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METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF RITONAVIR, LOPINAVIR AND EFAVIRENZ BY RP- HPLC

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ABSTRACT

A novel HPLC method was developed and validated for simultaneous estimation of ritonavir, lopinavir and efavirenz in tablet dosage form. In this method, an analytical shimadzu (150mm x 3.0mm x 3µm) column was used for chromatographic separation with a mixture of ACN and 0.02M potassium Di hydrogen Ortho phosphate as the mobile phase in the ratio 60:40%V/V. The flow rate of mobile phase was maintained at 1.4ml/min. The UV detection was performed at 233nm. Injection volume was set at 20µl. Run time was maintained at 10minutes. Retention time of Ritonavir, Lopinavir and Efavirenz were 5.919, 6.957, 8.176.The % RSD of Ritonavir, Lopinavir and Efavirenz was found to be 0.32, 0.44 and 0.39. The method was fully validated in terms of system suitability, specificity, linearity, precision, accuracy, and robustness, limit of detection and limit of quantification according to official guidelines of ICH Q2 (R1).

Keywords: ICH, Ritonavir, Lopinavir and Efavirenz, shimadzu

1. INTRODUCTION:

Efavirenz:

Efavirenz is a non- nucleoside reverse transcriptase inhibitor (NNRTI). It is used as a highly active antiretroviral therapy (HAART) for the treatment of a human immune deficiency virus type-1. Efavirenz

is chemically -6-chloro-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3, 1-benzoxazine-2-one. Efavirenz empirical formula is C14H9ClF3NO2. It is a white crystalline powder with a molecular mass is 315.7g/mol. It is practically insoluble in water, freely soluble in methanol and acetonitrile **(Figure 1)**.

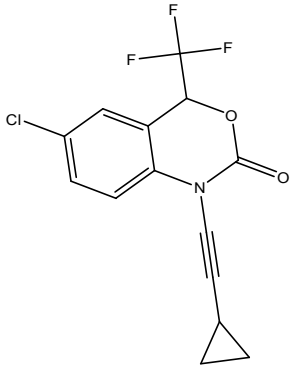

Figure 1: Structure of Efavirenz

Lopinavir:

The chemical name of lopinavir is N-[(1S,3S,4S)-4-[[2-2,6-dimethyl phenoxy) acetyl] amino] -3-hydroxy -5-phenyl-1-(phenylmethyl)pentyl] tetra hydro – α S-(1-methyl ethyl)-2-oxo -1(2H)-pyrimidine acetamide. Its empirical formula is $C_{37}H_{48}N_4O_5$. It is practically insoluble in water **(Figure 2)**.

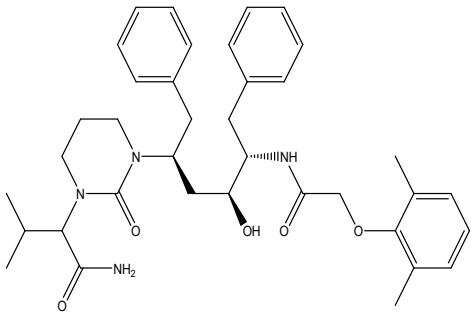

Figure 2: Structure of Lopinavir

Ritonavir:

Ritonavir is chemically 1,3-thiazol-5-yl methyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propane-2-yl)-1,3-thiazol-4yl] methyl}) carbomoyl] amino} butanamido] -1,6-diphenylhexane-2-yl]carbamate. Its empirical formula is $C_{37}H_{48}N_6O_5S_2$. It is practically insoluble in water.Ritonavir is an antiretroviral drug used to treat HIV infection and AIDS. Ritonavir is a protease inhibitor class and it inhibits the same host enzymes that metabolize other protease inhibitors. This inhibition of the protease results in increased plasma concentrations of these drugs. So the simultaneous determination with other HIV protease inhibitors likes lopinavir. Non – nucleotide reverse transcriptase inhibitors (NNRTIs) and boosted protease inhibitors (efavirenz) work equally well for people starting HIV treatment for the first time, with similar viral suppression, CD4 cell gains, and disease progression, according to a large meta-analysis presented at IDWEEK 2014, United States **(Figure 3)**.

