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Original Article

Analytical Method Development and Validation of *Efavirenz* by Using RP- HPLC

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ABSTRACT

Background: *Efavirenz* is a widely used antiretroviral medication for managing HIV infection, known for its role as a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI). By targeting and inhibiting the reverse transcriptase enzyme, it effectively hinders the replication of HIV, making it a crucial component of combination therapy for the virus. **Materials and Methods:** The purpose of this research is to develop an accurate, selective and sensitive HPLC method for the regular quantification of *efavirenz* in laboratory settings. In this research, tert butyl methyl ether is used as a diluent and methanol is used as a mobile phase while the assay test involved analyzing the Active Pharmaceutical Ingredient (API) and market formulations of *efavirenz*. EFZ was extracted from market formulations, diluted in tert-butyl methyl ether and analyzed using the developed HPLC method. The results indicated that the method accurately quantified EFZ in both the API and market formulations. **Results:** The Separation process utilized using a (X-Bridge® C8 5 µm 4.6×250 mm), with mobile phase consisting of Methanol. The Flow rate was maintained at 0.8 mL/min and EFZ detection occurred at a wavelength of 252 nm, using a manual injection volume of 10 µL and retention time was found to be 3.7 min. Linearity was examined and proven at different concentrations in the range of EFZ (2-64 µg/mL), respectively and correlation coefficient R^2 was found to be 0.9998. The method achieved very good validation parameters LOQ about 0.03 mg/mL and LOD about 0.01 mg/mL for EFZ. The high value of recoveries obtained for EFZ indicates that the proposed method was found to be accurate. **Conclusion:** The developed HPLC method for quantifying *efavirenz* in pharmaceutical formulations is straightforward, accurate and reproducible. It satisfies all validation criteria, making it suitable for routine quality control and ensuring the consistency and safety of *efavirenz* products.

Keywords: *Efavirenz*, HIV/AIDS, RP-HPLC, LOD, LOQ, Rt.

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INTRODUCTION

Efavirenz, an antiretroviral medication primarily used in the treatment of HIV/AIDS. It belongs to the class of drugs known as Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs). It works by inhibiting the activity of the enzyme reverse tran scriptase, which is essential for the replication of HIV. By blocking reverse transcriptase, *Efavirenz* prevents HIV from converting its RNA to DNA, thereby halting the virus's ability to replicate and reducing the viral load in the body. *Efavirenz* is typically administered orally in the form of tablets or capsules. The recommended dosage for adults is usually 600 mg once daily, preferably taken on an empty stomach. Some formulations of efavirenz may be taken with food to reduce the risk of gastrointestinal side effects. *Efavirenz* can interact with a wide range of medications, including certain antibiotics, antifungals, antidepressants and anticonvulsants. It's crucial for patients to inform their healthcare provider about all the medications, supplements and herbal remedies they are taking to avoid potential interactions that could affect the efficacy or safety of *Efavirenz*. Antiretroviral drugs from different classes, *Efavirenz* helps to control the progression of HIV infection and improve immune function.[1,2] *Efavirenz* may include dizziness, drowsiness, trouble sleeping, vivid dreams and rash. Some individuals may experience psychiatric symptoms such as mood changes, depression, anxiety, or hallucinations, particularly during the initial weeks of treatment. Most side effects tend to be temporary and diminish over time as the body adjusts to the medication. However, it's essential for patients to report any severe or persistent side effects to their healthcare provider promptly.[3-5] *Efavirenz* is not recommended for use during pregnancy, particularly during the first trimester, due to concerns about potential adverse effects on fatal development. Pregnant individuals living with HIV should

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discuss the risks and benefits of *Efavirenz* with their healthcare provider to determine the most appropriate treatment regimen that minimizes the risk to the fetus. Similarly, caution is advised for breastfeeding individuals, as *Efavirenz* can pass into breast milk and potentially affect the infant. All antiretroviral drugs Strict adherence to the prescribed treatment regimen is crucial for preventing the development of resistance and maintaining viral suppression. Regular monitoring of viral load and CD4 cell count is necessary to assess treatment efficacy and detect any signs of virology failure or drug resistance.[6] Effective treatment with *Efavirenz* can significantly prolong the life expectancy and improve the quality of life for individuals living with HIV/AIDS. *Efavirenz* plays a vital role in the management of HIV/AIDS by effectively suppressing viral replication and improving immune function. However, patients should be aware of their side effects, drug interactions and the importance of adherence to treatment to maximize its benefits while minimizing risks. Regular communication with healthcare providers is essential for individualized management and optimal outcomes. *Efavirenz*, chemically known as (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2H-3,1-benzoxazin-2-one, is a synthetic compound with the chemical structure shown on Figure 1. *Efavirenz* molecular weight is 315.67 g/mol.[7-12]

