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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING METHOD FOR SIMULTANEOUS ESTIMATION OF EFAVIRENZ AND PYRAZINAMIDE BY USING RP-HPLC METHOD

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Abstract:
A basic and specific LC strategy is depicted for the assurance of Efavirenz and pyrazinamide measurements structures. Chromatographic partition was accomplished on a c18 segment utilizing portable stage comprising of a blend of Sodium di hydrogen phosphate cushion (KH2PO4 and K2HPO4) Acetonitrile (40:60v/v), with identification of 252nm. Linearity was seen in the range 100-500 µg/ml Efavirenz (r2 =0.99) and 100-500µg/ml for pyrazinamide (r2 =0.999) for the measure of medications assessed by the proposed strategies was in acceptable concurrence with the name guarantee. The proposed techniques were approved. The exactness of the techniques was evaluated by recuperation learns at three distinct levels. Recuperation tests showed the nonattendance of obstruction from usually experienced pharmaceutical added substances. The strategy was seen as exact as demonstrated by the repeatability examination, indicating %RSD under 2. Every single factual datum demonstrates legitimacy of the techniques and can be utilized for routine investigation of pharmaceutical measurement structure. 400 volumes of pottasium di hydrogen phosphate and Di potasium hidrozen phosphate support and 600 volumes of ACN were readied. The versatile stage was sonicated for 10min to evacuate gases.

Keywords: *Efavirenz, Pyrazinamide, RP-HPLC, Simultaneous estimation.*

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INTRODUCTION:

Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for the treatment of a human immunodeficiency virus (HIV) type 1.[1] For HIV infection that has not previously been treated, efavirenz and lamivudine in combination with zidovudine or tenofovir is the preferred NNRTI-based regimen. Efavirenz is also used in combination with other antiretroviral agents as part of an expanded postexposure prophylaxis regimen to prevent HIV transmission for those exposed to materials associated with a high risk for HIV transmission.[2] IUPAC Name is (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1H-3,1-benzoxazin-2-one. Molecular formula is $C_{14}H_9ClF_3NO_2$. Molecular weight is 315.6 g/mol.

Pyrazinamide is an antituberculosis agent used as a component of tuberculosis (TB) treatment. Pyrazinamide diffuses into active M. tuberculosis that express pyrazinamidase enzyme that converts pyrazinamide to the active form pyrazinoic acid. Pyrazinoic acid can leak out under acidic conditions to be converted to the protonated conjugate acid, which is readily diffused back into the bacilli and accumulate intracellularly. The net effect is that more pyrazinoic acid accumulates inside the bacillus at acid pH than at neutral pH. Pyrazinoic acid was thought to inhibit the enzyme fatty acid synthase (FAS) I, which is required by the bacterium to synthesise fatty acids. However, this theory was thought to have been discounted.[3] However, further studies reproduced the results of FAS I inhibition as the putative mechanism first in whole cell assay of replicating M. tuberculosis bacilli which have shown that pyrazinoic acid and its ester inhibit the synthesis of fatty acids.[4] IUPAC Name is pyrazine-2-carboxamide. Molecular formula is $C_5H_5N_3O$. Molecular weight is 123.1 g/mol.

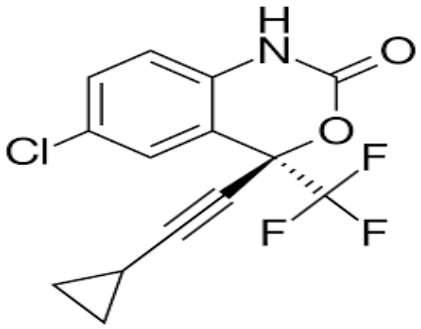

Figure 1: Structure of Efavirenz

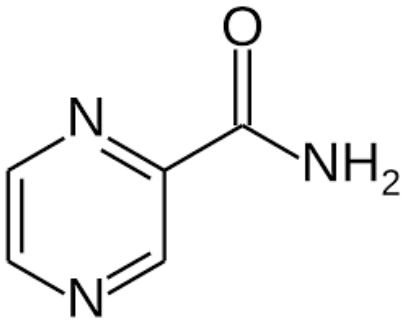

Figure 2: Structure of Pyrazinamide

The literature survey revealed that There are Various analytical methods were carried out for the estimation of Efavirenz and Pyrazinamide as a single or combined with other drugs in pharmaceutical dosages Literature survey reveals that the retention time for the simultaneous estimation of Efavirenz and Pyrazinamide is more. Hence the present study, we had made an attempt to develop simple, accurate, precise, less time consuming and with less retention time using RP-HPLC for the simultaneous estimation of Efavirenz and Pyrazinamide in bulk and pharmaceutical dosage form by RP-HPLC [5-12]. To validate the developed method in accordance with ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the drug in its dosage form.