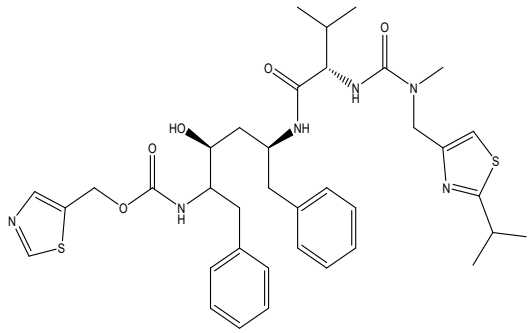

Figure 3: Structure of Ritonavir

Method development and simultaneous estimation of lopinavir, ritonavir, and efavirenz by reverse-phase high-performance liquid chromatography and method were developed and validated.

2. MATERIALS AND METHODS

2.1 Instrumentation: The spectrophotometric measurements were carried out using a Shimadzu double beam UV- visible spectrophotometer model 1700 with 1cm matched quartz cell. PDA detector and auto sampler was used for sampling. P^H meter manufactured by LABINDIA, Analytical balance manufactured by ESSAE.

2.2 Ingredients: Ritonavir, Lopinavir and efavirenz pharmaceutical formulation were kindly procured from national scientific laboratories, commercial pharmaceutical formulations which are claimed to obtained to contain 50mg of Ritonavir, 200mg of Lopinavir and 50mg of Efavirenz were used in analysis.

2.3 Chemicals and Reagents:

Methanol, HPLC grade water, Acetonitrile, 0.1ml TFA dissolved in 0.02M KH_2PO_4 in 100ml.

2.4 Preparation of buffer solution: weighed and transferred about 0.2721gm of potassium dihydrogen orthophosphate into a beaker containing 100ml of HPLC grade water and dissolved completely. The pH of the solution was adjusted to 4.5±0.05 and filtered through a 0.45µm membrane filter.

2.5 Preparation of mobile phase: Mobile phase is prepared by mixing 60ml of Acetonitrile and 40ml of a buffer with their ratio of 60:40%V/V.

2.6 Preparation of standard stock solution:

Accurately weighed amounts of 10mg ritonavir, 10mg lopinavir, and 10mg of efavirenz were taken into 10ml cleaned and dried volumetric flask. This was diluted with 10ml of Diluent – A(methanol: water 30:70V/V) and it was Sonicated to obtain a stock solution of 1000µg/ml.

2.7. Preparation of sample solution:

Take 20 tablets were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to 1 tablet powder of Ritonavir, Lopinavir and Efavirenz dissolved in sufficient diluents (Methanol: Water **(30:70%V/V)**). Filter it by using 0.45µ membrane filter and Sonicated for 5min.

3. RESULTS AND DISCUSSION:

3.1 Optimized chromatographic conditions: (Table 1, Figure 4)

Stationary phase: Shimadzu column (150mmX3.0mmX3µm)

Diluents:

Diluent- A - methanol: water (30:70%V/V)

Diluent- B –acetonitrile: buffer (60:40%V/V)

Injection volume: 20µl

Run time: 10min

Flow rate: 1.4ml/min

Detection wavelength: 233nm

3.2 Method validation:

3.3 System suitability: system suitability is a test to determine the suitability and effectiveness of chromatographic system prior to use. As per ICH " the checking of a system, before or during analysis of unknowns, to ensure system performance." Number of theoretical plates(N), tailing factor, resolution and relative standard deviation of peak area or repetitive injections were studied. The % RSD values are below 2%, theoretical plate count is above 2000 and the tailing factor is less than 2, indicating that the method is suitable and the results were shown in the **Table 2**.

3.4 Specificity: specificity is the ability of the analytical method to distinguish between the analyte and the other components in the sample matrix. It is assured by complete separation of peaks of analyte from other peaks originated from the sample matrix. Specificity evalution was done by injecting separately 20µl solution of standard, sample, placebo, and blank into the chromatographic system **(Figure 5-7)**.

