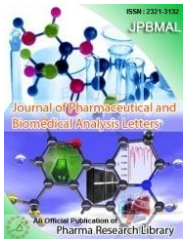

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Method Development and Validation for the Simultaneous Estimation of Sequinavir and Tipronavir by using HPLC

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A B S T R A C T

This study focused on developing and characterizing floating microspheres of Propranolol HCl, aiming to enhance gastric retention and sustained drug release. A UV-Visible spectrophotometric method was validated for drug quantification in 0.1N HCl at 256 nm, exhibiting strong linearity over the concentration range of 2 to 10 µg/ml. Compatibility studies using FTIR spectroscopy confirmed no chemical interactions between the drug and polymers, ensuring formulation stability. The prepared microspheres showed high percentage yields between 81.58% and 95.50%, with drug entrapment efficiency improving as polymer concentration increased due to higher solution viscosity reducing drug diffusion during formation. Further evaluations revealed that the microspheres exhibited satisfactory swelling behavior and mucoadhesive properties essential for prolonged gastric residence. In vitro release experiments demonstrated efficient and controlled drug liberation over 12 hours, with release kinetics fitting diffusion-controlled models such as zero-order, first-order, Higuchi, and Korsmeyer-Peppas. Optimized formulations showed promising potential for sustained release and enhanced gastroretention, which could improve therapeutic effectiveness and patient adherence.

Keywords: Propranolol HCl, floating microspheres, UV-Visible spectrophotometry, sustained release, mucoadhesion, drug release kinetics, FTIR compatibility.

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

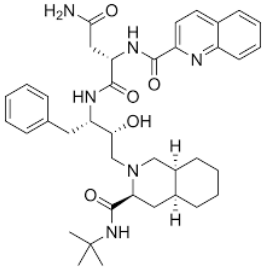

Fig.1: Sequinavir

Table 1: Sequinavir drug profile

IUPAC Name	(2S)-N-[(2S,3R)-4-[(2S)-2-[(2S)-2-[(methoxycarbonyl)amino]-3-methylbutanoyl]amino]-3-hydroxy-5-phenylpentyl]-3-methyl-2-[(2-quinolinylcarbonyl)amino]butaneamide
Molecular Formula	$C_{38}H_{50}N_6O_5$
Molecular	670.84 g/mol

Weight	
M.P	120-122°C
pKa	Not readily available
Category	Protease in hi Protease inhibitorbitor
Solubility	Slightly soluble in water, soluble in DMSO and methanol
Uses	Used in combination with ritonavir and other antiretroviral agents for the treatment of HIV-1 infection
Dosage	Typically, 1000 mg of Saquinavir with 100 mg of ritonavir, taken twice daily with food
Side Effects	Common side effects include nausea, diarrhea, fatigue, and headache. Serious side effects can include liver problems and changes in heart rhythm
Drug Interactions	Saquinavir can interact with many drugs, including other antiretrovirals and medications metabolized by CYP3A4. Always consult a healthcare provider for a full list of interactions
Storage	Store at room temperature away from moisture and heat
Mechanism of Action	Saquinavir inhibits the HIV-1 protease enzyme, preventing the virus from maturing and replicating

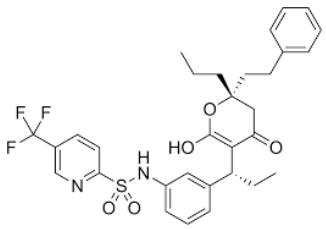

Fig.2: Tipranavir

Table 2: Tipranavir drug profile

IUPAC Name	(3S,6S)-3-[(1R)-1-[(2S)-2-[(2-isopropyl-1,3-thiazol-4-yl)methylcarbamoyl]pyrrolidin-1-yl]-2-methylpropyl]-6-(2-methylpropyl)-2,3,6,7-tetrahydro-1H,4H-thieno[3,2-d][1,3]oxazine-8-carboxamide
Molecular Formula	C31H48N6O5S
Molecular Weight	602.84 g/mol
Melting Point	Not readily available
pKa	Not readily available
Category	Protease inhibitor
Solubility	Soluble in DMSO, slightly soluble in water
Uses	Used in combination with ritonavir and other antiretroviral agents for the treatment of HIV-1 infection.
Dosage	Typically, 500 mg of Tipranavir with 200 mg of ritonavir, taken twice daily with food.
Side Effects	Common side effects include nausea, vomiting, diarrhea, headache, and skin rash. Serious side effects can include liver damage and bleeding in the brain
Drug Interactions	Tipranavir can interact with many drugs, including blood thinners and other antiretrovirals. Always consult a healthcare provider for a full list of interactions.
Storage	Store at room temperature away from moisture and heat
Mechanism of Action	Tipranavir inhibits the HIV-1 protease enzyme, preventing the virus from maturing and replicating