While looking through the literature, we only came across a handful of analytical spectrophotometry, high-performance thin-layer chromatography and liquid chromatography-mass spectrometry in order to test *Efavirenz*, all of the methods that are mentioned are used. In 2021, Quantification and validation of stability-indicating RP-HPLC Method for *Efavirenz* in bulk and tablet dosage form using Quality by Design (QBD) studied by Gurumukhi *et al*. (2021). In 2021, Saha *et al*. they optimised the chromatographic conditions, adjusting the level of acetonitrile in the mobile phase to 51. 4 and 17% v/v, the phosphate buffer had a pH of 4. 04, while the flow rate was at 1. 25 mL/min. Using these optimized parameters, it was possible to achieve a retention time of 11. 031 min in RP-HPLC.

Analytical Method Validation

Detailed experimental procedures for testing the efficacy of an analytical parameters are provided. These approaches are classified in accordance with the ICH Guidelines.

Specificity

Specificity in the development of an analytical method pertains to its capacity to precisely and selectively detect the desired analyte amid the presence of other elements like impurities, degradation byproducts, or interferences from the matrix. In simple terms, it guarantees that the method can differentiate and quantify the intended analyte without being affected by other substances.

Linearity

Linearity in the validation of an analytical method signifies the method's capability to yield test outcomes that vary in direct accordance with the concentration of the substance being analysed within a predetermined range. Essentially, it implies that as the concentration of the substance rises or falls, the instrument's response, such as peak area or signal intensity, changes in proportion.

Precision

Precision in the validation of an analytical method measures how closely the results agree when repeatedly analysing the same sample using the same method under specific conditions. It evaluates the consistency and reproducibility of results obtained through multiple analyses under identical conditions.

Accuracy

Accuracy in analytical method validation is about how closely the obtained values match the true or accepted reference values. It evaluates how accurately the method reflects the actual concentration or amount of the substance being analysed. In simple terms, accuracy shows how near the measured values are to the true values.

Detection Limit (LOD)

The detection limit, or LOD (Limit of Detection), in analytical method validation, is the minimum concentration or quantity of an analyte that can be detected with confidence under specified experimental conditions. It signifies the lowest level of analyte in a sample that can be differentiated from background noise or baseline signal with a reasonable degree of certainty.

Quantification Limit (LOQ)

The Limit of Quantification (LOQ) refers to the minimum concentration of an analyte within a sample that can be reliably determined with both acceptable precision and accuracy under the specified experimental conditions.

Robustness

Upon assessing the results, it is evident that alterations in the mobile phase had a notable impact on the method. This suggests that the method remained unaffected despite variations in the mobile phase. Furthermore, the system suitability parameters remained within acceptable limits.

Ruggedness

Ruggedness, in the context of flow rate, refers to the ability of a system or device to maintain consistent performance despite variations or changes in operating conditions. In other words, it describes how resistant a system is to fluctuations or disturbances in the flow rate.[13-16]

MATERIALS AND METHODS

The API *Efavirenz* was gifted from KIET School of Pharmacy and a commercial tablet (Duovir-E Kit, Mfg- Cipla Pvt. Ltd.,) was purchase from the pharmacy. We used HPLC grade methanol and tert-Butyl Methyl Ether solvent and reagents during the research and was obtained from Thermo Fisher Scientific India Pvt. Ltd., in Mumbai, India, while the latter was procured from Central Drug House Pvt. Ltd., in New Delhi, India.

Instrumentation

The High-Performance Liquid Chromatography Analysis were performed using a water Shimadzu HPLC (model 2489) system which is manual, a column (X-Bridge® C8 5 µm 4.6×250 mm), Shimadzu UV Detection will be used in this step to identify, tell apart and determine exact amounts of particular components among mixtures and using a digital precision balance which was manufactured by Shimadzu Japan.

Chromatographic Condition

A reverse phase column [X-Bridge® (C8 5 µm 4.6×250 mm)], maintained in the presence of mobile phase (methanol) we kept the mobile phase flow rate at 0.8 mL/min and the eluent were measured at 252 nm.

Method development

Preparation of standard solution

A standard solution of 1000ppm was prepared by dissolving 10 mg EFZ in Tertiary Butyl Methyl Ether (TBME). The EFZ was accurately weighed and transfer to a 100 mL volumetric flask that had been thoroughly cleaned and dried and fill up to the mark with TBME and prepared 100 µg/mL has been sonicated raised to a final volume of 10 mL by subjecting to sonication for 10 min and drug concentration was adjusted to a range of 2-64 µg/mL by repeatedly diluting a standard stock solution.