3.5 Linearity:

To evaluate the linearity of the method , mixed standard solution of ritonavir, lopinavir and efavirenz were prepared by diluting stock standard solution with the mobile phase to obtain linearity range of lopinavir 50,100,150,200,250µg/ml and 10,20,30,40,50µg/ml of ritonavir and 10,20,30,40,50µg/ml. From series of volumetric flasks 20µL of solution was injected into the HPLC system and chromatograms were recorded **(Figure 8-10)**.

3.6 Accuracy: Accuracy of an analytical method is the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Prepared 50%W/V, 100%W/V, 150%W/V level of solutions. Three injections from each concentration were analyzed under the same chromatographic conditions **(Table 3-5)**.

Acceptance criteria: The % mean recovery should be within 98.00-102.00%.

3.7 Precision: The precision of an analytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variation (%CV) or relative standard deviation (RSD) of the replicate measurements. Perform the intermediate precision and method precision. The results were given in the **Table 6, 7**.

$$\%\ \boldsymbol{RSD} = \frac{\textbf{satndard deviation}}{\textbf{mean}} \times \mathbf{100}$$

Acceptance criteria: The %RSD for the peak area of six standard injections should not more than 2.0%.

3.8 Limit of detection (LOD): The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample. Which can be detected but not necessarily quantified. The detection limit can be calculated based on the Standard Deviation of the Response and the slope. The parameter LOD was determined based on the response and slope of the regression equation. The detection limit (DL) may be expressed as:

$$\mathbf{LOD} = \mathbf{3.3} \times \frac{\boldsymbol{\sigma}}{\mathbf{S}}$$

The LOD for this method was found to be Lopinavir 5µg/ml, Ritonavir 1µg/ml, Efavirenz 1µg/ml.

3.9 Limit of quantification (LOQ): The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit can be calculated based on the Standard Deviation of the response and slope. The parameter LOQ was determined based on the response and slope of the regression equation. The quantification limit was expressed as:

$$\boldsymbol{LOQ} = \mathbf{10} \times \frac{\boldsymbol{\sigma}}{\boldsymbol{S}}$$

The LOQ for this method was found to be Lopinavir 15µg/ml, Ritonavir 3µg/ml, Efavirenz 3µg/ml.

1. **Robustness:**

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in the method parameters and provides an indication of its reliability during normal usage. Chromatograms were recorded for flow rate and mobile phase ratio variations and chromatographic parameters were evaluated. In this present robustness of the proposed method was demonstrated between different flow rate and different mobile phase ratios. Theoretical plates of Ritonavir, Lopinavir and Efavirenz was found to be 5457, 5926, 8010 respectively. Results were shown in the **Table 8, 9**.

Acceptance criteria: The %RSD of peak area by changing flow rate and mobile phase ratio should not more than 2.0%.

1. **Assay:**

Take 20 tablets each tablet contains 200mg of lopinavir, 50mg of ritonavir and 50mg of efavirenz were weighed and taken into mortar and crushed into a fine powder and uniformly mixed. Weight equivalent to 1tablet powder dissolved in sufficient mobile phase and filtered with 0.45µ membrane filter and sonicated for 5min. peak area of both standard and test was determined. The % of assay was calculated from the peak area of both standard and sample. The % of assay was calculated by using the formula and the results were shown in the **Table 10**.

$$Assay = \frac{sample\ area}{standard\ area} \times \frac{weight\ of\ standard}{dilution\ of\ standard} \times \frac{dilution\ of\ sample}{weight\ of\ sample} \times \frac{potency\ of\ API}{100} \times \frac{Average\ weight}{labelled\ claim} \times 100$$