2. Materials and Methods

Table 3: Instruments used

S.No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil

Table 4: Chemicals used

S.No	Chemical	Brand
1	Sequinavir	Supplied by MSN LAB
2	Tipranavir	Supplied by MSN LAB
3	Ortho phosphoric acid	FINAR chemical LTD
4	Water and Methanol for HPLC	Standard solutions Ltd
5	Acetonitrile for HPLC	Standard solutions Ltd
6	HCl, H_2O_2, NaOH	MERCK

Wave length selection:

UV spectrum of 10µg/ml Saquinavir and Tipronavir in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm and the isobestic λmax of both the drugs obtained at 248 nm.

Optimization of Column:

Platsil C18-EP (4.6 x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

Optimization of Chromatographic Conditions

Optimized Chromatographic Conditions

Instrument used: High performance liquid chromatography equipped With Auto Sampler and PDA detector
Temperature : Ambient
Column : PLATSIL C18-EP (4.6 x 250mm, 5µm)
Mobile phase: Acetonitrile: Triethylamine PH: 4 (80:20ml)
Flow rate : 1ml/min
Wavelength : 248 nm
Injection volume : 10 µl
Run time : 10 min.
Retention time : Saquinavir-2.089, for Tipronavir– 3.259

Preparation of buffer and mobile phase:

Preparation of Triethylamine pH 4:

To prepare Trimethylamine buffer solution, by adding 3.5ml of Trimethylamine in a 250ml water. Adjust this solution to pH 4 by using Acetic Acid.

Preparation of mobile phase:

Mix a mixture of above 200 ml Triethylamine PH- 4(20%) and Acetonitrile 800ml (80%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

Acetonitrile: Triethylamine PH: 4 (80:20) ratio.

System Suitability:

Tailing factor for the peaks due to Saquinavir and Tipronavir in Standard solution should not be more than 2.0. Theoretical plates for the Saquinavir and Tipronavir peaks in Standard solution should not be less than 2000

Validation parameters:

1. Assay:

Standard Solution Preparation:

Accurately weigh and transfer 25mg of Saquinavir and 12.5mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

Sample Solution Preparation:

Accurately weigh and transfer 25mg of Saquinavir and 12.5mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

Procedure: Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for the Saquinavir and Tipronavir peaks.

Calculation: (For Saquinavir and Tipronavir)

Where:

AT = average area counts of sample preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P= Percentage purity of working standard
LC = Label Claim mg/ml.

Acceptance criteria of System Suitability:

1) Tailing factor should be less than 2
2) Theoretical Plates should be above 2000

Linearity:

Preparation of stock solution:

Accurately weigh and transfer 25mg of Saquinavir and 12.5mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (25ppm of Saquinavir and 12.5ppm Tipronavir):

0.25 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – II (50ppm of Saquinavir and 25ppm Tipronavir): 0.5 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – III (75ppm of Saquinavir and 37.5ppm Tipronavir):

0.75 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – IV (100ppm of Saquinavir and 50ppm Tipronavir):

1.0ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – V (125ppm of Saquinavir and 62.5ppm Tipronavir):

1.25ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Procedure:

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.

Precision:

Preparation of stock Solution:

Accurately weigh and transfer 25mg of Saquinavir and 12.5mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

Procedure:

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 to be within the specified limits.

Intermediate precision/ Ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day within the laboratory.

Preparation of stock solution:

Accurately weigh and transfer 25mg of Saquinavir and 12.5mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

Procedure:

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 to be within the specified limits.

Accuracy:

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

Preparation of Standard stock solution:

Accurately weigh and transfer 25mg of Saquinavir and 12.5mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration): Accurately weigh and transfer 12.5mg of Saquinavir and 6.23mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

For preparation of 100% solution (With respect to target Assay concentration): Accurately weigh and transfer 25mg of Saquinavir and 12.34mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

For preparation of 150% solution (With respect to target Assay concentration):

Accurately weigh and transfer 37.5mg of Saquinavir and 18.6mg Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents (75ppm Saquinavir & 37.5ppm Tipronavir).

