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# RP – HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ANTIRETROVIRAL DRUGS LOPINAVIR AND RITONAVIR IN TABLET DOSAGE FORM

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A simple reverse phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated for the quantitative estimation of antiretroviral drugs Lopinavir (LPV) and Ritonavir (RTV). The different analytical parameters such as linearity, precision, accuracy, and specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined. Chromatography was carried out by binary gradient technique on a reversed-phase phenomenex-Luna $C_{18}$ column using Ambroxol (ABM) as the internal standard. The calibration curve for each analyte in the desired concentration range (r2 > 0.999) was found to be linear. The recovery values was found to be 99.9 and 100.24% and relative standard deviation was <2% for LPV and RTV respectively. The proposed method is highly sensitive, precise and accurate, which was evident from the LOD value of 30 ηg/ml for LPV and 25 ηg/ml for RTV hence the present method applied successfully for the quantification of active pharmaceutical ingredient content (API) in the combined formulations of LPV and RTV.

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## 1. Introduction

Lopinavir (LPV) [1-2] is chemically known as [1S-[1R*, (R*), 3R*, 4R*]]-*N*-[4 [[(2,6dimethyl-phenoxy) acetyl] amino]-3-hydroxy-5-phenyl 1(phenyl methyl) pentyl] tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2*H*) pyrimidineacetamide. Ritonavir (RTV) [1-2] is chemically known as 10-Hydroxy-2-methyl-5- (1-methylethyl) -1- [2-(1-methylethyl) -4-thiazolyl] -3, 6-dioxo-8, 11-bis (phenylmethyl) -2, 4,7,12 -tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*, 8R*, 10R*, 11R*)] both the drugs were used as an antiretroviral agents.

There are many methods reported for the determination of determination of LPV and RTV in pharmaceutical preparations and in human plasma individually or in combination with other antiviral drugs using HPLC [1-10] UV/Visible or Mass Spectroscopy detector. However, no references are reported so far for the simultaneous determination of both drugs in combined dosage form or any such pharmaceutical preparations by HPLC. In this communication we report a new simple, rapid and precise RP-HPLC method for the simultaneous determination of LPV and RTV in combination dosage form, and also used for the API content, which can be used for its routine analysis in ordinary laboratories.

## 2. Experimental

### 2.1. Reagents

LPV and RTV reference standard drugs (pure) were otained as gift samples from Heterolab, Hyderabad and tablet formulation is from Emcure pharmaceuticals (Emletra) were used

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for this present study. Water HPLC grade, Acetonitrile HPLC grade, and (AR grade) Glacial acetic acid, were purchased from E-merck.

### 2.2. Instrumentation

The present work was carried out on gradient high pressure liquid chromatograph [Shimadzu HPLC] with LC-20 AT Prominence solvent delivery system (pump) for constant flow and constant pressure delivery. SPD – M20 A Prominence Diode array detector connected to software LC solution class M20A for controlling the instrumentation as well as processing the data generated was used. The Luna $C_{18}$ RP column (250×4.6mm i.d, 5u) was used for all chromatographic separation.

### 2.3. Experimental Condition

The HPLC system was operated by binary gradient mode at a flow rate of 1.2 ml / min at 25 ± 2º c. The most suitable mobile phase for analysis was found to be Acetonitrile, Triethylamine (0.5%) pH 5.0 adjusted with glacial acetic acid, (67:33) %v/v. Detection was carried out at 240 nm using AMB as internal standard.

### 2.4. Preparation of Mobile Phase

Triethylamine (0.5%) was prepared by using HPLC water. Acetonitrile and Triethylamine (0.5%) in the ratio of (67:33 % v/v) was prepared, filtered and degassed. The pH was adjusted to pH 5 with glacial acetic acid.

### 2.5. Preparation of mixed standard solution

LPV and RTV (1mg/ml) standard stock solution was prepared using Acetonitrile as a solvent, the internal standard Ambroxol (AMB) (1mg/m) stock solution was prepared by using methanol as a solvent.