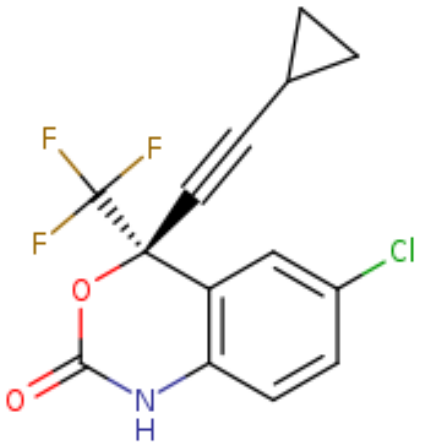

Figure 1: Chemical Structure of Efavirenz.

Preparation Of Sample Solution

Twenty tablets of EFZ-600 were measured by weight and the average weight of each pill was determined and transfer into mortar pestle and crushed the tablet to make a powder. The powder was transferred into the centrifugal tube for 20 min. Take the EFZ solution was filtered through the Whatman filter paper. The Solution equal to 200 ppm further produce dilution to obtain final concentration of 2-64 ppm. These solutions were injected into under chromatographic condition and peak areas were measured and chromatogram was recorded 3.7 min respectively.

Selection of the Solvent

The selection of the solvent is based on the solubility examine by the maximum absorbance observed through UV spectroscopy. Tertiary butyl methyl ether showed the high solubility for EFZ. As a result, tertiary butyl methyl ether was selecting as the preferred solvent and diluent for this research.

Selection of wavelength

The selection of wavelength of efavirenz was assessed using a variety of solvents, such as water, ethanol, methanol, tertiary butyl methyl ether, n-hexane and a mixture of solvents. To quantify the solubility in each solvent, using a UV spectrophotometer, the absorbance of efavirenz solutions at specific wavelengths was measured. The drug profile of efavirenz is as follows: the solubility of the drug is higher in methanol and tertiary butyl methyl ether with its maximum wavelength (λ_{max}) at 250 nm and 252nm.

Analytical Method Validation of HPLC and Result Discussion

Linearity

Calibration curve of EFZ was prepared from the stock solution, in a series of (2, 4, 8, 16, 32 and 64 ppm) separately in 10 mL volumetric flask and diluted with tertiary butyl methyl ether as a diluent, a volume of 10 µL from each solution was injected several times at each concentration level, the peak area that resulted was recorded in Table 1 and Figure 2 for API.

Similarly, the result of market formulation is shown in Table 2 and Figure 3.

Accuracy

In order to determine how precise the developed method is, the non-exist sample was spiked with three distinct concentrations of the efavirenz standard, which are 80%, 100% and 120% of the precise concentration of 24 ppm, 32 ppm, 40 ppm. The outcome was examined in three different versions and the percent recoveries and the relative standard deviation of the concentration were calculated. The accuracy result of EFZ API was recorded in Table 3 and similarly the accuracy result of EFZ Market formulation was recorded in Table 4.

Table 1: Linearity Result for API.

Linearity				
Sl. No.	**CONC.(PPM)**	**RT**	**AUC**	**Height**
1		3.75	283832	31644
2	4	3.754	454224	58939
3	8	3.775	725705	87267
4	16	3.754	1342034	177816
5	32	3.745	2269076	306684
6	64	3.751	4549698	640256
	SUM	22.529	9624569	1302606
	MEAN	3.754833	1604095	217101
	SD	0.01042	1613959	230171.6593
	RSD	0.277497	100.6149	106.0205431

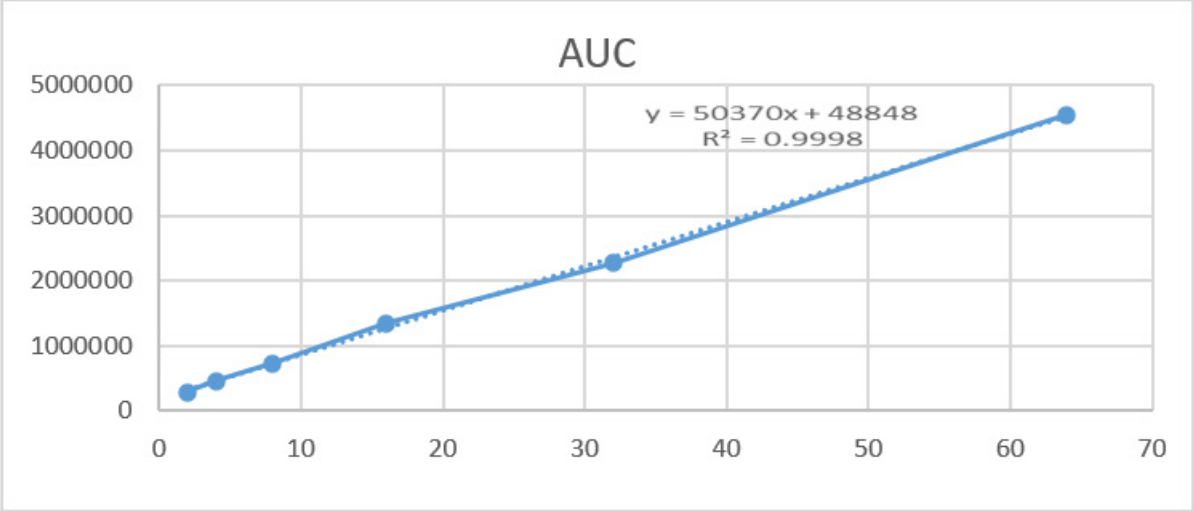

Figure 2: Peak Area for API.