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculate the Amount found and Amount added for Saquinavir and Tipronavir vand calculate the individual recovery and mean recovery values.

Limit of detection:

Preparation of Saquinavir and Tipronavir solution:

Preparation of 0.09µg/ml Saquinavir solution:

Accurately weigh and transfer 25mg of Saquinavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 0.1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 2.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Preparation of 0.09µg/ml Tipronavir solution:

Accurately weigh and transfer 12.5mg of Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 0.1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 2.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Limit of quantification:

Preparation of Saquinavir and Tipronavir solution:

Preparation of 0.27µg/ml solution Saquinavir:

Accurately weigh and transfer 25mg of Saquinavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 0.1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 3.6ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Preparation of 5.10µg/ml solution Tipronavir:

Accurately weigh and transfer 12.5mg of Tipronavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.75ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 5.91ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 2.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

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 0.8 ml/min to 1.2 ml/min.
- Standard solution 75µg/ml of Saquinavir and 37.5µg/ml Tipronavir prepared and analysed using the varied flow rates along with method flow rate.
- b)The Organic composition in the Mobile phase was varied from 72% to 88%

Standard solution 75µg/ml of Saquinavir and 37.5µg/ml Tipronavir was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

3. Results and Discussion

Optimized Chromatographic Conditions

Instrument used: High performance liquid chromatography equipped With Auto Sampler and PDA detector
Temperature : Ambient
Column : PLATSIL C18-EP (4.6 x 250mm, 5µm)
Mobile phase: Acetonitrile: Triethylamine PH: 4 (80:20ml)
Flow rate : 1ml/min
Wavelength : 248 nm
Injection volume : 10 µl
Run time : 10 min