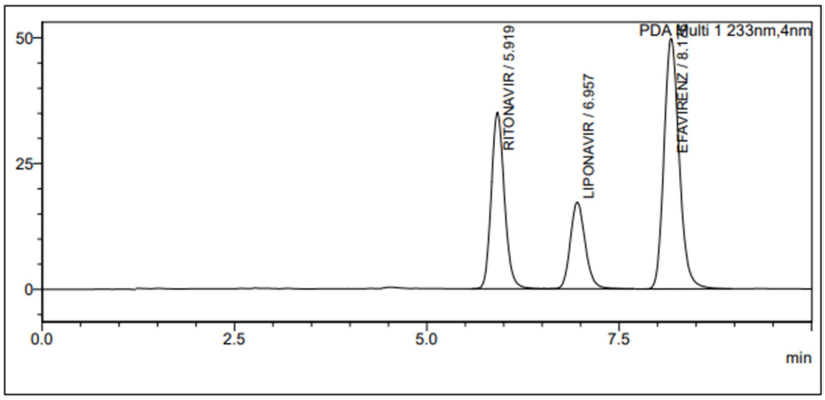

Figure 4: Method optimization peak

Table 1: Method optimization parameters were shown in the table

S.No.	Parameters	Ritonavir	Lopinavir	Efavirenz
1.	Retention time	5.919	6.957	8.176
2.	Tailing factor	1.152	1.164	1.173
3.	Theoretical plates	5457	5926	8010
4.	Peak area	416366	232901	682700

Table 2: System suitability tests for Ritonavir, Lopinavir and Efavirenz

Drugs	Standard peak area	Sample peak area	Tailing factor	Retention time	Theoretical plates	% RSD
Ritonavir	416366	426553	1.175	6.042	5476	0.324637
Lopinavir	232901	243301	1.193	7.022	5838	0.448629
Efavirenz	682700	695723	1.192	8.186	7819	0.362271

