impurities the limit of detection and limit of quantitation were 0.04 ppm and 0.125 ppm, respectively. The liquid chromatography-mass spectrometry/mass spectrometry method developed was specific, sensitive, precise and accurate. The developed method could be used for quantification and monitoring of genotoxic impurities of efavirenz.

Key words: Efavirenz-related genotoxic impurities, liquid chromatography-mass spectrometry/mass spectrometry method, trace-level, validation

Efavirenz (EFV, (S)-6-chloro-(cyclopropylethynyl)-1,4-dihydro-4(trifluoromethyl)-2H-3,1-benzoxazin-2-one) is an effective antihuman immunodeficiency virus type 1 agent that acts as a non-nucleoside reverse transcriptase inhibitor (NNRTI). Normally, NNRTI combination is the best suited for treating naive HIV patients. Some well-known NNRTI combinations are, combinations of EFV with antiretroviral (ARV) drugs like tenofovir, lamivudine, zidovudine and emtricitabine[1]. Furthermore, the mechanism of action of ARV drugs is by diffusion and circulation into the genital tract[2-4].

Pharmacopeia of Brazil, USA, British and Japan, indicated restrictions for active pharmaceutical ingredients (APIs) and formulations for allowable levels of impurities. Moreover, the Food and Drug Administration (FDA) and International Council for Harmonisation (ICH) mentioned strategies for the identification and quantification of impurities along with residual solvent in any in novel dosage forms[5-9]. Moreover, some impurities in trace levels could affect the efficacy and safety of API, as well as be carcinogenic[10,11]. Hence, monitoring and control of trace impurities in any API turn into a very tough assignment. Therefore, the process of minimizing such carcinogenic substances became important in pharmaceutical toxicology[12].

Based on the above documented facts, researchers mainly focused on minimizing the production of impurities in any API manufacturing process[13]. However total elimination of impurities in any process is difficult. So, the method development for accurate identification of impurities is the only option for pharmaceutical industries. Furthermore, USFDA and

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Accepted 14 April 2020
Revised 17 February 2020
Received 08 December 2019
Indian J Pharm Sci 2020;82(3):429-434

agency of European medicines proposed toxicological threshold limits to be 1.0-1.5 µg/d[14,15] for genotoxic impurities.

To date, several analytical methods are defined for the quantification of EFV in biological fluids, high-performance liquid chromatography-ultraviolet/visible (HPLC-UV/Vis) detection[16-20], HPLC[21], liquid chromatography-mass spectrometry (LC-MS)[22], LC-MS/MS[23,24] and ultra-performance liquid chromatography-mass spectrometry/mass spectrometry (UPLC-MS/MS) methods[25,26]. In the present work, we aimed to determine genotoxic impurities in EFV, EFV-related compound C (CPP), 8-hydroxy EFV (CPE), and EFV impurity 1 (EFI, fig. 1) using LC-MS/MS method for quantification of these impurities in EFV formulations.

MATERIALS AND METHODS

HPLC grades of ammonium acetate and methanol were purchased from Merck (Mumbai, India). CPE, EFV, CPP and EFI were obtained from Perfomics Analytical Labs (Hyderabad, India). A Shimadzu LC-MS/MS-8050 system associated with the Nexera X2 HPLC and Lab Solutions software v.5.6 was used. Separations were accomplished on a 5 µm particle size of Hypersil C18 column (4.6×250 mm) purchased from Thermo Fisher Scientific.

Method development:

Generally, for any analysis, sample preparation plays an important role; it affects the sensitivity, as well as better recovery of impurities. So, preferable combinations of acetonitrile, water, ammonium acetate, and methanol were used as diluents for chromatographic efficiency. In the present work, 0.01 M ammonium acetate in methanol was chosen as a diluent with column oven at 40º due to good response and recovery for impurities CPE, CPP, and EFI. Also, both isocratic and gradient modes of elution were performed.

Nevertheless, from the observations, it was noticed that all the impurities were effectively separated by the gradient method. Similarly, a column of dimensions, namely Zorbax C8, Hypersil C18 column Phenomenox, Kromasil C8 and C18, were also investigated for resolutions. Finally, Hypersil C18 column was selected due to its better response, peak shape, linearity, and reproducibility even at a lower concentration. Moreover, the positive mode of ESI resulted in improved signal intensities and lesser noise background for impurities, when compared to negative mode.