Aliquots of mixed standard solutions were diluted in Acetonitrile to get a final concentration of 40, 80, 120, 160, and 200, µg /ml of LPV 10, 20, 30, 40, and 50 µg/ml RTV and 25µg/ml of AMB.

### 2.6. Preparation of sample solution

#### *2.6.1. Tablet formulation*

Twenty tablets of each containing 200 mg of LPV and, 50 mg of RTV were weighed, and crushed into fine powder. A quantity of powder equivalent to 50 mg was dissolved in 25 ml of Acetonitrile and sonicate for 15 min. Then volume was made up to 50 ml with Acetonitrile and filtered through whatmann filter paper. The final mixed sample solution were prepared, correspond to 80 µg /ml of LPV and 20 µg/ml of RTV and 25 µg/ml of AMB.

### 2.7. Recording of chromatograms

With the optimized chromatographic conditions a steady baseline was recorded. After the stabilization of the baseline for 20 min. Standard solutions containing 40-200 µg /ml of LPV and 10 -50 µg/ml of RTV along with 25 µg/ml of AMB were injected and chromatograms were recorded. Retention time of LPV, RTV and AMB were found to be 9.1, 8.2 and 5.2 mins respectively. Similarly chromatograms were recorded for sample solution along with AMB.

Calibration curves were plotted using peak area retentions of standard drug peaks to the internal standard peak area against concentration of corresponding standard solutions.Peak area of

the sample chromatograms were reordered and the amount[13] of LPV and RTV were calculated from the regression equation.

### 2.8. Method validation [9-12]

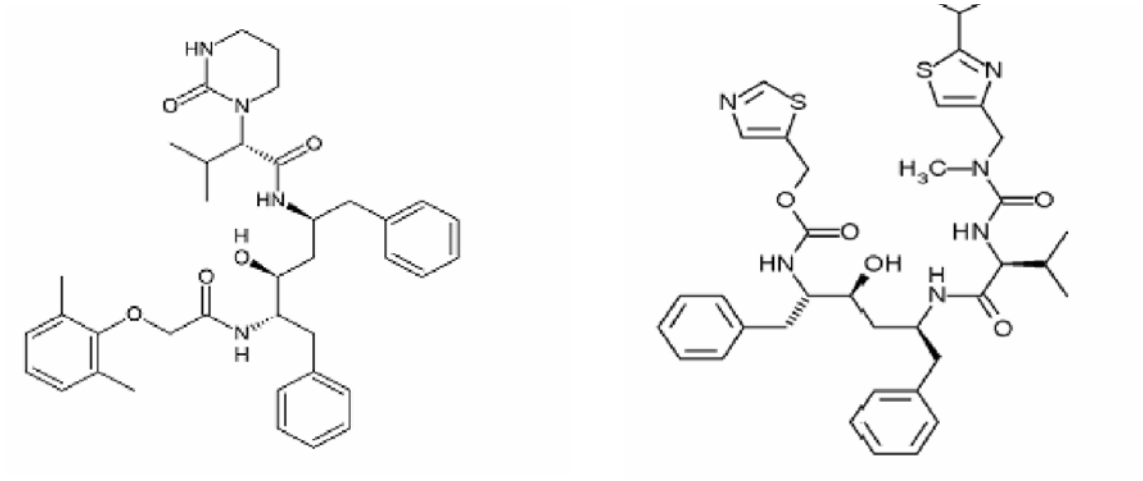
The accuracy, precision, Linearity, Specificity, ruggedness and robustness were determined by analyzing 40-200 µg/ml and 10-50 µg/ml of LPV and RTV drugs respectively.

#### *2.8.1. Recovery studies (accuracy)*

To study the accuracy of the above method, standard solution was added to pre-analyzed sample solution at different levels i.e. 80%, 100%, and 120% separately [9-11]. Results of recovery studies were recorded in table-2.