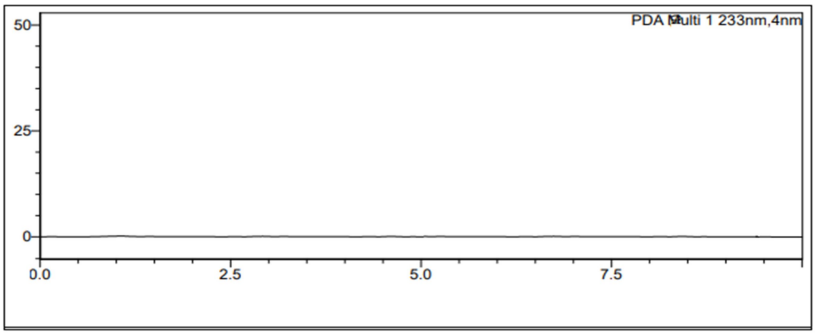

Figure 5: Blank peak

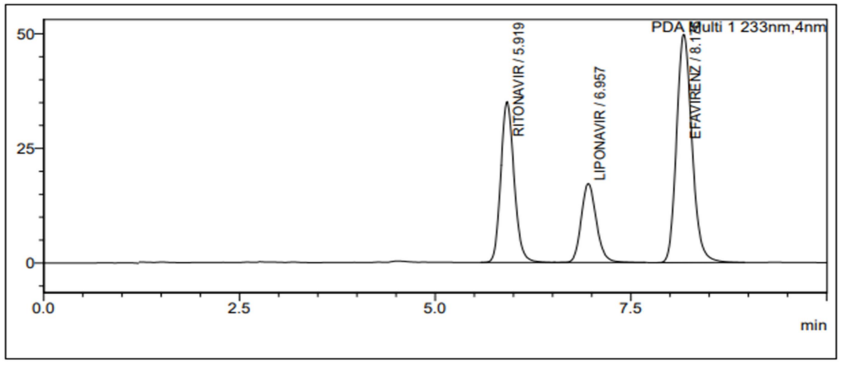

Figure 6: Standard chromatogram of Ritonavir, Lopinavir and Efavirenz

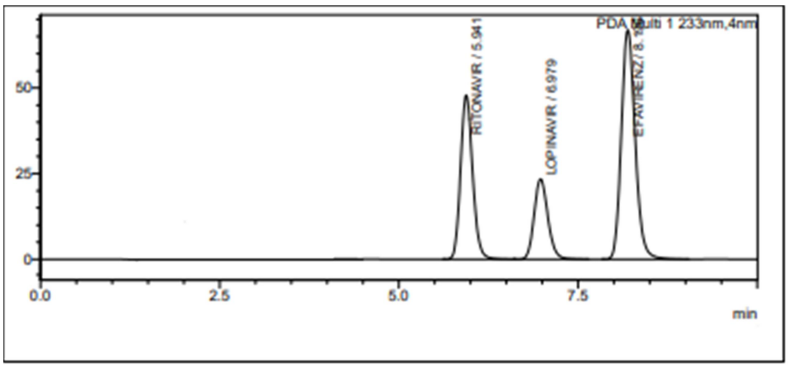

Figure 7: Sample chromatogram of Ritonavir, Lopinavir and Efavirenz

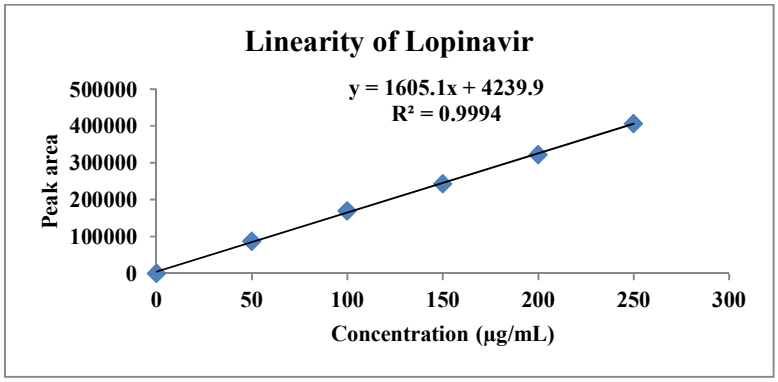

Figure 8: Calibration curve of Lopinavir

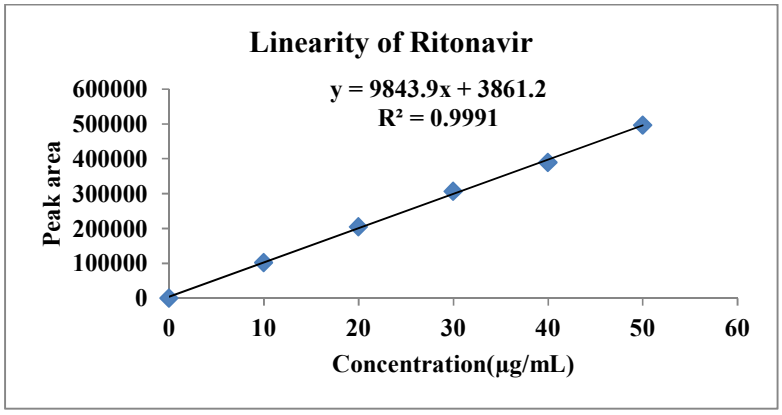

Figure 9: Calibration curve of Ritonavir

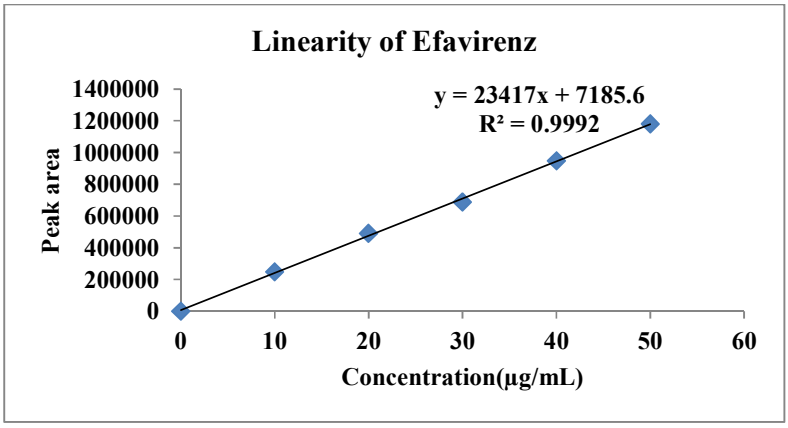

Figure 10: Calibration curve of Efavirenz

Table 3: Accuracy data of Ritonavir

Recovery level	Accuracy of Ritonavir				
	Peak area		% Recovery	% of Mean recovery	Average % recovery
	sample	Standard			
50	212369	424411	99.79	100.26	100.11
	214258	424411	100.62		
	213896	424411	100.39		
100	425465	424411	100.05	100.15	
	426545	424411	100.26		
	425894	424411	100.14		
150	635894	424411	99.68	99.92	
	638456	424411	100.09		
	637965	424411	100.01		