MATERIALS AND METHODS:

Chemicals and Reagents: Efavirenz and Pyrazinamide were Purchased from market. NaH_2PO_4 was analytical grade supplied by Finerchem limited, Orthophosphoric acid (Merck), and Water and Methanol for HPLC (Lichrosolv (Merck).

Equipment and Chromatographic Conditions: The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, UV detector and Empower 2 software. Analysis was carried out at 252 nm with column INERTSIL column, C18(150x4.6 ID) 5µm, dimensions at 25^0C temperature. The optimized mobile phase consists of Mixed phosphate buffer: Acetonitrile (30:70). Flow rate was maintained at 1 ml/min.

Determination of Working Wavelength (λmax)

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately.

Preparation of standard stock solution of Efavirenz

50mg of Efavirenz was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 0.2ml to 10ml with methanol.

Preparation of standard stock solution of pyrazinamide

50mg of Pyrazinamide was weighed in to 100ml volumetric flask and dissolved in Methanol and then dilute up to the mark with methanol and prepare 10 µg /ml of solution by diluting 0.2 ml to 10ml with methanol.

Preparation of mixed standard solution

Weigh accurately 80mg of Efavirenz and 100 mg of Pyrazinamide in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase From above stock solution 80µg/ml of Efavirenz and 100 µg/ml of Pyrazinamide is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Preparation of sample solution:

5tablets (each tablet contains 8mg of Efavirenz and 10mg of Pyrazinamide) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of Efavirenz (80µg/ml) and Pyrazinamide (100µg/ml) were prepared by dissolving weight equivalent to 80mg of Efavirenz and 100 mg of Pyrazinamide and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 80µg/ml of Efavirenz and 100 µg/ml of Pyrazinamide was made by adding 1.0ml of stock solution to 10 ml of mobile phase.

METHOD:

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System suitability parameters: To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.0 ml/min to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 20 µL of standard into INERTSIL column, C18(150x4.6 ID) 5µm, the mobile phase of composition Mixed phosphate buffer: Acetonitrile (30:70) was allowed to flow through the column at a flow rate of 1.0 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Assay of pharmaceutical formulation: The proposed validated method was successfully applied to determine Efavirenz and Pyrazinamide in their pharmaceutical dosage form. The result obtained for was comparable with the corresponding labeled amounts and they were shown in Table-2.

Validation of Analytical method:

Linearity: The linearity study was performed for the concentration of 100ppm and 500ppm level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are shown in table 3.

Accuracy studies: The accuracy was determined by help of recovery study. The recovery method carried out at three level 80%, 100%, 120% and 80%, 100%, 120% Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Efavirenz and Pyrazinamide and calculate the individual recovery and mean recovery values. The results are shown in table 4.

Precision Studies: precision was calculated from Coefficient of variance for six replicate injections of the standard. The standard solution was injected for six times and measured the area for all six Injections in HPLC. The %RSD for the area of six replicate injections was found. The results are shown in table 5.

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. The flow rate was varied at 0.6 ml/min to 0.8 ml/min. The results are shown in table 6.

LOD and LOQ: The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The results are shown in table 7.

