Ind. J. Pharm. Edu. Res., 2024; 58(2s):s558-s565.
https://www.ijper.org

Original Article

Stability Indicating RP-UPLC Method for Impurity Profiling of Darunavir and Ritonavir in Fixed Dose Drug Combination Product

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ABSTRACT

Background: The goal of the proposed study was to develop and validate stability indicating mass compatible reverse phase UPLC method for impurity profiling of Darunavir Ethanolate and Ritonavir degradation impurities in fixed-dose drug combination products. **Materials and Methods:** The optimized chromatographic condition includes use of Zorbax Bonus C_{18} column (150x2.1 mm, 1.8 µm) with mobile phase A (Buffer (55): Methanol (45)) and mobile phase B (Acetonitrile: (30) and Methanol (70)), flow rate of 0.22 mL/min and detection at 240 nm with gradient program of 50 min. The method was validated as per ICH quality guideline Q2 (R1) including specificity by forced degradation study to confirm suitability for intended use. **Results:** The observed retention time for Darunavir was 11.4 min and for Ritonavir was 30.0 min. The Limit of Quantitation (LOQ) for all degradation impurities found is 0.05%, equivalent to the reporting threshold level. The method was found linear and accurate in the range of LOQ to 150% with an observed range of % accuracy of 90.1 to 106.3 for all known impurities. The method was found precise based on less than 10.0% RSD and robust for deliberate changes. Considering the use of a volatile mobile phase, the method can be applied for LC-MS-based analysis for mass identification. **Conclusion:** The developed UPLC method was applied for impurity profiling during the release and stability study of Darunavir Ethanolate and Ritonavir in fixed-dose drug combination products.

Keywords: Method Validation, Forced degradation, Linearity, Accuracy, Robustness, Impurity profiling.

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Received: 19-04-2023;
Revised: 17-09-2023;
Accepted: 06-01-2024.

INTRODUCTION

The anti-retrovirus drug products are used for the treatment of HIV disease. There are various anti retrovirus drug products available including some fixed dose drug combination also.[1,2] Considering requirement of higher dosage regime of anti-retroviral product for HIV treatment, the formulation having two active in one tablet helps to maintain dose along with ease of therapy from patient perspective. Further refer to the literature reference on bioavailability, observed high relative bioavailability of Ritonavir in presence of other drug such as Darunavir, Atazanavir confirming better efficacy of fixed dose drug combination product.[3,4]

This research article involves analytical method for impurity profile in fixed dose drug combination drug product i.e. Darunavir Ethanolate and Ritonavir tablets (800 mg+100 mg). The product as bilayer tablets having Darunavir ethanolate and excipients in one layer and Ritonavir with excipients in second layer followed by film coating. Considering the poor water solubility of active ingredient Ritonavir, increase oral bioavailability of poorly water-soluble drugs was one of the most challenging aspects of drug product development. Therefore, formulation approach followed was by Hot Melt Extrusion (HME) to convert crystalline Ritonavir to Amorphous nature to increase solubility and hence bioavailability.[5-7] Further Ritonavir HME process blend was compressed with Darunavir to get fixed dose drug combination tablet.

Individual monograph of Darunavir and ritonavir tablet found in Indian pharmacopeia and US Pharmacopeia which include multiple known impurities and its method of analysis, however fixed dose drug combination product monograph was not available hence developing common method to capture both

DOI: 10.5530/ijper.58.2s.58

active and all degradation impurities was challenging task for analytical. Further, literature methods are available for estimation of individual Darunavir in bulk or formulation and impurity based literature method available by UPLC and MS compatible methods for formulation containing Darunavir.[8-11]

Similarly, for Ritonavir, available literature methods are for impurity profiling of individual Ritonavir based product and its degradation impurities.[12,13] There were some literatures found for simultaneous estimation which include assay of multiple HIV drugs by single method and different fixed dose drug combination-based impurity method for HIV drugs like Atazanavir with Ritonavir, Lopinavir with Ritonavir.[14-16]

Considering no literature found for the fixed dose drug combination product containing Darunavir and Ritonavir for its impurity profile, same activity considered under this study. The method was developed using UPLC instrument so as to have possible short runtime to achieve better separation of impurities in single chromatogram. The developed method found capable to detect and quantify all possible degradation impurities related to Darunavir Ethanolate, Ritonavir and other possible impurities if generated due to manufacturing process and/or interaction with excipients. The developed method was validated as per available method validation guidance.[17,18] The objective of this study was to have validated stability indicating Mass compatible UPLC method for impurity profiling of Darunavir Ethanolate and Ritonavir in fixed dose drug combination product.

