Development and Validation of New Analytical Method for The Simultaneous Estimation of Darunavir And Ritonavir in Pharmaceutical Dosage Form

Pemra Raju*, K. Thejomoorthy2, P.Sreenivasa Prasanna3
1 Department of Pharmaceutical analysis, M.L.College of Pharmacy, S. Konda-523101.
2 Head, Department of Pharmaceutical analysis, M.L.College of Pharmacy, S. Konda-523101.
3 Principal, M.L.College of Pharmacy, S.Konda-523101.

Abstract
A simple, Accurate, precise method was developed for the simultaneous estimation of the Darunavir and Ritonavir in Tablet dosage form. The chromatogram was run through Agilent C18 150 x 4.6 mm, 5m. Mobile phase containing Buffer 0.1% Formic acid: Acetonitrile, taken in the ratio 70:30 was pumped through the column at a flow rate of 0.95 ml/min. The temperature was maintained at 30°C. The optimized wavelength selected was 293 nm. The retention times of Darunavir and Ritonavir were found to be 2.369 min and 2.911. %RSD of the Darunavir and Ritonavir were and found to be 0.7 and 0.5 respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Darunavir and Ritonavir were 1.49, 5.191 and 0.37, 1.11 respectively. Regression equation of Darunavir is y = 5421x + 640.7, and y = 3870.x + 5191 of Ritonavir. Retention times were decreased and run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control tests in Industries.

Keywords:
Darunavir, Ritonavir, RP-HPLC.
Ritonavir is chemically 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[2S]-3-methyl-2-[(methyl(2-(propan-2-yl)-1,3-thiazol-4-y1)methyl)] carbamoyl] amino] butanamido] 1,6-diphenylhexan-2-yl|carbamate. It is an HIV protease inhibitor that interferes with the reproductive cycle of HIV.

Although it was initially developed as an independent antiviral agent, it has been shown to possess advantageous properties in combination regimens with low-dose ritonavir and other protease inhibitors. There are few methods reported in the literature of Darunavir and Ritonavir alone or in combination with other drugs in the pure and pharmaceutical formulation by UV, HPLC and UPLC-MS. In view of the need of suitable, cost effective RP HPLC method for routine analysis of simultaneous estimation of RTV and DRV in bulk and synthetic mixture (tablet dosage form), attempts were made to develop a simple, accurate, precise and cost effective analytical method for the estimation of RTV and DRV. The purpose of stability testing is to check the drug quality under the action of many environmental factors like temperature, acid, base and oxidative condition. This is necessary for establishment of re-test period for the drug products and for recommendation conditions for their storage. ICH guidelines therefore emphasize stability-indicating analytical methods. Efforts were therefore made to develop a novel, fast and validated stability indicating HPLC procedure for determining simultaneously both the drugs in tablet dosage forms. The proposed method will be validated as per ICH guidelines.

Experimental work
Materials and Methods
Materials

Ritonavir and Darunavir pure drugs (API), Combination Ritonavir and Darunavir, Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

**Instruments**
Electronics Balance-Denver , pH meter -BVK enterprises, India , Ultrasonicator-BVK enterprises, WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Auto sampler integrated with Empower 2 Software.UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Ritonavir and Darunavir solutions.

**Methods**

**Diluent**
Based up on the solubility of the drugs, diluent was selected, Methanol and Water taken in the ratio of 50:50

**Preparation of Standard stock solutions**
Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

**Preparation of Standard working solutions (100% solution)**
1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (50µg/ml Ritonavir of and 400µg/ml of Darunavir)

**Preparation of Sample stock solutions**
5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to tablet was transferred into a 100 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

**Preparation of Sample working solutions (100% solution)**
1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent.(50µg/ml of Ritonavir and 4000µg/ml of Darunavir)

**Preparation of buffer**
0.1%OPA Buffer
1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

**Method Validation**

**System suitability parameter**
The system suitability parameters were determined by preparing standard solutions of Ritonavir (50ppm) and Darunavir (400ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and
USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

Specificity
Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision
Preparation of Standard stock solutions Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

Preparation of Standard working solutions (100% solution) 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (50µg/ml of Ritonavir and 400µg/ml of Darunavir)

Preparation of Sample stock solutions 5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to tablet was transferred into a 10 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

Preparation of Sample working solutions (100% solution) 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent.(50µg/ml of Ritonavir and 400µg/ml of Darunavir)

Linearity
Preparation of Standard stock solutions Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