$LOD = 3.3\sigma/S$ and

LOQ = 10 σ/S, where

σ= Standard deviation of y intercept of regression line,

S = Slope of the calibration curve

RESULTS AND DISCUSSION:

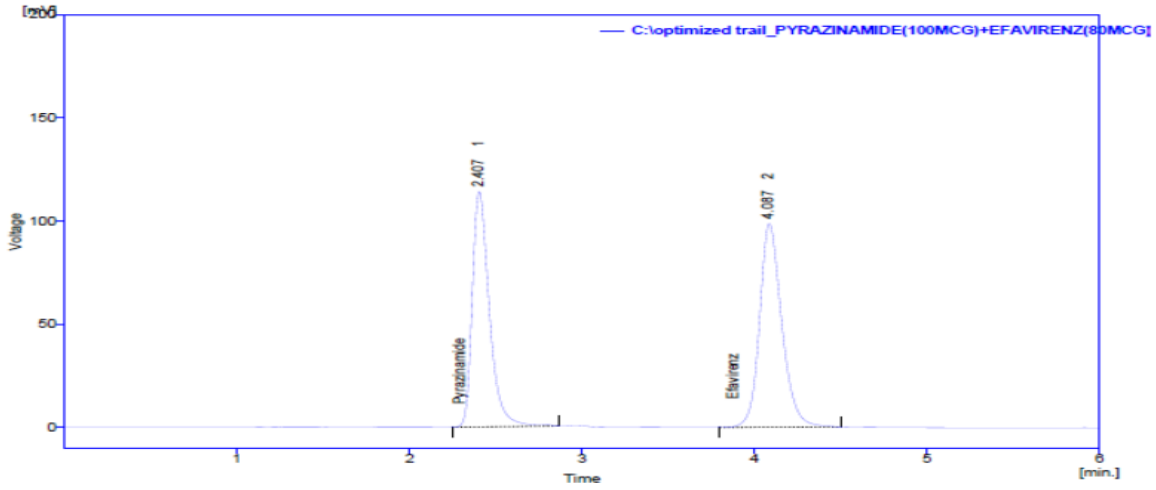

Figure 3: Standard chromatogram

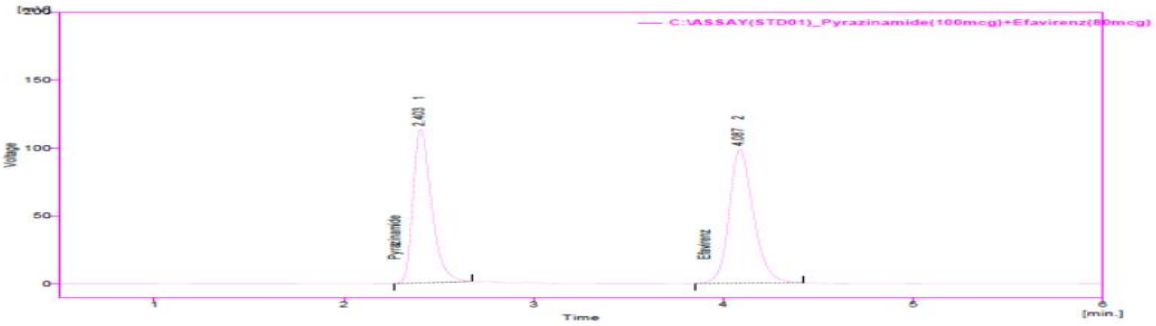

Figure 4: Sample chromatogram

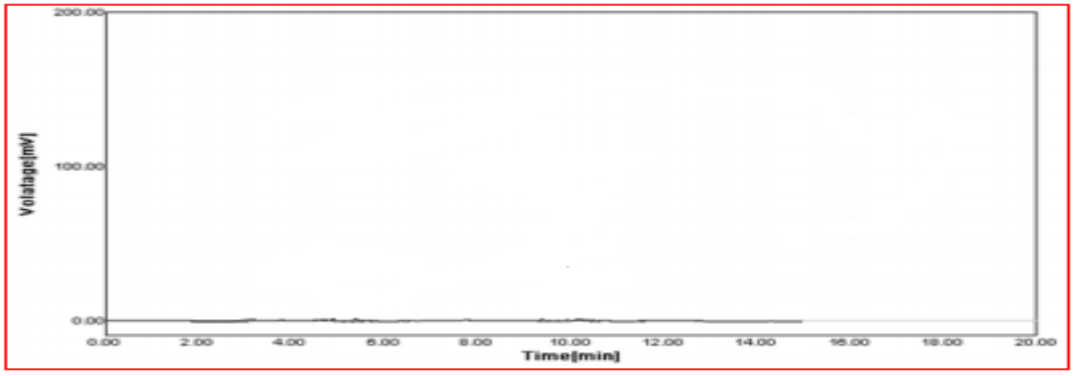

Figure 5: Blank chromatogram

Table 1: System suitability parameters

Parameter	Efavirenz (n= 5)	Pyrazinamide (n= 5)
Retention time (Rt) (min)	2.40	4.08
Theoretical plates (N)	3535.2	6511.6
Tailing factor (N) f	1.51	1.17