MATERIALS AND METHODS

Materials

The Darunavir Ethanolate and Ritonavir was gifted by Emcure Pharmaceuticals Ltd. Ammonium acetate of Emparta grade Merck and high purity solvent Acetonitrile, Methanol of HPLC grade were of J. T. Baker supplied by Avantor. Additionally, concentrated hydrochloric acid (35%), Sodium hydroxide flakes (98%) and Hydrogen peroxide (30%) were from Merck Millipore. The HPLC grade water from Merck Milli Q system was used for preparation of mobile phase and diluent. The drug product as fixed dose drug combination film coated tablet containing Darunavir ethanolate equivalent to 800 mg Darunavir and 100 mg Ritonavir was developed by Emcure Pharmaceuticals Ltd. including placebo blend (excipient blend without any active substance).

Instrumentation

The Ultra high performance liquid chromatography system of Acquity waters make with Photo Diode Array detector, Quaternary gradient pump and column oven connected through Emower-3 software was used for impurity method optimization. The polar-embedded chemistry based Agilent make HPLC column Zorbax bonus, C_{18} (150x2.1 mm, 1.8 µm) was selected to achieve impurity separation of both Darunavir ethanolate and Ritonavir in single chromatographic method.

Selection of wavelength

The UV Scan of active ingredient Darunavir ethanolate and ritonavir shows UV maxima (λ_{max}) as 267 nm and 240 nm respectively. As per product label claim, concentration of Ritonavir was lower (100 mg/tablet) as compare to Darunavir (800 mg/tablet) and multiple known degradation impurities of Ritonavir needs to consider for impurity profiling. Based on this a common wavelength as 240 nm was selected for impurity profiling of Darunavir and Ritonavir in fixed dose drug combination product.

Chromatographic condition optimization

The chromatographic conditions followed for impurity profiling in Darunavir ethanolate and Ritonavir fixed dose drug combination product include use of mobile phase A: 0.02 M Ammonium acetate and Methanol in the ratio 55:45 (%v/v) and mobile phase B include mixture of Acetonitrile and Methanol in the ratio 30:70 (%v/v). The method was optimized with flow rate of 0.22 mL/min, injection volume 5.0 µL and column oven temperature was maintained at 55°C throughout the analysis.

The method was optimized with gradient program as follows, time (min)/A(v/v): B(v/v); T0.01/100:0, T1/100:0, T3/95:5, T35/60:40, T38/0:100, T42/0:100, T44/100:0 and T50/100:0. The diluent used for standard and sample preparation include mixture of 0.02M Ammonium acetate and Methanol in the ratio 30:70 (%v/v).

Preparation of standard solution

The initial standard stock solution containing Darunavir Ethanolate and Ritonavir was prepared at concentration 0.4 mg/mL and 0.1 mg/mL respectively in method diluent. This was further diluted to 0.004 mg/mL 0.0005 mg/mL using diluent resulting to get final concentration of 0.1% with respect to test concentration.

Preparation of Placebo solution

Weighed accurately and transferred tablet placebo (containing all excipients without Darunavir ethanolate and Ritonavir) equivalent to 50 mg of Ritonavir in to 100 mL clean and dry volumetric flask. Added 80 mL of diluent and sonicate for 45 min with intermittent shaking. The solution was allowed to attain room temperature and dilute to final volume with diluent and mix well. The final solution was filtered through 0.20 µ Nylon (Millipore Millex-GN) filter by discarding first 2 mL of filtrate.

Preparation of sample solution

First average weight of 20 tablets was determined. Tablets were crushed to very fine powder. Weighed accurately and transferred tablet powder equivalent to 50 mg of Ritonavir in to 100 mL clean

and dry volumetric flask. Added 80 mL of diluent and sonicated for 45 min with intermittent shaking. The solution was allowed to attain room temperature and finally diluted to volume with diluent and mix well. The resulting solution was filtered through 0.20 µ Nylon (Millipore Millex-GN) filter by discarding first 2 mL of filtrate. The concentration of final solution was 4 mg/mL Darunavir and 0.5 mg/mL Ritonavir.