25% Standard solution 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (12.5µg/ml of Ritonavir and 100µg/ml of Darunavir)

50% Standard solution 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (25µg/ml of Ritonavir and 200µg/ml of Darunavir)

75% Standard solution 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (37.5µg/ml of Ritonavir and 300µg/ml of Darunavir)

100% Standard solution 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (50µg/ml of Ritonavir and 400µg/ml of Darunavir)

125% Standard solution 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (62.5µg/ml of Ritonavir and 500µg/ml of Darunavir)

150% Standard solution 1.5ml each from two standard stock solutions was pipetted out and made up to 10ml (75µg/ml of Ritonavir and 600µg/ml of Darunavir)

Accuracy
Preparation of Standard stock solutions Accurately weighed 12.5 mg of Ritonavir, 100mg of Darunavir and transferred to 25ml volumetric flask and 3/4 th of diluents was added to these flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (500µg/ml of Ritonavir and 4000µg/ml of Darunavir)

Preparation of 50% Spiked Solution 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria
The % Recovery for each level should be between 98.0 to 102

Robustness Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Robustness conditions like Flow minus (1ml/min), Flow plus (1.2ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus(35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Ritonavir, Darunavir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Ritonavir, Darunavir, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies [23]

Oxidation
To 1 ml of stock solution of Ritonavir and Darunavir, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60°C.
For HPLC study, the resultant solution was diluted to obtain 50 μg/ml & 400 μg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Acid Degradation Studies**
To 1 ml of stock solution Ritonavir and Darunavir, 1 ml of 2N Hydrochloric acid was added and refluxed for 30 mins at 60°C. The resultant solution was diluted to obtain 50 µg/ml & 400 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Alkaline Degradation Studies**
To 1 ml of stock solution Ritonavir and Darunavir, 1 ml of 2N sodium hydroxide was added and refluxed for 30 mins at 60°C. The resultant solution was diluted to obtain 50 µg/ml & 400 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Dry Heat Degradation Studies**
The standard drug solution was placed in oven at 105°C for 1 hr study dry heat degradation. For HPLC study, the resultant solution was diluted to 50 µg/ml & 400 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Photo Stability studies**
The photochemical stability of the drug was also studied by exposing the 500 µg/ml & 4000 µg/ml solution to UV Light by keeping the beaker in UV Chamber for 1 day or 200 Watt hours/m² in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 200 µg/ml & 300 µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Neutral Degradation Studies**
Stress testing under neutral conditions was studied by refluxing the drug in water for 1 hr at a temperature of 60°C. For HPLC study, the resultant solution was diluted to 50 µg/ml & 400 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

**Results and Discussion**

**Optimized method**

**Chromatographic conditions**

| Parameter              | Value                     |
|------------------------|---------------------------|
| Mobile phase           | 70% Formic acid (0.1%): 30% Acetonitrile |
| Flow rate              | 1 ml/min                  |
| Column                 | Azilent C18 (4.6 x 150 mm, 5 μm) |
| Detector wave length   | 260 nm                    |
| Column temperature     | 30°C                      |
| Injection volume       | 10 μl                     |
| Run time               | 6 min                     |

**Diluent**
Water and Acetonitrile in the ratio 50:50

**Results** Both peaks have good resolution, tailing Factor, theoretical plate count and resolution.

**System suitability** All the system suitability parameters were within the range and satisfactory as per ICH guidelines.

**Table: 1 System suitability parameters for Darunavir and Ritonavir**

| S no | Linj | RT (m in) | Tailing | RT (m in) | Tailing | Resolution |
|------|------|-----------|---------|-----------|---------|------------|
| 1    |      | 2.404     | 1.19    | 2.986     | 1.13    | 4.4        |
| 2    |      | 2.405     | 1.16    | 2.986     | 1.12    | 4.4        |
| 3    |      | 2.405     | 1.20    | 2.988     | 1.15    | 4.3        |
| 4    |      | 2.413     | 1.17    | 2.998     | 1.09    | 4.2        |
| 5    |      | 2.421     | 1.18    | 3.013     | 1.13    | 4.4        |
| 6    |      | 2.433     | 1.18    | 3.036     | 1.10    | 4.5        |

**Discussion**

According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

**Validation**
Specificity

Figure No. 5. Chromatogram of blank

Linearity:

Table 2 Linearity table for Darunavir and Ritonavir.