Table 2: Assay results for Efavirenz and Pyrazinamide

	Label Claim (mg)	**% Assay**
Efavirenz	8	100.03
Pyrazinamide	10	99.75

Table 3: Linearity results of Efavirenz and Pyrazinamide

	Efavirenz			Pyrazinamide	
Conc. (µg/ml)	Area mean ± SD	% RSD	Conc. (µg/ml)	Area mean ± SD	% RSD
5	152.064 ± 0.52	0.33	80	1101.8 ± 3.73	0.34
10	274.652 ± 1.20	0.43	160	2318.865 ±1.58	0.07
15	396.602 ± 3.26	0.81	240	3620.355 ± 3.11	0.09
20	504.71 ± 2.98	0.58	320	4881.735 ± 2.28	0.05
25	625.31 ± 1.44	0.22	400	6274.02 ± 6.39	0.10

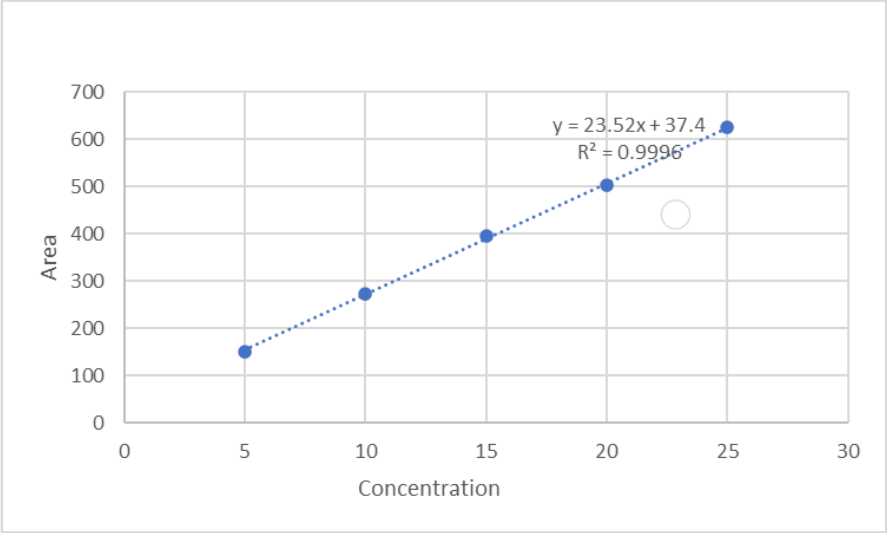

Figure 6: Linearity graph for Efavirenz

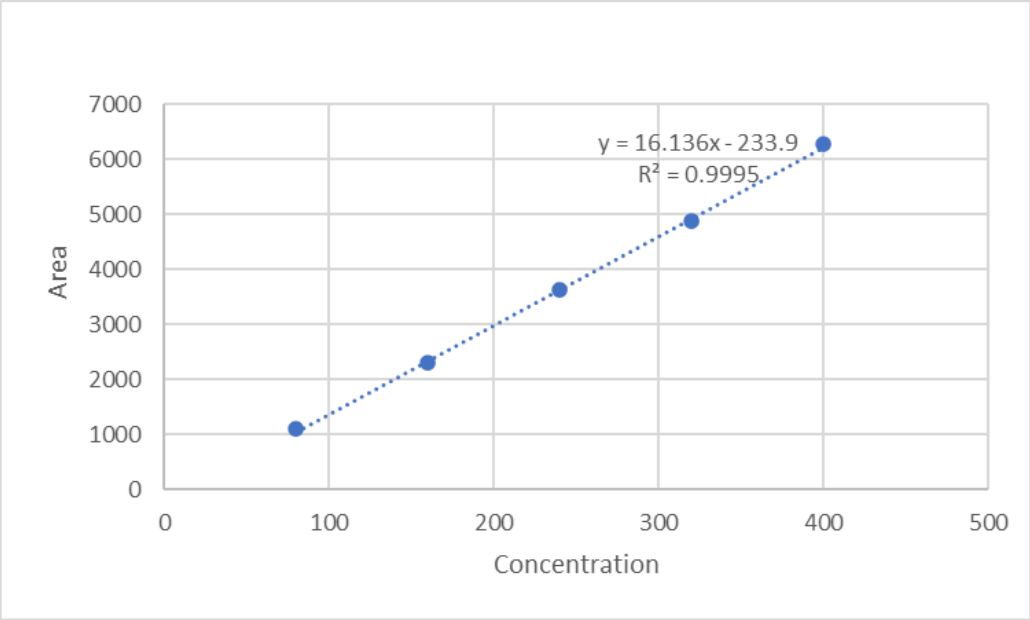

Figure 7: Linearity graph for Pyrazinamide

Table 4: Showing accuracy results for Efavirenz and Pyrazinamide

Drug	Conc. Level (%)	Sample amount (µg/ml)	Standard amount added (µg/ml)	Total amount found (µg/ml)	Total amount recovered (µg/ml)	% Recovery ± SD
Efavirenz	80%	10	8	18	8.01 ± 0.12	100.37 ± 1.55
	100%	10	10	20	10.55 ± 0.13	100.02 ± 1.78
	120%	10	12	22	12.31± 0.21	101.54 ± 1.07
Pyrazinamide	80%	160	128	288	127.43 ± 0.95	99.57 ± 0.75
	100%	160	160	320	162.81 ± 2.41	101.77 ± 1.51
	120%	160	192	352	196.35 ± 0.41	102.21 ± 0.41

Table 5: Precision results for Efavirenz and Pyrazinamide

Parameters	Efavirenz			Pyrazinamide		
	Conc. (µg/ml)	Area Mean ± SD	%RSD	Conc. (µg/ml)	Area Mean ± SD	%RSD
Repeatability	20	510.82 ± 10.15	1.98	320	4829.06 ± 26.44	0.54