Preparation of sample solution spiked with impurities

The sample solution was prepared as described above. All individual known impurities stock was prepared at known concentration and spiking of each impurity solution was performed in sample solution before volume make up. The spiking concentration was considered as per ICH guideline-based specification limit of impurities refers to qualification threshold.

Procedure for UPLC (Ulter High Pressure Liquid Chromatography) analysis

The evaluation of diluent as blank, placebo solution, replicate injections of standard solution followed by sample solution were considered for impurity profile study during batch analysis and validation. The outcome of blank and placebo was evaluated to check any peaks disregard and replicate injections of standard were used for system suitability check during every analysis. Further spiked sample solution was used for selectivity study by peak identification.

Evaluation of System suitability criteria

To confirm reproducibility of method as per good chromatography practice, following system suitability criteria were considered for Darunavir and ritonavir peak in standard solution for evaluation before sample analysis. This includes monitoring of USP Plate count (N)=not less than 3000, USP tailing factor (T) control as not more than 2.0 and % RSD for the peak area obtained by replicate injections (n=6) as not more than 10.0%.

Analytical Method Validation

The optimized impurity method was validated for its suitability to use for release and stability testing of drug product. The in-house specification criteria was designed for known and unknown impurities refer to maximum daily dose based calculation and available monograph in US Pharmacopiea. The method validation was carried out in accordance to ICH guidance. This includes parameters as stated below.

Specificity

The specificity was carried out by evaluating separation of known impurities from each other and also from the peak of Darunavir and Ritonavir. Further evaluation of blank and placebo peaks separation from all impurity and API peaks also verified. Addition to visual separation, peak purity was monitored for each known impurity and API peak.

Forced degradation study

The forced degradation of drug product was carried out at different conditions and outcome was monitored by PDA detector to confirm peak purity of known impurity and Darunavir, Ritonavir peak. The forced degradation study confirms method suitability for intended use. The optimized stress conditions followed for acid degradation (0.1 N HCl/10 min/30C), Base degradation (0.1 N NaOH/10 min/30C), Peroxide degradation (5% H_2O_2/10 min/30C), thermal degradation (2 hr/60C), Photolytic degradation (1.2 million lux hr).

Sensitivity of method by LOQ

According to the maximum daily dose for each API, the reporting threshold level as 0.05% was considered for LOQ level hence each impurity and Ritonavir API solution at 0.05% level was injected to evaluate Signal to Noise (S/N) ratio which should be ≥10.

Refer to the very high response of Darunavir, very low level i.e. 0.005% level was considered for LOQ and 0.002% for LOD level. Also, all other impurities and Ritonavir LOQ level solution was further diluted to 0.02% to prepare LOD level solution and injected to evaluate S/N ratio which should be moe than 3. The derived LOQ level precision was confirmed by six replicate preparation of each impurity and both API to evaluate % RSD for the peak area obtained which should be less than 10.0%.

Linearity

The linearity of method was established from LOQ level to 150% level of specification limit for each known impurity. The outcome was verified for correlation coefficient through graphical evaluation.

Precision

Precision study includes system precision and method precision both. In case of system precision, standard solution injected for six time and the outcome of System Suitability (SST) criteria were monitored as stated for evaluation of SST. The method precision was carried out by analysing six set of tablet sample preparation spiked with all known impuritie at limit level. The outcome was interpretted as % RSD for the result obtained for each known impurity, single maximum unknown impurity and total impurities.

Accuracy/Recovery

The accuracy was carried out by spiking each known impurity in tablet sample solution at LOQ, 100% and 150% level with respect to specification limit. The outcome as % accuracy was derived by calculating amount added and amount found for each impurity. Three replicate determination was carried out for each impurity

and each level to have total 9 determination and % RSD was derived for replicates of each level.

Robustness

The robustness was performed by deliberate change in method parameters to monitor impact on impurity separation. The major two parameters i.e. change in flow rate and change in column oven temperature was considered for system suitability evaluation and also for separation of impurities from each other and from both API peak.

RESULTS

The UPLC method developed for impurity profile of Darunavir ethanolate and Ritonavir tablets as fixed dose drug combination using single chromatography for control of all possible degradation impurities. The method was optimized with mobile phase ratio in gradient program so as to achieve desired system suitability and impurity separation.

The optimized method was validated as per ICH and the observed outcome recorded for each parameter against acceptance criteria refer to guidance based requirement. The system suitability chromatogram containing Darunavir Ethanolate and Ritonavir and the sample chromatogram showing impurity profile in sample solution prepared using Darunavir Ethanolate and Ritonavir tablet (800 mg+100 mg) was recorded (Figure 1(A) and 1(B)).