Table 2: Linearity Result for Market Formulation.

Linearity				
Sl. No.	**CONC (PPM)**	**RT**	**AUC**	**Height**
1	2	3.75	362826	31644
2	4	3.754	496477	58939
3	8	3.775	901619	87267
4	16	3.754	1584979	177816
5	32	3.745	2993785	306684
6	64	3.751	5786973	640256
	SUM	22.529	12126659	1302606
	MEAN	3.754833333	2021109.833	217101
	SD	0.010419533	2080658.682	230171.6593
	RSD	0.277496549	102.946344	106.0205431

Robustness

The robustness was measured by changing the flow rate (± 10%). The system approaches must be satisfied as part of the method considerations. The robustness was carried out at different flow rate. The robustness results of EFZ for API were shown in Table 5 and similarly the robustness result of EFZ for Market Formulation were shown in Table 6.

Limit of detection and limit of quantification

The HPLC method of *Efavirenz* detection serves as the basis for the limit of detection. The statistical equation is used to compute the LOD and LOQ values and the LOQ value is multiplied by 3x. The LOQ and LOD result of EFZ API were shown in Tables 7 and 8.

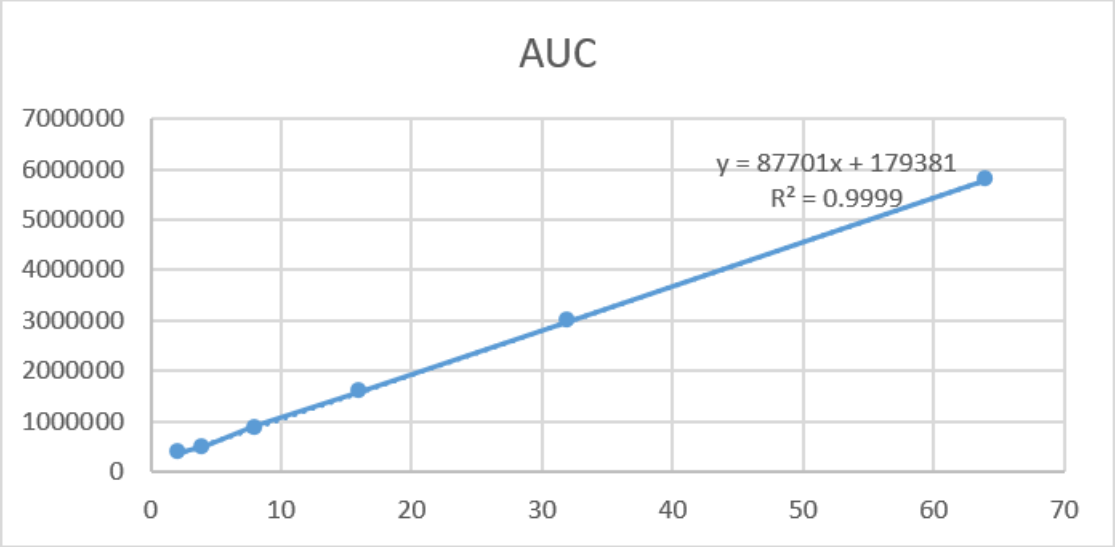

Figure 3: Peak Area for Market formulation.

Table 3: Accuracy Result for API

Conc. (µg/mL)	Mean±SD	%RSD	% Recovery
80%	1877506±10801.41	0.5753	100
100%	2273655±44133.426	1.9410	102
120%	2731049±49304.82	1.8053	99.2

Table 4: Accuracy Result for Market formulation.

Conc. (µg/mL)	Mean±SD	%RSD	% Recovery
80%	1468349±11016.8	0.7502	98.4
100%	1761059±3339.59	1.8961	99.3
120%	2150049 ± 38549.30	1.792	102

Table 5: Robustness Result for API.

	0.7 mL/min				0.8 mL/min			0.9 mL/min	
CONC. (PPM)	RT	AUC	Height	RT	AUC	Height	RT	AUC	Height
32	4.26	3085919	365040	3.75	2319866	322061	3.35	2058122	317079
32	4.20	3016100	364220	3.71	2397170	329501	3.34	2115875	330822
32	4.20	3118518	377473	3.73	2378292	337668	3.33	2116436	327883
Mean		3073512	368911		2365109	329743.3		2096811	325261.3
SD		42722.4	6063.49		32907.08	6373.83		27358.21	5908.88
RSD%		1.3900	1.6432		1.391356	1.9329		1.304753	1.8166

Table 6: Robustness Result for Market Formulation.