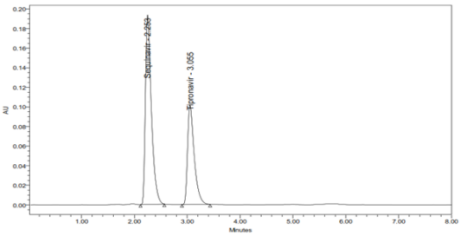

Fig.3: Sample Chromatogram for system suitability

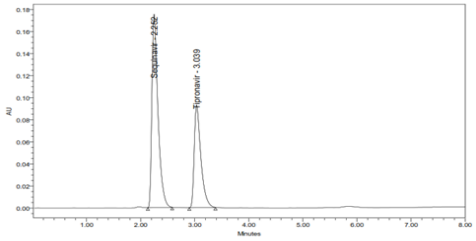

Fig.4: Standard Chromatogram for system suitability

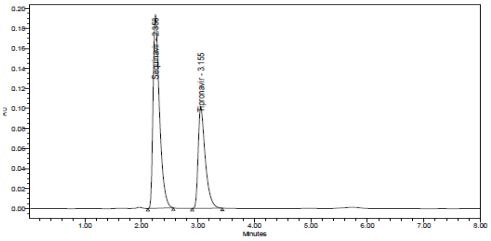

Fig.5: Assay Chromatogram for Sample

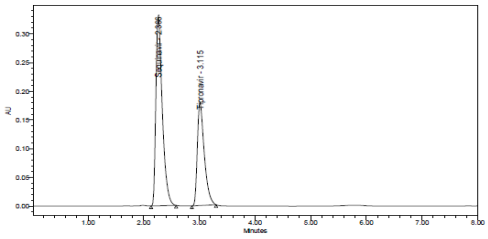

Fig.6: Chromatogram for linearity-5

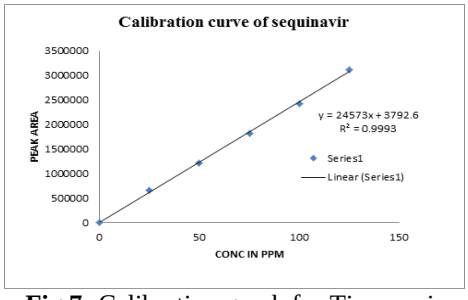

Fig.7: Calibration graph for Tipronavir

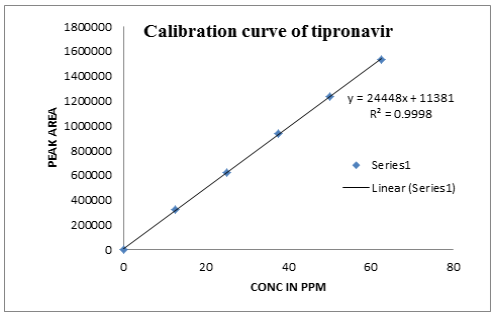

Fig.8: Calibration graph for Tipronavir

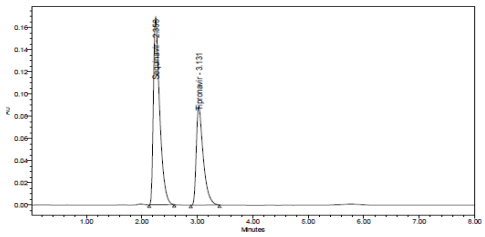

Fig.9: Chromatogram for Precision -6

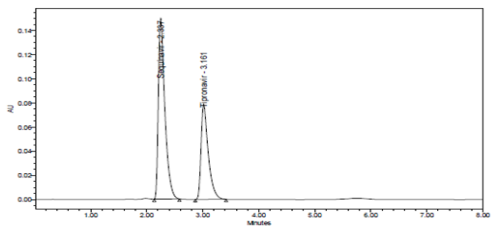

Fig.10: Chromatogram for ID Precision -6

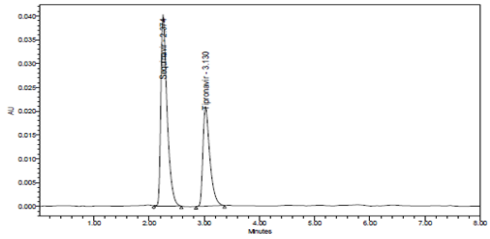

Fig.11: Chromatogram for Accuracy 50%-3

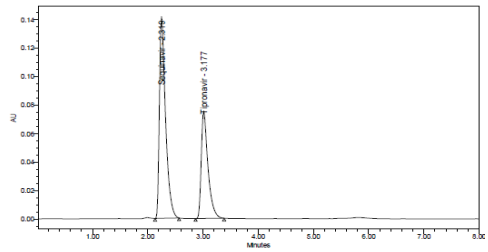

Fig.12: Chromatogram for Accuracy 100%-3

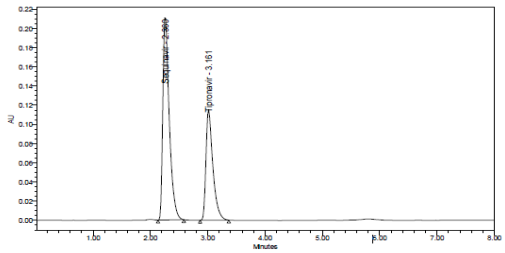

Fig.13: Chromatogram for Accuracy 150%-3

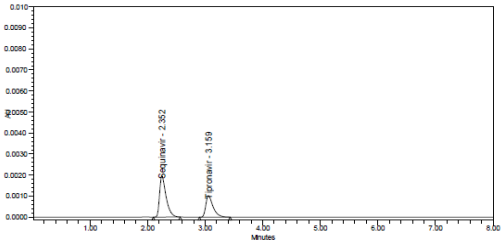

Fig.14: Chromatogram of Saquinavir and Tipronavir showing LOD

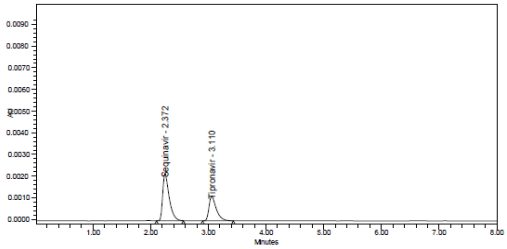

Fig.15: Chromatogram of Saquinavir and Tipronavir showing LOQ

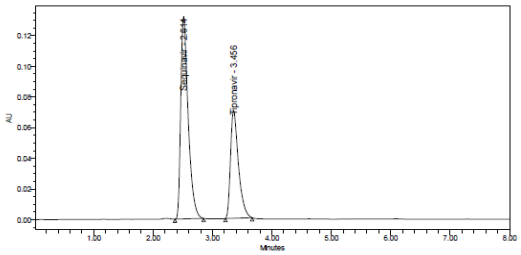

Fig.16: Chromatogram showing less flow

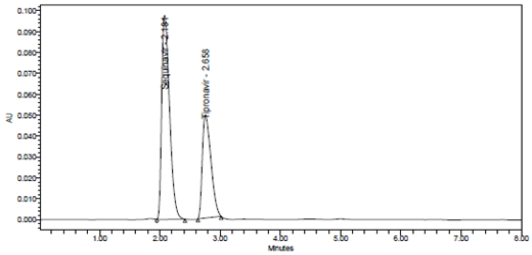

Fig.17: Chromatogram showing more flow

Table 5: Results of system suitability parameters

S.No	Name (sample)	RT (min)	Area (µVsec)	Height (µV)	USP tailing	USP plate count
1	Saquinavir	2.253	1806547	198465	1.60	2186.81
2	Tipronavir	3.055	916584	99232	1.43	2216.21

S.No	Name	RT(min)	Area (µVsec)	Height (µV)	USP tailing	USP plate count
1	Saquinavir	2.252	1846982	198396	1.54	2176.50
2	Tipronavir	3.039	926582	99198	1.40	2212.18

Table 6: Results of Precision for Saquinavir and Tipronavir

Injection	Saquinavir Area	Tipronavir Area
Injection-1	1826542	946598
Injection-2	1838746	943254
Injection-3	1837423	942586
Injection-4	1831513	946421
Injection-5	1839875	942589
Injection-6	1834761	943654
Average	1834810	944183.7
Standard Deviation	5043.937	1847.972
%RSD	0.3	0.2

Table 7: Results of Intermediate precision for Saquinavir and Tipronavir

Injection	Saquinavir Area	Tipronavir Area
Injection-1	1846521	952541
Injection-2	1849654	951589
Injection-3	1831254	959856
Injection-4	1846587	957456
Injection-5	1849512	953256
Injection-6	1843578	957542
Average	1844518	955373.3
Standard Deviation	6876.461	3345.484
%RSD	0.4	0.4

Table 8: Results of LOD

Drug name	Baseline noise(µV)	al obtained(µV)	S/N ratio	Conc. In ppm