Method Validation study

Specificity

The Specificity study was carried out by spiking all possible degradation impurities in sample solution. These includes below listed impurities which as a part of drug product specification.[19]

Ritonavir Impurity E (Hydroxy-Ritonavir): Thizol-5-ylmethyl-[(1S,2S,4S)-1-benzyl-2-hydroxy-4-[[(2S)-2-[[[[2-(1-hydroxy-1-methylethyl)thiazol-4-yl-methyl]carbamoyl]amino]-3-methylbutanoyl]-amino-5-phenyl pentyl]carbamate.

Ritonavir impurity F (Hydantoin amino alcohol): Thiazol-5-ylmethyl[(1S,2S,4S)-1-benzyl-4-[[(2S)-1-benzyl-2-hydroxy-4-[(4S)-4-(1-methylethyl)-2,5-dioxoimidazolidin-1-yl]-5-phenylpentyl] carbamate.

Ritonavir Impurity-L (Oxazolidinone derivative): 4*S*,5*S*)-4-benzyl-5-[(2*S*)-2-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]-butanoyl]amino]-3-phenylpropyl] oxazolidin- 2-one.

Ritonavir Impurity-O (3*R*-Epimer): Thiazol-5-ylmethyl [(1S,2R,4S)-1-benzyl-2-hydroxy-4-[[(2S)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]-butanoyl]amino]-5-phenylpentyl]carbamate.

Thiazolyl N-methyl Methanamine impurity: 1-(2-isopropyl-1,3-thizol-4-yl-)N-methylmethaenamine acetate salt.

All the listed impurities were spiked at limit level concentration. Recording of purity angel and purity threshold using Empower software was carried out for each impurity and API peaks. The retention time and peak purity outcome of specificity was compiled (Table 1A and Table 1B) and spiked sample chromatogram (known impurities spiked in tablet sample) was recorded (Figure 2).

Forced Degradation (FD) Study

Addition to selectivity study, forced degradation was also carried out by applying different stress condition to drug product sample solution. The similar exposure was also carried out for diluent blank and placebo solution for comparative evaluation of degradation impurities against sample solution. The evaluation of each FD samples were carried out using photo diode array detector and the outcome was monitored for satisfactory result of peak purity. The evaluation was carried out for individual known impurity and each API ensuring peak purity angle was lower than peak purity threshold. The overall outcome of Forced degradation study was recorded (Table 1).

Sensitivity of method by LOD/LOQ

The LOD at 0.02% and LOQ at 0.05% level (of test concentration) was verified for individual impurity and Ritonavir API peak and the outcome was recorded as S/N ratio (not less than 10). For Darunavir ethanolate the LOD level was considered as 0.002% and LOQ level as 0.005%. Further % RSD for peak area of six replicate injections of each impurity and both API at LOQ level (not more than 10.0%) was recorded (Table 5).

Linearity

The linearity was performed from LOQ to 150% of each impurity specification level along with Darunavir and Ritonavir API. According to the range selected, the concentration considered for Darunavir (0.20-12.0 µg/mL), Ritonavir (0.25-20.0 µg/mL), Impurity E (0.25-2.25 µg/mL), Impurity F (0.25-19.5 µg/mL), Impurity L (0.25-2.25 µg/mL), Impurity O (0.25-2.25 µg/mL) and for N-thiazole impurity (0.25-6.07 µg/mL). The outcome of linearity was evaluated by plotting calibration curve for average area (X axis) verses concentration for each component (Y axis). The final outcome interpretted by statistical evaluation for correlation coefficient, slope and Y-intercept for each impurity and API. The outcome of each linearity to meet correlation coefficient ≥0.995 was verified. The obtained corellation coefficient outcome of Linearity for each impurity was recorded (Table 5).