Intra-day	10	278.85 ± 3.30	1.17	160	2343.58 ± 25.39	1.08
	15	380.10 ± 7.23	1.9	240	3622.95 ± 29.16	0.8
	20	513.44 ± 5.96	1.16	320	4852.93 ± 10.42	0.21
Inter-day	10	269.23 ± 4.31	1.6	160	2341.07 ± 35.47	1.52
	15	385.62 ± 6.54	1.7	240	3607.29 ± 7.43	0.21
	20	494.91 ± 3.92	0.79	320	4882.17 ± 12.66	0.26

Table 6: Robustness results for Efavirenz and Pyrazinamide

Parameters	Change level	Area (n=2)		Area(n=2)	
		Efavirenz		Pyrazinamide	
		Area (Mean ± SD)	% RSD	Area (Mean ± SD)	% RSD
Flow rate	0.6 ml/min	528.38 ± 6.71	1.27	5545.68 ± 41.51	0.75
(± 0.1 ml/min-1)	0.8 ml/min	635.10 ± 5.73	0.9	6001.63 ± 52.09	0.87
Wavelength (± 1ml/min-1)	273 nm	629.8 ± 5.35	0.85	6115.60 ± 20.30	0.33
	275 nm	678.37 ± 12.61	1.86	6007.1 ± 8.29	0.14
Mobile phase composition	71:29 ml	710.1 ± 8.92	1.26	5643.3 ± 49.53	0.88
(± 1ml/min-1)	69:31 ml	810.86 ± 7.21	0.89	6060.05 ± 39.89	0.66

Table 7: LOD, LOQ of Efavirenz and Pyrazinamide

S.NO	**Drug**	**LOD**	**LOQ**
1	Efavirenz hydrochloride	0.262	0.68
2	Pyrazinamide	0.6	2.11

CONCLUSION:

The Developed HPLC method was validated and it was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Efavirenz and Pyrazinamide in its pure form and in its pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of Efavirenz and Pyrazinamide in pure and its pharmaceutical dosage forms.

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