Table 4: Accuracy data of Lopinavir

Recovery level	Accuracy of Lopinavir				
	Peak area		% recovery	% of mean recovery	Average % recovery
	sample	standard			
50	121254	240317	100.62	100.38	100.21
	121012	240317	100.37		
	120854	240317	100.17		
100	241324	240317	100.22	100.16	
	240854	240317	99.98		
	241524	240317	100.29		
150	361254	240317	100.01	100.1	
	361584	240317	100.11		
	361854	240317	100.18		

Table 5: Accuracy data of Efavirenz

Recovery level	Accuracy of Efavirenz				
	Peak area		% recovery	% of mean recovery	Average % recovery
	Sample	Standard			
50	345689	693930	99.35	99.77	100.08
	348965	693930	100.23		
	347456	693930	99.73		
100	694258	693930	99.85	100.04	
	695468	693930	99.98		
	697485	693930	100.30		
150	1044581	693930	100.15	100.43	
	1049561	693930	100.63		
	1048251	693930	100.51		

Table 6: precision data of Ritonavir, Lopinavir

S. No.	Retention time	Peak area		Retention time	Peak area	
		I.P	M.P		I.P	M.P
1.	6.015	428580	428880	7.001	242930	243030
2.	6.030	426861	427261	7.014	241985	242085
3.	6.042	425853	425953	7.022	243001	243201
4.	6.049	427619	427819	7.026	243015	243215
5.	6.055	429262	429462	7.029	244227	244427
6.	6.062	429679	429479	7.035	244990	245090
Avg	428142.3	427975.7	428142.3	243508	243358	243508
SDV	1396.197	1469.464	1396.197	1075.248	1070.7	1075.248
% RSD	0.326106	0.343352	0.326106	0.441566	0.439969	0.441566

Table 7: Precision data of Efavirenz

S.No.	Efavirenz		
	Retention time	Peak area	
		I.P	M.P
1.	8.214	696028	696228
2.	8.202	695499	695699
3.	8.186	695223	695423
4.	8.172	697821	698021
5.	8.159	701556	701256
6.	8.152	701208	701408
Avg	698005.8	697889.2	698005.8
SDV	2730.998	2854.829	2730.998
%RSD	0.39125	0.409066	0.391257

Table 8: Robustness data of Ritonavir, Lopinavir

S.No.	Parameters	Ritonavir				Lopinavir			
		Rt. (min)	Average	Tailing	%RSD	Rt.(min)	Average	Tailing	%RSD
1.	Change in flow rate - 1.3ml/min	4.987	462652	1.194	0.150	5.621	263520	1.214	0.718
	Change in flow rate - 1.5ml/min	4.333	402114	1.198	0.184	4.879	228202	1.209	0.830
2.	Change in mobile phase ratio -55:45	8.448	431919	0.272	0.032	10.108	244844	1.292	0.064
	Change in mobile phase ratio – 65:35	4.594	433469	1.198	0.018	5.180	242299	1.197	0.018

Table 9: Robustness data of Efavirenz

S.No.	Parameters	Efavirenz			
		Rt.(min)	Average	Tailing	%RSD
1.	Change in flow rate – 1.3ml/min	6.554	751068	1.209	0.160
	Change in flow rate – 1.5ml/min	5.675	652235	1.208	0.038
2.	Change in mobile phase ratio – 55:45	11.349	701687	1.321	0.414
	Change in mobile phase ratio – 65:35	6.051	692966	1.211	0.109

Table 10: Assay of ritonavir, lopinavir and efavirenz

Drugs	Sample peak area	Standard peak area	%Assay
Ritonavir	5.941	5.919	100.30%
Lopinavir	6.979	6.957	100.14%
Efavirenz	8.196	8.176	100.29%

4. CONCLUSION

The present developed isocratic RP-HPLC method was found to be specific, simple, accurate, and rapid for the determination of Ritonavir, Lopinavir, and Efavirenz in pharmaceutical formulation. It also provides satisfactory accuracy and precision with lower limits of detection and quantification. The shorter duration of analysis for Ritonavir, Lopinavir and Efavirenz was reported methods suitable for routine quantitative analysis in pharmaceutical dosage forms.

5. Acknowledgment

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