Saquinavir	83	245	2.96	0.09
Tipronavir	83	237	2.86	0.09

Table 9: Results of LOQ

Drug name	Baseline noise(µV)	ignal obtained(µV)	S/N ratio	Conc. In ppm
Saquinavir	83	826	9.95	0.27
Tipronavir	83	820	9.88	5.10

Table 10: Results for variation in flow for Saquinavir and Tipronavir

S. No	Flow Rate (ml/min)	System Suitability Results Saquinavir	
		USP Plate Count	USP Tailing
1	0.8	3295	1.42
2	1.0	3269	1.5
3	1.2	3271	1.32

4. Conclusion

A simple, precise, and robust RP-HPLC method was successfully developed and validated for the simultaneous estimation of Saquinavir and Tipranavir in bulk and pharmaceutical dosage forms, following ICH Q2(R1) guidelines. Chromatographic separation was achieved on a PLATSIL C18-EP column (4.6 × 250 mm, 5 µm) using a mobile phase of acetonitrile and triethylamine buffer (pH 4) in the ratio of 80:20 v/v, at a flow rate of 1.0 mL/min, with UV detection at 248 nm. The method demonstrated excellent system suitability parameters, with USP plate counts above 2000 and tailing factors below 2 for both drugs. Linearity was established in the range of 20–100 µg/mL for Saquinavir and 10–50 µg/mL for Tipranavir, with correlation coefficients (R^2) of 0.9993 and 0.9998, respectively. Precision studies showed %RSD values below 0.5%, confirming reproducibility, while accuracy studies yielded mean recoveries within the acceptable range of 98–102%. The LOD and LOQ were found to be 0.09 ppm and 0.27 ppm for Saquinavir, and 0.09 ppm and 5.10 ppm for Tipranavir, respectively. Robustness testing indicated that small changes in flow rate and mobile phase composition did not significantly affect chromatographic performance. The developed RP-HPLC method fulfills all validation parameters outlined in ICH Q2(R1), demonstrating specificity, precision, accuracy, linearity, robustness, and sensitivity. The short retention times, high sensitivity, and reproducible results make the method suitable for routine quality control and batch release testing of Saquinavir and Tipranavir in combined dosage forms. Its robustness under varied chromatographic conditions ensures reliability in different laboratory settings, while the simplicity and cost-effectiveness of the procedure make it practical for widespread pharmaceutical industry applications. This validated method can be confidently applied for regulatory compliance, ensuring consistent product quality and therapeutic efficacy.

5. References

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