#### *2.8.2. Specificity*

The specificity of the RP-HPLC method was determined by complete separation of LPV and RTV as shown in [Figure -1] with parameters like retention time ($t_R$), resolution (Rs) and tailing factor (T), peak purity curve and peak purity index. Tailing factor for peaks of LPV and RTV was less than 2% and resolution was satisfactory. The average retention time ± standard deviation for LPV and RTV were found to be 9.1±0.0148 and 8.2±0.0217 respectively for six replicates. The peaks obtained for LPV and RTV were sharp and have clear baseline separation.



Lopinavir (a) Ritonavir (b)

*Fig. 1. Structures of Lopinavir (LPV) and Ritonavir (RTV).*

#### *2.8.3. Limit of detection (LOD) and limit of quantification (LOQ)*

The LOD and LOQ were separately determined and reported in table – 3, based on the calibration curve of standard solution. The residual standard deviation of the regression line or the standard deviation of y – intercepts of regression lines may be used to calculate LOD and LOQ. LOD = 3.3 × D/S and LOQ = 10 × D/S, where, D is the standard deviation of y – intercepts of regression line and S is the slope of the calibration curve.

## 3. Results and discussion

The validated HPLC method was adopted for the quantification of LPV and RTV in their combined tablet dosage form and the typical chromatograms of the formulation was presented in

[Fig. 2]. The peak area ratios of each of the drugs to the internal standard were calculated and the amount of each drug present per tablet was estimated from the respective calibration curves. The mean assay results, expressed as a percentage in the label claim, were shown in Table 1. The results indicated that the amount of each drug in the tablet is within the requirements of 95 to 105% of the label claim.

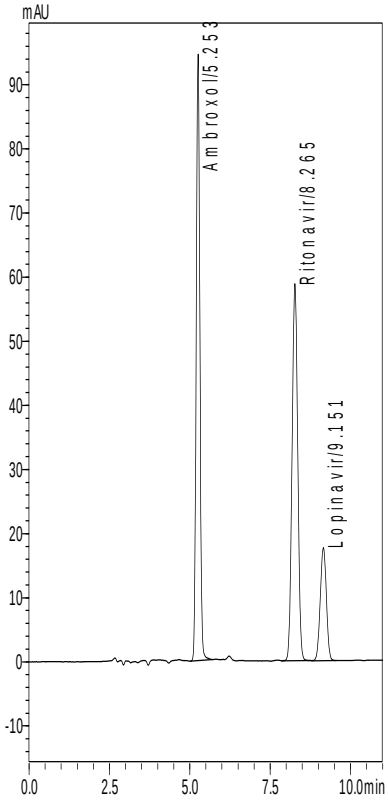



*Fig. 2. Chromatogram for Formulation.*

*Table 1. Analysis of formulations.*

| Drug | Label Claim (mg/tablet) | Estimated Amount (mg/tablet) | % Label claim | %RSD* |
|---|---|---|---|---|
| LPV | 200 | 198.64 | 99.09 | 0.53 |
| RTV | 50 | 49.26 | 98.52 | 0.35 |

*-Each value is a mean of six observations.

The Linearity and correlation coefficient of LPV and RTV was found to be 40-200 ug /ml, and 10–50 ug/ml, 0.999, and 0.9989 respectively.

Accuracy of the method was ascertained by recovery study (n=6) table 2. The concentration of standard spiked to the sample was 80%, 100%, and 120% of the assay level. The retention time of Lopinavir and Ritonavir was found to be 8.2, and 9.1 minutes respectively.

*Table 2. Accuracy (recovery studies).*

| Drug | Label Claim mg/tab | Estimated Amount mg/tab | Spike Level (%) | Amount of drug added (mg) | Amount of drug recovered (mg) | Percentage Recovery ± SD* |
|---|---|---|---|---|---|---|
| LPV | 200 | 199.41 | 80 | 80 | 79.48 | 99.35 ± 0.0694 |
| | | | 100 | 100 | 98.92 | 100.54 ± 0.3499 |
| | | | 120 | 120 | 120.46 | 100.39 ± 0.3081 |
| RTV | 50 | 48.87 | 80 | 80 | 80.23 | 98.26 ± 0.190 |
| | | | 100 | 100 | 99.59 | 99.59 ± 0.3426 |
| | | | 120 | 120 | 120.42 | 99.89 ± 0.3901 |

*-Each value is a mean of six observations.