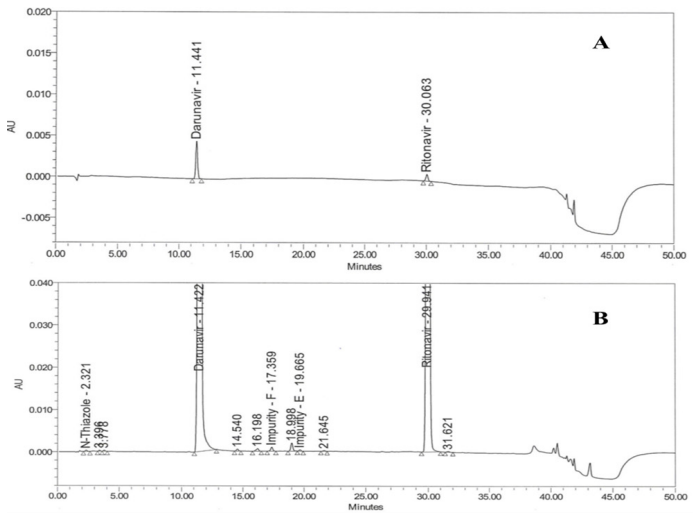

Figure 1: (A) Standard chromatogram containing Darunavir Ethanolate and Ritonavir (B) Sample chromatogram prepared using drug product Darunavir Ethanolate and Ritonavir tablet (800 mg+100 mg).

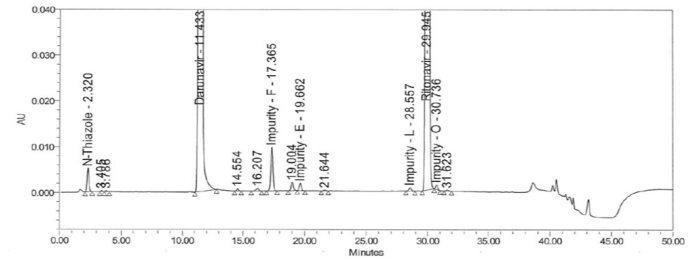

Figure 2: Selectivity study chromatogram of tablet sample solution spiked with known impurities.

Precision

System precision

System precision was established by evaluating system suitability criteria as per method. This includes % RSD of peak area (less than 10.0%) obtained from six replicate injections of standard preparation for Darunavir and Ritonavir peak. The USP plate count observed for Darunavir was more than 30000 and for Ritonavir more than 150000. Also tailing factor observed for both component peaks lies between 0.8 to 1.5. The compiled outcome of % RSD for peak is of Darunavir and Ritonavir was recorded (Table 2).

Method precision

The method precision study was carried out using drug product sample solution spiked with all known impurities at specification level. The outcome of all six-sample preparation for each known, single maximum unknown impurity and total impurities were calculated and % RSD derived by statistical calculation. The six-replicate analysis outcome of % impurity result was evaluated to ensure below 10.0% RSD (Table 2).

Accuracy/Recovery

The accuracy study was carried out in the range of LOQ to 150% level by spiking known impurities in sample preparation for other than LOQ level and for LOQ level all known impurities were spiked in placebo solution. Considering minimum requirement of 9 determinations for each impurity, triplicate preparation

Table 1A: Specificity and Forced degradation study outcome.

Sl. No.	Stress type	% Impurity / % Degradation						
		Ritonavir impurity E	Ritonavir impurity L	Ritonavir impurity F	Ritonavir impurity O	Impurity N-Thiazole	Any unspecified impurity	Total Impurities/ Degradation
1	Untreated sample	0.07	ND	0.31	ND	0.06	0.05	0.65
2	Acid degradation	0.57	1.64	0.23	ND	0.06	0.18	2.91
3	Base degradation	0.39	16.26	0.21	ND	0.05	0.05	17.09
4	Peroxide degradation	0.07	ND	0.32	ND	0.06	0.05	0.64
5	Thermal degradation	0.07	ND	0.35	ND	0.08	0.07	0.77
6	Humidity degradation	0.08	ND	0.33	ND	0.07	0.05	0.67
7	Photolytic degradation	0.07	ND	0.31	ND	0.07	0.05	0.63
8	Retention time	19.662	28.557	17.365	30.736	2.32	-	-

ND= Not Detected

Table 1B: Peak Purity data.

Sl. No.	Stress type	Purity Angle/Purity Threshold					
		Ritonavir impurity E	Ritonavir impurity L	Ritonavir impurity F	Ritonavir impurity O	Impurity N-Thiazole	Conclusion
1	Untreated	23.612/57.040	NA	43.521/53.400	NA	11.792/60.145	Pass
2	Acid degradation	9.750/52.309	0.948/51.127	4.063/54.310	NA	6.812/56.518	Pass
3	Base degradation	5.313/52.333	0.145/50.121	5.129/54.965	NA	4.477/56.327	Pass
4	Peroxide degradation	20.684/56.549	NA	25.304/52.730	NA	10.396/58.817	Pass
5	Thermal degradation	21.450/26.703	NA	21.069/22.425	NA	7.808/26.250	Pass
6	Humidity degradation	19.013/30.111	NA	27.213/27.447	NA	7.161/31.576	Pass
7	Photolytic degradation	20.846/31.226	NA	17.856/27.277	NA	12.880/32.232	Pass

Interpretation: Peak purity passes if purity Angle is less than purity threshold. NA = Not Applicable.