	0.7 mL/min				0.8 mL/min			0.9 mL/min	
CONC. (PPM)	RT	AUC	Height	RT	AUC	Height	RT	AUC	Height
16	4.26	2086675	285735	3.72	1663565	239937	3.33	1598566	249998
16	4.25	2015395	278471	3.72	1699087	247153	3.35	1607926	251491
16	4.26	2083972	273689	3.73	1692014	251366	3.34	1593271	247298
Mean		2062014	279298.3		1684888.1	246152		1599921	249595.1
SD		32983.07	4952.43		15352.10	4719.25		6059.11	1735.26
RSD%		1.5995	1.7731		0.911164	1.9172		0.37871	0.69523

Table 7: LOQ Result for API.

Sl. No.	CONC (PPM)	RT	AUC	Height
1	0.03	3.745	164179	13641
2	0.03	3.734	165138	13670
3	0.03	3.744	166978	13714
4	0.03	3.724	161168	13546
5	0.03	3.733	161693	13563
6	0.03	3.751	168232	13735
	SUM	22.455	987388	81869
	MEAN	3.7425	164564.6	13644.83
	SD	0.00418	2811.93	77.49430
	RSD	0.11177	1.70870	0.567938

Table 8: LOD Result for API.

Sl. No.	CONC (PPM)	RT	AUC	Height
1	0.01	3.815	100894	8966
2	0.01	3.784	106122	9180
3	0.01	3.811	109584	9290
	SUM	11.41	316600	27436
	MEAN	3.80333	105533.3	9145.33
	SD	0.01686	4374.805	164.758
	RSD	0.44335	4.145425	1.80155

Table 9: LOQ Result for Market Formulation.

Sl. No.	CONC (PPM)	RT	AUC	Height
1	0.03	3.752	155332	13133
2	0.03	3.761	151758	13008
3	0.03	3.726	156243	13159
4	0.03	3.740	149270	12923
5	0.03	3.770	157920	13191
6	0.03	3.735	151413	12998
	SUM	22.608	921936	78412
	MEAN	3.768	153656	13068.6
	SD	4.8648	3333.27	106.918
	RSD	1.2911	2.16931	0.8181

Table10: LOD Result for Market Formulation.

Sl. No.	CONC (PPM)	RT	AUC	Height
1	0.01	3.442	7068	868
2	0.01	3.447	7489	1085
3	0.01	3.469	7073	1244
	SUM	10.358	22630	3197
	MEAN	3.4526	7543.33	1065.66
	SD	0.0143	504.698	188.744
	RSD	0.4160	6.69065	17.7113

Table 11: Precision Result for API.

Sl. No.	CONC. (PPM)	Interday 1		Interday 2		Interday 3	
		Mean± SD	%RSD	Mean± SD	%RSD	Mean ±SD	%RSD
1	2	213725.5±188.5	0.08	210906± 459	0.21	212315±1452.75	1.23
2	4	413725±5022.57	1.21	426424.5±7114.20	1.66	426789±2542.756	0.59
3	8	658114.5±7252.79	1.1	671821±2527.19	0.37	661682.5±2683.47	0.4
4	16	1354547±13454.82	0.99	1346703±18035.46	1.33	1352085.5±5617.9	0.41
5	32	2765512±69559.50	2.51	2713539±17686.15	0.65	2737404.5±27391.19	1
6	64	5183508.5±39298.87	0.75	5108917±106489.51	2.08	5149588±69399.70	1.34