Method optimization:

Mobile phase A used was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml Milli-Q water by sonication followed by filtration (0.22 µm). Pure HPLC grade methanol was used as a mobile phase B. An LC-MS/MS system, coupled with an 8050 triple quadrupole detector, was used. Separation was achieved on a 5 µm Hypersil C18 column (250×4.6 mm) with injection volume 10 µl, 1 ml/min flow rate, sample cooler temperature at 15° and column oven temperature 40°. Table 1 summarized conditions of MRM, Valco

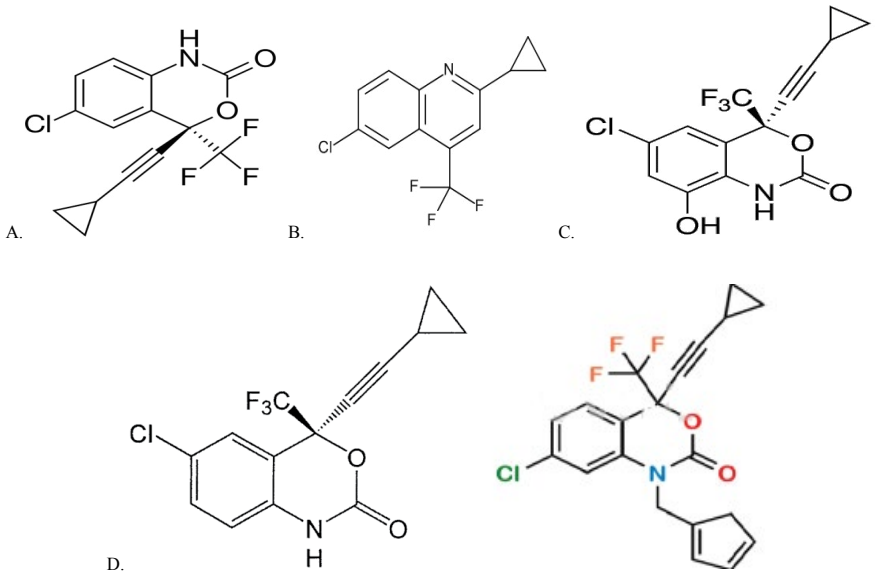

Fig. 1: Chemical structures of analytes
Chemical structures of A. efavirenz (EFV), B. efavirenz-related compound C (CPP), C. 8-hydroxy efavirenz (CPE), D. Efavirenz impurity 1 (EFI)

TABLE 1: GRADIENT PROGRAMME

Gradient program		
Time (min)	**Mobile phase A**	**Mobile phase B**
0.01	45	55
8.00	45	55
10.00	20	80
13.50	Total Flow	0.8 ml
15.00	45	55
17.00	45	55
17.00	Total Flow	0.8 ml
20.00	Total Flow	1 ml
21.50	20	80
32.00	45	55
37.00	Controller	Stop

Multiple reactions monitoring conditions					
		Parameters			
Impurity	**MRM**	**Q1 Prebias**	**CE**	**Q3 Prebias**	**Dwell time (milliseconds)**
CPP IMP	212.10>57.10	-26.0	-22.0	-24.0	100
CPE IMP	305.70>69.05	20.0	22.0	24.0	100
Efavirenz Impurity 1	364.15>291.25	22.0	22.0	30.0	100
Efavirenz	314.15>244.10	20.0	19.0	26.0	100

Valco Valve Condition for sample method		
Time (min)	**Command**	**Value**
17.00	FCV2=	1
24.00	FCV2=	0

valve, and source gas parameters for mobile phases A and B under gradient mode against the blank solution (diluent). In order to safeguard the ESI sourced from high EFV concentrations, the valve ailment is kept under enabling transferral of eluent to unwanted.

Standard preparation:

Separately, 2.6 mg of CPE, CPP and EFI were weighed accurately and dissolved completely in 100 ml diluent via sonication. One milliliter of the above impurity/ intermediate standard stock solution was further diluted to 100 ml with diluent. One hundred milligrams of accurately weighed EFV was diluted to 5 ml. To evaluate the system suitability parameters, 10 µl of the above-prepared solution was separately injected namely blank, standard, and sample preparations and their peak area responses were monitored. As per the pharmacopeias, the average peak area response of % relative standard deviation (RSD) of CPE, CPP, and EFI impurities should not be more than 15.0.

Method validation:

Method was validated according to the USFDA and ICH guidelines. The appropriateness and efficacy of the chromatographic scheme were obtained from the system suitability test and it is proficient in the investigation without any bias. To guarantee the capacity of the chromatographic systems, these must placate pre-defined acceptance conditions to implement the examination of various samples. In the contemporary experiment, CPE, CPP, EFI impurity solutions were injected into the LC-MS/MS system for determining system suitability parameters such as peak area and its RSD and retention time, which were detailed after data incorporation using software (Table 2; fig. 2).

The CPE, CPP and EFI impurities were also checked for specificity by injecting them against the blank solution. The outcomes showed that the chosen method is unbiased concerning the presence of further components and interestingly, no nosiness was recorded at the RTs of CPE, CPP, and EFI impurities (Table 3 and fig. 3).