Table 2: System precision and Method precision study outcome.

SST Solution/ Test solution (Impurity Spiked)	Darunavir peak area	Ritonavir peak area	Ritonavir Impurity E	Ritonavir Impurity F	Ritonavir Impurity L	Ritonavir Impurity O	N-Thiazole Impurity	Single maximum unknown impurity	Total impurities
TS-1	49965	10545	0.40	3.01	0.35	0.28	0.89	0.07	5.15
TS-2	49898	10022	0.40	3.00	0.34	0.30	0.89	0.06	5.15
TS-3	49843	10571	0.41	3.03	0.37	0.29	0.89	0.07	5.27
TS-4	50365	10723	0.40	3.00	0.34	0.29	0.90	0.08	5.15
TS-5	50257	10340	0.41	3.03	0.33	0.28	0.91	0.06	5.18
TS-6	50370	10499	0.39	2.99	0.32	0.28	0.90	0.07	5.11
Average	50116	10450	0.40	3.01	0.34	0.29	0.90	0.07	5.17
SD (±)	241.35	243.26	0.006	0.016	0.017	0.007	0.008	0.007	0.055
% RSD	0.48	2.33	1.55	0.52	5.04	2.29	0.93	9.59	1.06

SST Solution=System suitability solution.
Note: In impurity spiked test solutions, resolution between critical pairs like Impurity L and Ritonavir peak found more than 3.5 and between Ritonavir and impurity O peak found more than 2.0.

for each level were analyzed and the outcome calculated using calculation formula as % Recovery=% impurity found/% impurity added. The general criteria as per industry practice of not less than 80.0% to not more than 120.0% was followed for data interpretation. The compiled outcome of % recovery for each known impurity was recorded (Table 3).

Robustness

The robustness study was considered for two critical deliberate changes i.e. flow rate and column oven temperature. The outcome of system suitability parameters and Retention Time (RT) for Darunavir, Ritonavir and each known impurities were evaluated against method precision data to identify impact of deliberate change. The comparative data evaluation for each condition was recorded (Table 4).

The outcome of robustness study indicates that deliberate change in flow rate and column oven temperature shows impact on peak retention however system suitability and separation of impurities were not compromised.

DISCUSSION

The available pharmacopeia monographs and literature includes drug product methods for content and impurity analysis of individual drug hence this study was aimed to have single

Table 3: Accuracy/Recovery study outcome.

Impurity/ Level	Impurity E		Impurity F		Impurity L		Impurity O		Impurity N-thiazole	
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
LOQ	99.7	8.1	94.2	6.6	95.7	9.6	97.4	6.2	99.8	1.4
50%	106.3	0.8	102.0	0.3	94.3	2.2	90.1	1.3	101.8	1.0
100%	104.1	2.1	102.9	0.7	100.8	2.5	95.0	2.3	102.1	0.8
150%	103.3	0.3	102.7	0.5	102.4	3.2	95.6	0.6	102.8	0.2

Table 4: Robustness study outcome.

Parameter	Condition	Darunavir Ethanolate			Ritonavir			RT of Imp E	RT of Imp F	RT of Imp L	RT of Imp O	RT of Thiazole Imp
		RT	N	T	RT	N	T					
Method Precision	Temp=55 Flow=0.22	11.34	37848	0.99	29.96	176077	1.08	19.568	17.27	28.46	30.64	2.23
Change in Column Oven Temp. (°C)	+5=60	11.43	36099	0.97	30.23	183715	0.95	17.40	19.86	28.70	30.94	2.38
	-5=50	13.03	43243	0.97	32.25	206189	1.02	21.66	19.51	30.87	32.99	2.47
Change in Flow rate (mL/min)	+10%= 0.24	11.39	35680	0.95	30.16	176822	0.98	19.76	17.44	28.65	30.87	2.21
	-10% = 0.20	13.11	41756	0.96	32.44	186198	0.94	21.84	19.52	31.02	33.17	2.65

Temp=Temperature, RT=Retention time, N=Theoretical plates, T=Tailing factor, Imp=Impurity.