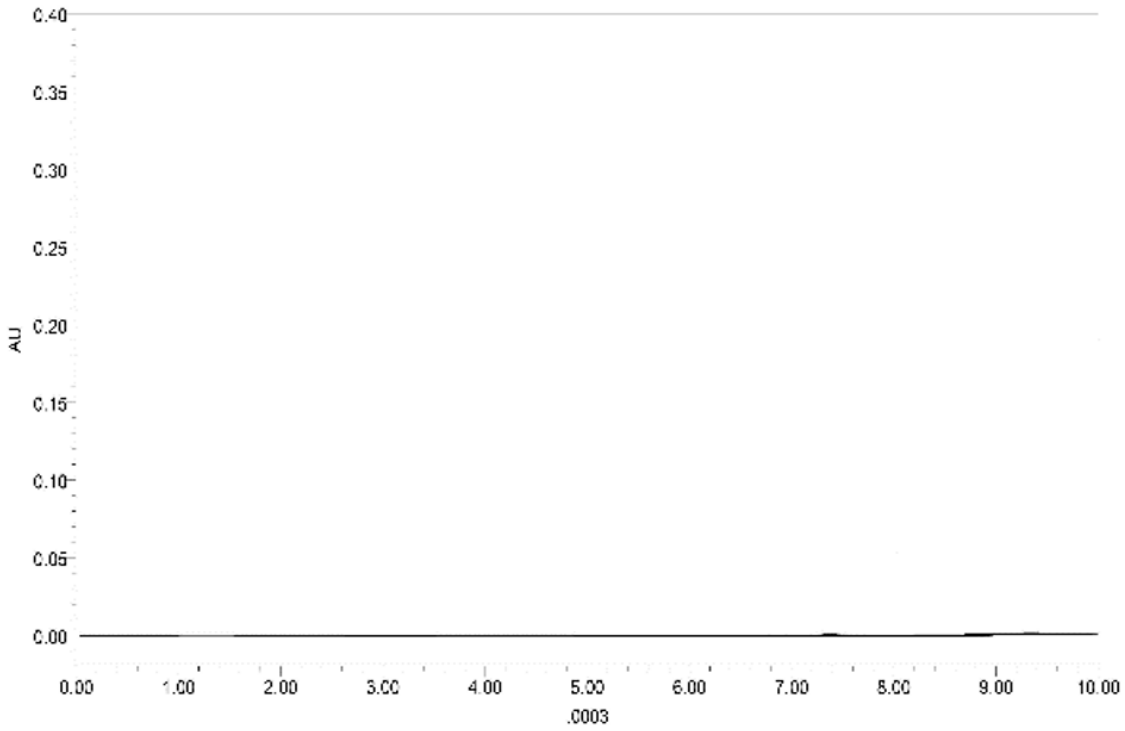

Figure 4: Blank.

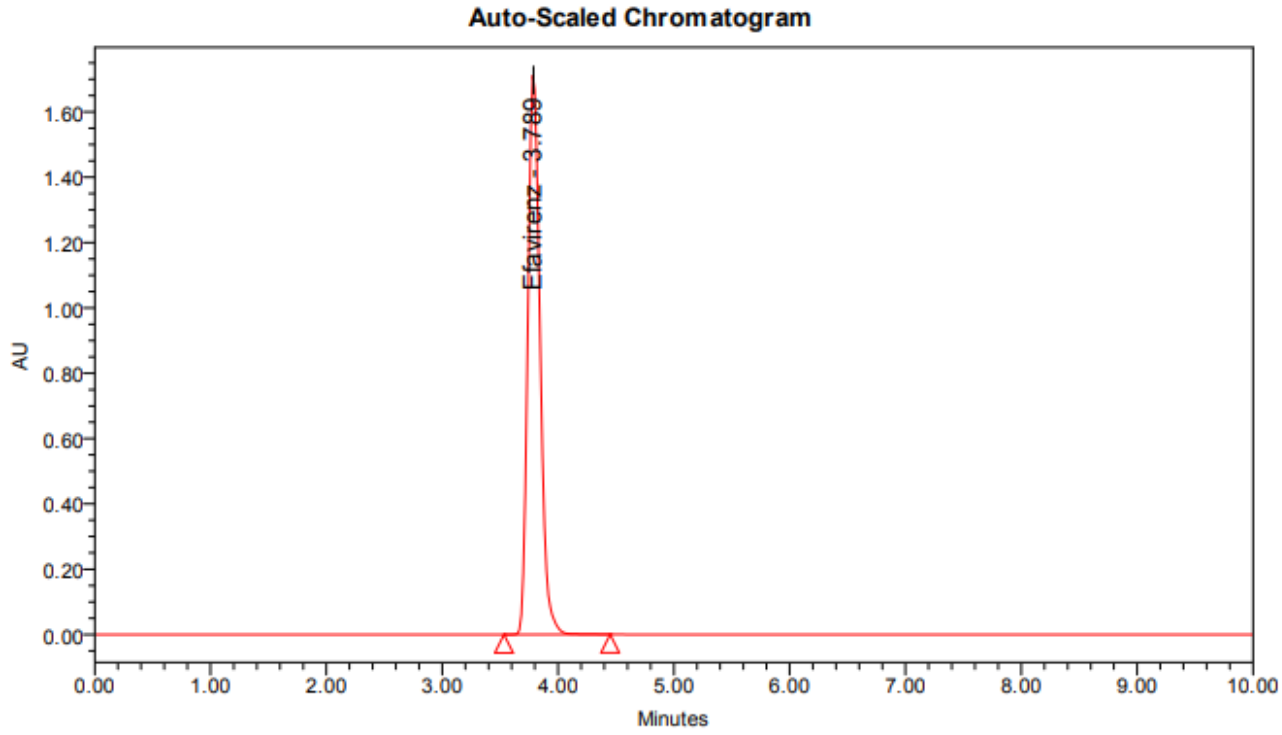

Figure 5: Standard..