RESULTS AND DISCUSSION

At limit of quantification (LOQ), 4 levels of the precision method, namely system precision, intermediate precision (ruggedness), method precision (repeatability) and precision were evaluated. System precision suggested inconsistency in the dimensions of the analytical system, while repeatability (method precision) indicated the reproducibility of the method. Standard solution was prepared with CPE, CPP, and

TABLE 2: RESULTS FOR SYSTEM SUITABILITY

System suitability parameters	CPP	CPE	EFI
% RSD of Peak areas obtained from six replicate injections of the standard solution	0.8	1.2	1.6

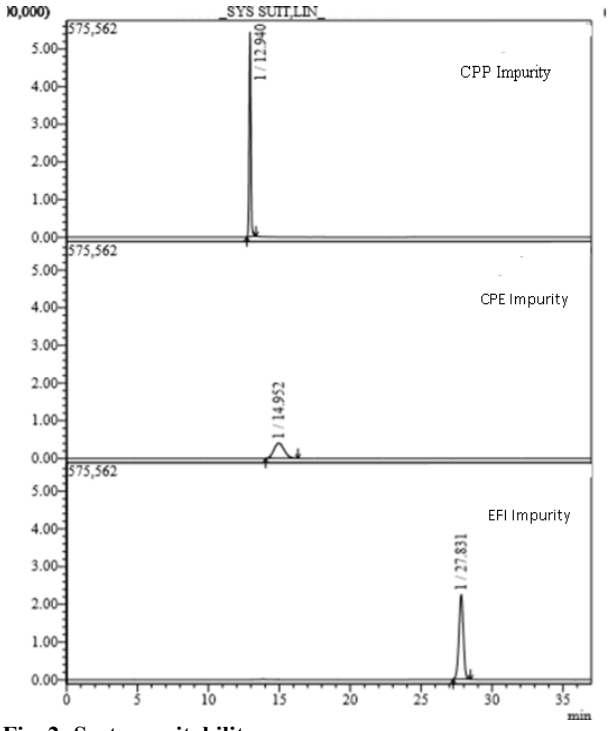

Fig. 2: System suitability

TABLE 3: BLANK INTERFERENCE RESULTS FOR CPP, CPE, AND EFI

Name	Retention time (min)	Interference found at the retention time of CPP, CPE, and EFI
CPP	12.940	No
CPE	14.952	No
EFI	27.831	No
Blank	NA	No

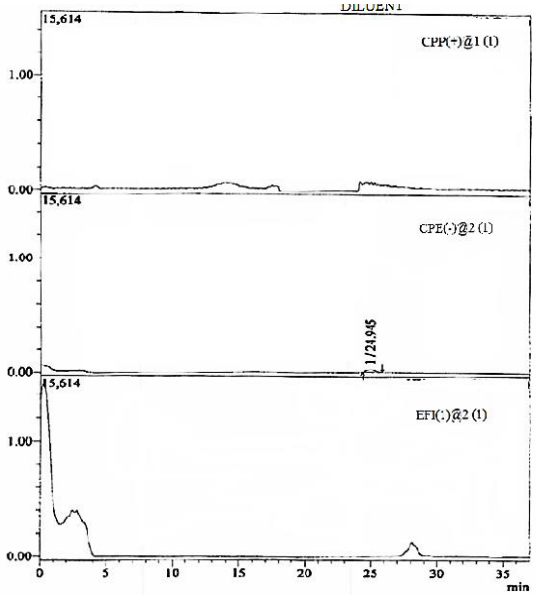

Fig. 3: Specificity

EFI impurities and injected (n=6) into the LC-MS/MS system from which peak area and RSD were derived, whereas form method precision, the % RSD data was obtained (Table 4). The results of % RSD of CPE, CPP and EFI impurities were set up to be within the acceptance limits (Table 5).

Furthermore, to create an in lines connection amongst the concentration/quantity of analyte existing in the sample taken and to the detector response, a set of standards of calibration were equipped. Moreover, linearity was calculated by formulating 5 to 150 % of standard concentrations.

The peak area of each sample was noted and plotted against respective concentrations. The Eqn.