| Conc (µg/mL) | Peak area | Conc (µg/mL) | Peak area |
|--------------|-----------|--------------|-----------|
| 0            | 0         | 0            | 0         |
| 100          | 390764    | 12.5         | 67733     |
| 200          | 786093    | 25           | 134305    |
| 300          | 1174300   | 37.5         | 205232    |
| 400          | 1563383   | 50           | 277599    |
| 500          | 1907925   | 62.5         | 338712    |
| 600          | 2341934   | 75           | 404125    |

Discussion

Six linear concentrations of Darunavir (100-600µg/ml) and Ritonavir (12.5-75µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Darunavir was \( y = 3870.x + 5191 \) and of Ritonavir was \( y = 5421.x + 640.7 \). Correlation coefficient obtained was 0.999 for the two drugs.

Precision

System Precision

Table 3 System precision table of Darunavir and Ritonavir

| S. No | Area of Darunavir | Area of Ritonavir |
|-------|-------------------|-------------------|
| 1.    | 1562412           | 272468            |
| 2.    | 1565061           | 277211            |
| 3.    | 1568363           | 271649            |
| 4.    | 1566157           | 270677            |
| 5.    | 1566158           | 273575            |
| 6.    | 1561519           | 272713            |
| Mean  | 1564945           | 273049            |
| S.D   | 2560.9            | 2264.3            |
| %RSD  | 0.2               | 0.8               |
Repeatability

Table 4 Repeatability table of Darunavir and Ritonavir

| S. No | Area of Darunavir | Area of Ritonavir |
|-------|-------------------|-------------------|
| 1.    | 1563796           | 273031            |
| 2.    | 1563323           | 274473            |
| 3.    | 1570928           | 275737            |
| 4.    | 1585721           | 275393            |
| 5.    | 1590713           | 273519            |
| 6.    | 1582460           | 272597            |
| Mean  | 1576157           | 274125            |
| S.D   | 11729.6           | 1282.7            |
| %RSD  | 0.7               | 0.5               |

Discussion: Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.7% and 0.5% respectively for Darunavir and Ritonavir. As the limit of Precision was less than “2” the system precision was passed in this method.

Intermediate precision (Day, Day Precision)

Table 5 Intermediate precision table of Darunavir and Ritonavir

| S. No | Area of Darunavir | Area of Ritonavir |
|-------|-------------------|-------------------|
| 1.    | 1508957           | 270706            |
| 2.    | 1506297           | 270017            |
| 3.    | 1488309           | 274639            |
| 4.    | 1509602           | 268996            |
| 5.    | 1502940           | 267494            |
| 6.    | 1499562           | 270510            |
| Mean  | 1502611           | 270394            |
| S.D   | 7958.4            | 2393.2            |
| %RSD  | 0.5               | 0.9               |

Discussion

Multiple sampling from a sample stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation and % RSD were calculated for two drugs and obtained as 0.5% and 0.9% respectively for Darunavir and Ritonavir. As the limit of Precision was less than “2” the system precision was passed in this method.

Accuracy

Table 6 Accuracy table of Darunavir

| % Level | Amount Spiked (μg/mL) | Amount recovered (μg/mL) | % Recovery | Mean % Recovery |
|---------|-----------------------|--------------------------|------------|----------------|
| 50%     | 200                   | 200.00                   | 100.00     |                |
|         | 200                   | 202.03                   | 101.02     |                |
|         | 200                   | 196.43                   | 98.22      |                |
| 100%    | 400                   | 396.33                   | 99.08      | 99.67%         |
|         | 400                   | 398.75                   | 99.69      |                |
|         | 400                   | 400.62                   | 100.15     |                |
| 150%    | 600                   | 596.41                   | 99.40      |                |
|         | 600                   | 590.94                   | 98.49      |                |
|         | 600                   | 605.68                   | 100.95     |                |

Table 7 Accuracy table of Ritonavir

| % Level | Amount Spiked (μg/mL) | Amount recovered (μg/mL) | % Recovery | Mean % Recovery |
|---------|-----------------------|--------------------------|------------|----------------|
| 50%     | 25                    | 24.94                    | 99.75      |                |
|         | 25                    | 24.80                    | 99.19      |                |
|         | 25                    | 25.03                    | 100.13     |                |
| 100%    | 50                    | 49.10                    | 98.21      | 99.57%         |
|         | 50                    | 49.30                    | 98.60      |                |
|         | 50                    | 49.57                    | 99.13      |                |
| 150%    | 75                    | 74.33                    | 99.10      |                |
|         | 75                    | 74.83                    | 99.78      |                |
Discussion
Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 99.67% and 99.57% for Darunavir and Ritonavir respectively.