Table 5: Discussion summary of method validation.

Components Name	Selectivity by Retention time	S/N Ratio/ RSD for LOQ Precision	Linearity Correlation Coefficient Range: LOQ to 150%	Method Precision % RSD	Accuracy/Recovery (Min and Max) Range: LOQ to 150%
N-Thiazole	2.320	76/4.3%	0.9999	0.93	Min=99.8, Max=102.8
Darunavir	11.433	47/8.6%	0.9996	N. A.	N. A.
Impurity F	17.365	35/2.7%	0.9999	0.52	Min=94.2, Max=102.9
Impurity E	19.662	46/4.7%	0.9998	1.55	Min=99.7, Max=106.3
Impurity L	28.557	25/6.3%	0.9994	5.04	Min=94.3, Max=102.4
Ritonavir	29.945	45/3.4%	1.0000	N. A.	N. A.
Impurity O	30.736	37 /7.6%	0.9999	2.29	Min=90.1, Max=97.4

Note: Considered 0.05% as LOQ level for all impurities and both active ingredient. N. A.=Not applicable, Min=Minimum, Max=Maximum.

chromatographic method for monitoring and control of all possible degradation impurities in fixed dose drug combination product.

Considering required elution and separation of each known and unknown impurities of Darunavir and Ritonavir, the conventional HPLC method requires very for long run time. By achieving desired separation and to have short and cost-effective method, UPLC method was developed and validated. Also the developed method uses volatile salts in mobile phase so that method can also be applied for LC-MS analysis if required for unknown impurity identification.

The developed method found selective for all possible known degradation impurities and outcome of forced degradation study confirm stability indicating nature of method for intended use. The manufacturing process followed for drug product involve hot melt extrusion where Ritonavir was exposed to high temperature in presence of excipient hence generates Ritonavir impurity F as major degradation product along with N-thiazole impurity. The said impurity also covered under evaluation by UPLC method. The method was validated as per ICH guideline for all parameters required to confirm suitability of method. The compiled outcome

for all validation parameters as discussion summary of method validation was recorded (Table 5).

CONCLUSION

A UPLC based impurity method developed and validated for estimation of all possible degradation impurities in fixed dose drug combination product. The developed short run time method found sensitive and capable to monitor all possible impurities generated during manufacturing process and stability of drug product. The observed validation outcome for linearity, accuracy, precision and robustness study indicates ruggedness and robustness of method and its suitability for intended use. The forced degradation study confirms specificity of method for all possible degradation impurities due to both active ingredients, manufacturing process followed and possible interaction with excipients. The method uses UPLC instrument so as to have short run time i.e. 50 min and very less flow rate i.e. 0.22 mL/min results in very low consumption of solvents hence method found economic. Further, the mobile phase contains all volatile salt and solvent hence same method can be directly applied for LC-MS application to identify mass of unknown impurity. Accordingly, the developed and validated UPLC method with PDA detector can be applied for impurity profiling of Darunavir ethanolate and Ritonavir impurities in fixed dose drug combination dosage form and the same can be applied for quality control routine and stability testing of drug product.

ACKNOWLEDGEMENT

The author wishes to thank the management of Emcure Pharmaceuticals Limited, Research Centre (Adalaj, Gandhinagar, India) for supporting this work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

UPLC: (Ultra High Performance / Pressure Liquid Chromatography); **API:** Active Pharmaceutical Ingredient; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **RSD:** Relative Standard Deviation; **USP:** United state Pharmacopoeia; **HPLC:** High Performance / Pressure Liquid Chromatography.

SUMMARY

In Pharmaceutical analysis, it is important for a chromatography user to develop specific and robust method with short run time. In this study we have developed a short run time UPLC, MS compatible method of analysis for quantitative estimation of all possible degradation impurities related to Darunavir Ethanolate and Ritonavir to facilitate fixed dose drug combination product analysis. The developed method was proved stability indicating through analytical method validation including forced degradation study.

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Cite this article: Buch M, Vaghani H. Stability Indicating RP-UPLC Method for Impurity Profiling of Darunavir and Ritonavir in Fixed Dose Drug Combination Product. Indian J of Pharmaceutical Education and Research. 2024;58(2s):s558-s565.