TABLE 4: METHOD PRECISION RESULTS FOR CPP, CPE AND EFI

Preparation	CPP (ppm)	CPE (ppm)	EFI (ppm)
1	2.5	2.8	3.0
2	2.6	2.9	3.1
3	2.7	3.0	3.2
4	2.7	2.9	3.2
5	2.8	3.1	3.4
6	2.5	2.7	3.1
Average	2.6	2.9	3.2
% RSD	4.6	4.9	4.3

TABLE 5: INTERMEDIATE PRECISION RESULTS FOR CPP, CPE, AND EFI

Preparation	CPP (ppm)	CPE (ppm)	EFI (ppm)
1	2.5	2.6	2.5
2	2.5	2.6	2.5
3	2.6	2.6	2.5
4	2.5	2.6	2.5
5	2.5	2.6	2.5
6	2.6	2.7	2.6
Average	2.5	2.6	2.5
% RSD	2.0	1.6	1.6
% RSD for twelve determinations (precision & intermediate precision)			
Preparation	**CPP (ppm)**	**CPE (ppm)**	**EFI (ppm)**
1	2.5	2.8	3.0
2	2.6	2.9	3.1
3	2.7	3.0	3.2
4	2.7	2.9	3.2
5	2.8	3.1	3.4
6	2.5	2.7	3.1
7	2.5	2.6	2.5
8	2.5	2.6	2.5
9	2.6	2.6	2.5
10	2.5	2.6	2.5
11	2.5	2.6	2.5
12	2.6	2.7	2.6
Average	2.6	2.8	2.8
% RSD	4.0	6.5	12.4

TABLE 6: LINEARITY OF CPP, CPE, AND EFI

Levels	Linearity results					
	CPP IMP		CPE IMP		EFI IMP	
	Conc. in ppb	Area	Conc. in ppb	Area	Conc. in ppb	Area
5 %	2.508	259187	2.531	117538	2.569	305901
10 %	5.224	298024	5.272	137684	5.352	388002
25 %	12.538	1214979	12.653	619137	12.845	1482624
50 %	25.075	2661829	25.306	1341802	25.690	3110678
75 %	37.613	3873591	37.958	1991286	38.534	4712314
100 %	50.046	4817911	50.506	2451722	51.272	5857968
125 %	62.688	6522284	63.264	3349419	64.224	8271495
150 %	75.748	8502919	76.444	4296518	77.604	11062322
Slope	107848		55129		129684	
Intercept	-165458.4		-87086.3		-234051.5	
Correlation	0.9974		0.9994		0.9976	

TABLE 7: LOD AND LOQ ESTABLISHMENT RESULTS

Impurity name	Limit of detection	Limit of quantification
	in ppm	in ppm
CPP	0.04	0.125
CPE	0.04	0.125
EFI	0.04	0.125

TABLE 8: LOQ PRECISION RESULTS

LOQ Preparation	CPP (ppb)	CPE (ppb)	EFI (ppb)
Preparation-1	1597	1268	1396
Preparation-2	1601	1301	1403
Preparation-3	1583	1342	1405
Preparation-4	1576	1296	1413
Preparation-5	1605	1304	1426
Preparation-6	1597	1344	1419
Average	1593.2	1309.2	1410.3
%RSD	0.7	2.2	0.8

TABLE 9: LOQ ACCURACY RESULTS

LOQ preparation	CPP (ppm)	CPE (ppm)	EFI (ppm)
Preparation-1	110.6	116.2	115.3
Preparation-2	114.1	117.1	112.6
Preparation-3	115.7	117.5	111.5
Preparation-4	112.9	115.1	111.6
Preparation-5	116.7	121.6	115.8
Preparation-6	113.7	116.4	115.0

y=mx+b, defined the linear relation between impurity concentration (x) and respective peak area (y). From this analysis, a correlation coefficient (must be above 0.99) and slope-intercept values were derived (Table 6). Hence, the coefficient for CPE, CPP and EFI impurity peak was found to be 0.99 each, which indicated good linearity (Table 6). Similarly, a signal-to-noise ratio for impurities of limit of detection (LOD) and LOQ were predictable at 3:1 and 10:1, respectively (Table 7). Also, Table 8 and 9 shows the % RSD values of impurities (n=6), and the RSD of peak areas, respectively. The S/N ratio for performing precision of LOQ and LOQ solutions (n=6) was found to be 10:1.

A novel LC-MS/MS method was established and validated for the ultra-trace-level identification of three PGI, namely CPE, CPP, and EFI in EFV. For this analysis, a switching valve was used not only to guard the ESI source but also to afford favorable analysis conditions. Furthermore, the MRM-mode method afforded good sensitivity and the LOD and LOQ values were measured to be minimal for all the 3 impurities. Based on the data of analysis, it could be justified that this method is validated completely with acceptable parameters like accuracy, linearity, and precision. Moreover, this method was also found to be simple and sensitive. Hence, this analytical method could be used as a tool for quality control, as well as checking of PGI in EFV.

Acknowledgments:

The authors would like to thank the following for providing necessary facilities to carry out this research work. Koneru Lakshmaiah Education Foundation, Green Fields, Vaddeswaram-522 502, Guntur, Andhra Pradesh, India, and Perfomics Analytical Labs LLP, Nacharam, Hyderabad, Telangana 500076, India.

Conflict of interest:

The authors report no conflict of interest in this work.

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