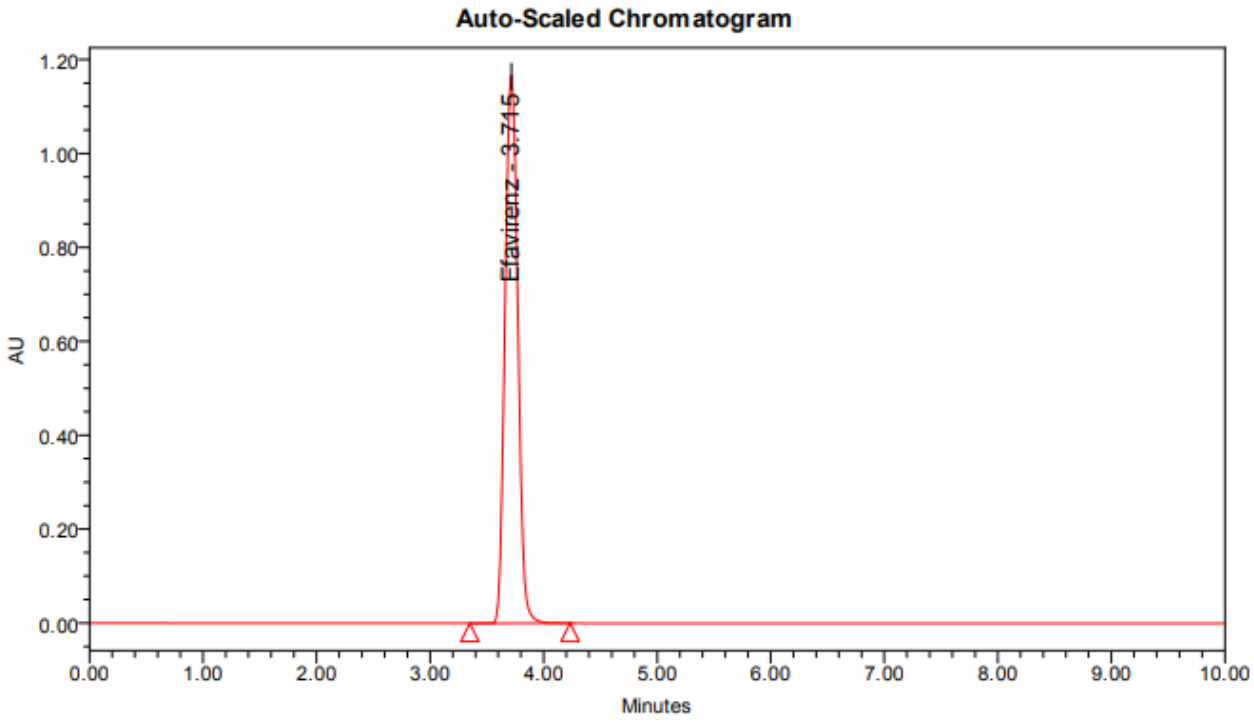

Figure 6: Sample.

Similarly, the LOQ and LOD result of EFZ Market formulation were shown in Tables 9 and 10 respectively.

$$\textbf{Equation: } LOD=\frac{3.3\Omega}{S} \; LOQ=\frac{10\Omega}{S}$$

Where, Ω =Standard deviation of the y-intercept of the calibration curve and

S=Slope of the calibration line.

Specificity

The specificity of the proposed method was assessed through chromatograms of *Efavirenz* API, the commercial product and blank. As expected, no interference was observed since the retention times of the sample and the standard were closely related. The blank chromatogram was shown in Figure 4 and standard chromatogram was shown in Figure 5 and sample chromatogram was shown in Figure 6 respectively.

Precision

Precision defines the random errors associated with reproducibility and repeatability of an experiment. The method to be emplaced is quantified using the terms %RSD (relative standard deviation). For percentage agreement, %RSD of less than 2% is considered acceptable. The precision studies associated with the developed analytical techniques also entailed determination of intra-day variability and inter-day variability. As a means to evaluate accuracy, the experiments were carried out based on repeatability and inter-day precision. The results were found to have %RSD in acceptable limit that is not more than 2%. The precision results for EFZ API were shown in Table 11 and for market formulation were shown in Table 12 respectively.

Ruggedness

The repeatability of the method validation was checked where the results of analyst A and analyst B did not differ significantly with the percentage of relative standard deviation being below 2% which means that this method is quite reliable. The outcomes referring to the ruggedness of the method for the Active Pharmaceutical Ingredient (API) at the levels of 32 ppm and 64 ppm. The ruggedness result for EFZ API for 32 ppm and 64 ppm were shown in Tables 13 and 14 respectively. Similarly, the result

Table 12: Precision Result for Market Formulation.