The method was found to be accurate with percent recoveries ranging from 99.99% to 100.24%. The percentage RSD values of LPV and RTV was found to be 0.3499 and 0.3926 which clearly indicates that the method is precise.

The results of specificity studies from peak purity curve, and peak purity index shown in [Fig. 3] clearly suggests no interference of the excipients and mobile phase.

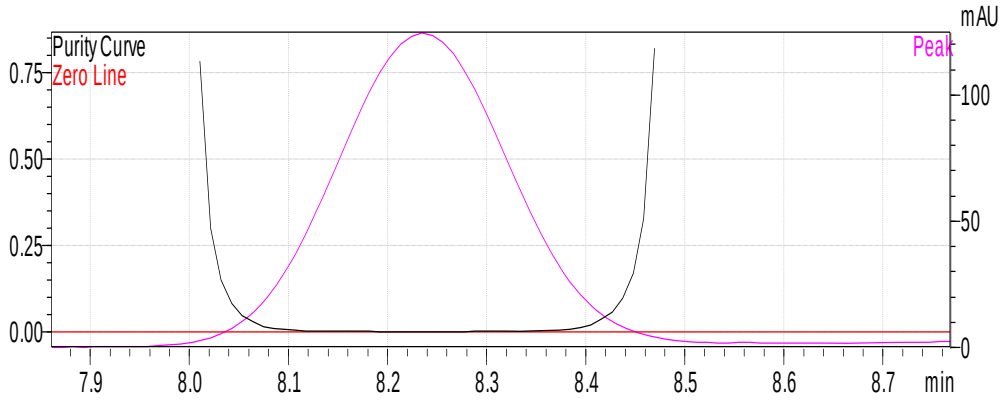



*Fig. 3. (a). Peak purity curve of standard (LPV).*

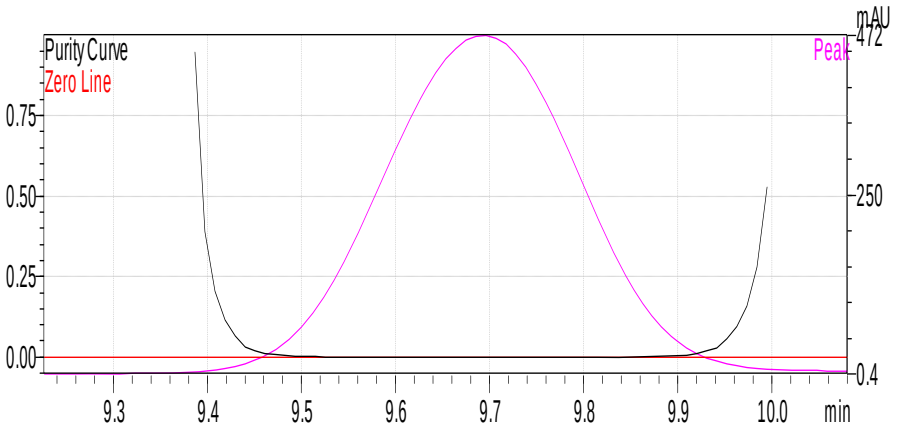



*Fig. 3. (b). Peak purity curve of standard (RTV).*

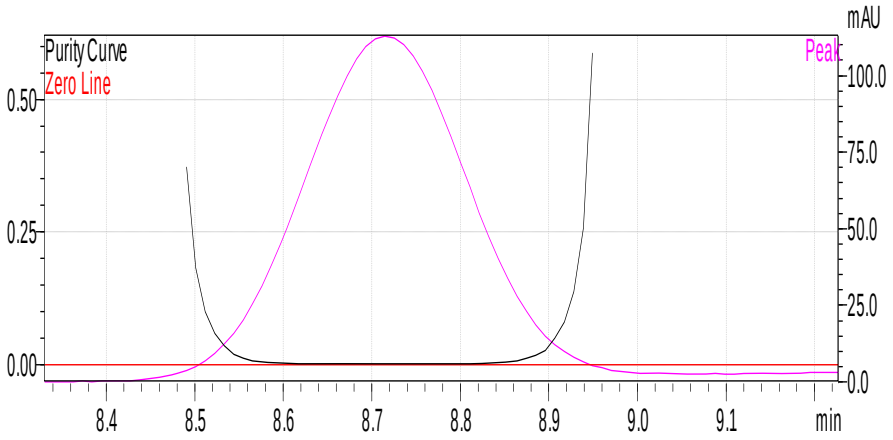



*Fig. 3. (c). Peak purity curve of sample (LPV).*

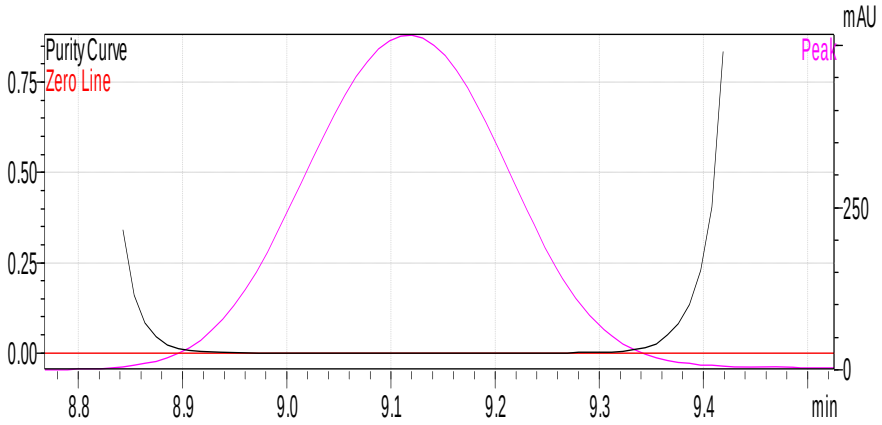



*Fig. 3. (d). Peak purity curve of sample (RTV).*

*Fig. 3: peak purity curves of Standard and Sample chromatograms.*

*Table 3. Results for validation parameters.*

| Validation Parameters | LPV | RTV |
|---|---|---|
| Linearity range (µg/ml) | 40-200 | 10-50 |
| Correlation co-efficient ($r^2$) | 0.9989 | 0.999 |
| LOD (µg/ml) | 40 | 20 |
| LOQ (µg/ml) | 160 | 50 |
| Intraday (%RSD)* | 0.0456 | 0.0648 |
| Interday (%RSD)* | 0.0647 | 0.0633 |
| Repeatability (%RSD)* | 0.065 | 0.067 |

| | | |
|---|---|---|
| Accuracy (%) | 99.99-101 | 100-102 |
| Peak purity index | 1.0000 | 1.0000 |
| Resolution factor (Rs) | - | 2.6 |
| No. of theoretical plates (N) | 10493 | 9533 |
| Capacity factor (K') | 0.841 | 0.65 |
| High equivalent to theoretical plates (HETP) | 14.7 | 15.6 |
| Tailing factor | 1.017 | 1.03 |

*-Each value is a mean of six observations.

## 4. Conclusions

The proposed method is accurate, selective and precise hence can be used for the routine quality-control analysis and quantitative simultaneous determination of Lopinavir and Ritonavir in combined tablet dosage forms and API. The mobile phase is easy to prepare and economical. The sample recoveries in all formulations were in good agreement with their respective label claims. The percentage RSD for all parameters was found to be less than 2, which indicates the validity of the method is in fair agreement. The method is also fast and requires approximately 10 min run time per sample for analysis.

The developed RP-HPLC method can be easily and conveniently adopted for routine analysis of LPV and RTV in multicomponent pharmaceutical preparation and for bioanalytical studies owing to the retention times i.e. more than 6 minutes is conceded to be advantageous for bioanalytical studies, since the plasma porteinous matters elute and interferes within 6 minutes of retention time. The method developed can be used for the rapid analysis of antiretroviral drugs.

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