Sensitivity
Table 8 Sensitivity table of Darunavir and Ritonavir

| Molecule       | LOD  | LOQ  |
|----------------|------|------|
| Darunavir      | 1.49 | 4.51 |
| Ritonavir      | 0.37 | 1.11 |

Robustness
Table 9 Robustness data for Darunavir and Ritonavir.

| S.no | Condition          | %RSD of Darunavir | %RSD of Ritonavir |
|------|--------------------|-------------------|-------------------|
| 1    | Flow rate (-) 0.9ml/min | 1.1               | 1.1               |
| 2    | Flow rate (+) 1.1ml/min      | 0.3               | 1.0               |
| 3    | Mobile phase (-) 65:35A | 0.8               | 1.0               |
| 4    | Mobile phase (+) 75B:25A | 0.5               | 0.7               |
| 5    | Temperature (-) 25°C    | 0.5               | 0.4               |
| 6    | Temperature (+) 35°C    | 0.8               | 0.7               |

Discussion
Robustness conditions like Flow minus (0.85ml/min), Flow plus (1.15ml/min), mobile phase minus (65B:35A), mobile phase plus (75B:25A), temperature minus (25°C) and temperature plus(35°C)was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Assay
Mylan pharmaceuticals(Durat R 450 Tablet), bearing the label claim Darunavir 400mg, Ritonavir 50mg. Assay was performed with the above formulation. Average % Assay for Darunavir and Ritonavir obtained was 100.62% and 100.29% respectively.

Table 10 Assay Data of Darunavir

| S.no | Standard Area | Sample area | % Assay |
|------|---------------|-------------|---------|
| 1    | 1562412       | 1563796     | 99.83   |
| 2    | 1565061       | 1563323     | 99.80   |
| 3    | 1568363       | 1570928     | 100.28  |
| 4    | 1566157       | 1585721     | 101.23  |
| 5    | 1566158       | 1590713     | 101.54  |
| 6    | 1561519       | 1582460     | 101.02  |
| Avg  | 1564945       | 1576157     | 100.62  |
| Stdev| 2560.9        | 11729.6     | 0.75    |
| %RSD| 0.2           | 0.7         | 0.7     |

Table 11 Assay Data of Ritonavir

| S.no | Standard Area | Sample area | % Assay |
|------|---------------|-------------|---------|
| 1    | 272468        | 273031      | 99.89   |
| 2    | 277211        | 274473      | 100.42  |
| 3    | 271649        | 275737      | 100.88  |
| 4    | 270677        | 275393      | 100.76  |
| 5    | 273575        | 273519      | 100.07  |
| 6    | 272713        | 272597      | 99.73   |
| Avg  | 273049        | 274125      | 100.29  |
| Stdev| 2264.3        | 1282.7      | 0.5     |
| %RSD| 0.8           | 0.5         | 0.5     |

Fig 08 Chromatogram of working standard solution
Degradation data

Table 12 Degradation data for Darunavir and Ritonavir

| Type of degradation | Darunavir | Ritonavir |
|---------------------|-----------|-----------|
|                     | AREA      | %RECOVERED | % DEGRADED | AREA      | %RECOVERED | % DEGRADED |
| Acid                | 1420809   | 90.70      | 9.30       | 256076   | 93.69      | 6.31       |
| Base                | 1471589   | 93.94      | 6.06       | 260788   | 95.41      | 4.59       |
| Peroxide            | 1453963   | 92.82      | 7.18       | 257555   | 94.23      | 5.77       |
| Thermal             | 1522949   | 97.22      | 2.78       | 265997   | 97.32      | 2.68       |
| Uv                  | 1552374   | 99.10      | 0.90       | 269020   | 98.43      | 1.57       |
| Water               | 1556561   | 99.36      | 0.64       | 270988   | 99.15      | 0.85       |

Conclusion

A simple, accurate, precise method was developed for the simultaneous estimation of the Darunavir and Ritonavir in Tablet dosage form. Retention time of Darunavir and Ritonavir were found to be 2.369 min and 2.911 min. %RSD of the Darunavir and Ritonavir were found to be 0.7 and 0.5 respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively. %Recovery was obtained as 99.67% and 99.78% for Darunavir and Ritonavir respectively.

Authors are Declared no Conflict of Interest.

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