Sl. No.	CONC. (PPM)	Interday 1		Interday 2		Interday 3	
		Mean± SD	%RSD	Mean± SD	%RSD	Mean ±SD	%RSD
1	2	364800.5±2792.3	0.7	366645.5± 14441.2	1.9	363663±4408.1	1.21
2	4	506736.5±366.98	0.7	503956±2429.61	0.4	518247.5±79.90	0.1
3	8	909272.5±10823.6	1.19	914098±1050.7	0.11	912796±5316.02	0.58
4	16	1662079.5±32384.7	1.9	1683196.5±15053.5	0.8	1685889.5±18386.8	1.09
5	32	2954172.5±56020.5	1.89	2992877±620.83	0.2	3068136.5±26645.9	0.8
6	64	5825363±5491.65	0.93	5770614.5±15585.3	0.27	5836441±16297.3	0.27

Table 13: Ruggedness Result for API for 32 ppm.

Ruggedness					
	Sl. No	CONC (PPM)	RT	AUC	Height
Analyst1 Ishu	1	32	3.771	2179503	283251
	2	32	3.798	2201015	251172
Analyst2 Abhinav	1	32	3.75	2189635	293972
	2	32	3.745	2276706	297293
		SUM	15.064	8846859	1125688
		MEAN	3.766	2211714.8	281422
		SD	0.02412	44209.574	21038.052
		RSD	0.64059	1.9988823	7.4756245

Table 14: Ruggedness Result for API for 64 ppm.

Ruggedness					
	Sl. No.	CONC (PPM)	RT	AUC	Height
Analyst1 Ishu	1	64	3.756	4913727	638446
	2	64	3.753	4897393	646778
Analylst2 Abhinav	1	64	3.751	5003542	658320
	2	64	3.747	5057956	639247
		SUM	15.007	19872618	2582791
		MEAN	3.7517	4968154.5	645697.75
		SD	0.0037	75908.118	9213.906
		RSD	0.1006	1.5278	1.4269689

Table 15: Ruggedness Result for Market Formulation for 32 ppm.

Ruggedness					
	Sl. No.	CONC (PPM)	RT	AUC	Height
Analyst1 Ishu	1	32	3.727	3575544	512756
	2	32	3.727	3576443	516270
Analylst2 Abhinav	1	32	3.721	3630747	507126
	2	32	3.753	3604224	490177
		SUM	14.928	14386958	2026329
		MEAN	3.732	3596739.5	506582.25
		SD	0.01428	26291.47472	11567.131
		RSD	0.38271	0.730980787	2.2833668

Table 16: Ruggedness Result for Market Formulation for 64 ppm.

Ruggedness					
	Sl. No.	CONC (PPM)	RT	AUC	Height
Analyst 1Ishu	1	64	3.717	5911714	858304
	2	64	3.725	6091111	854901
Analyst 2 Abhinav	1	64	3.722	6098924	852333
	2	64	3.717	6198726	882939
		SUM	14.881	2430047	3448477
		MEAN	3.72025	6075118.70	862119.25
		SD	0.00394	119446.37	14093.63
		RSD	0.10611	1.966156	1.634766

for EFZ market formulation for 32 ppm and 64 ppm were shown in Tables 15 and 16 respectively.

CONCLUSION

In conclusion, the present work has demonstrated that the analysis time of the HPLC method has been reduced thus making the technique more efficient. HPLC is one of the most commonly used analytical techniques for creating new techniques and in the separation of compounds in various samples. This technique mostly uses a C-8 stationary phase and a mobile phase that usually consists of methanol. The sample with a volume of 10 microliters is delivered by a mobile phase to a detector that determines the retention time 3.7 min and height and width of the peak and the area under the curve. In the previous literature review Rt was found to be 6- 11 min. These factors are important when evaluating the method for its recovery studies, such as repeatability, linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ). Furthermore, the modifications made to this HPLC method are aimed to provide better accuracy and reproducibility in several analytical processes, making it even more valuable in the field of chemical analysis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

EFZ: Efavirenz; **TBME:** Tert Butyl Methyl Ether; **PPM:** Parts per million; **API:** Active Pharmaceutical Ingredient; **HPLC:** High Performance Liquid Chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **CONC:** Concentration; **Rt:** Retention Time; **RSD:** Relative Standard Deviation; **SD:** Standard Deviation.

SUMMARY

This research demonstrates an improved HPLC method for analyzing the antiretroviral drug efavirenz, used in the treatment of HIV. The modified method reduces the retention time to 3.7 min from the previously reported 6-11 min, enhancing efficiency. Efavirenz, typically analyzed using a C-8 stationary phase and a methanol mobile phase, now benefits from better accuracy and reproducibility with this new method. This advancement is particularly valuable for chemical analysis, including recovery studies and determining parameters such as repeatability, linearity, LOD and LOQ.

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