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Experimental Article

Development and validation of a stability indicating HPLC method for the simultaneous analysis of lopinavir and ritonavir in fixed-dose combination tablets

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الملخص

الأهداف: تحديد الاستقرار الدوائي بطريقة بسيطة، ودقيقة، وسريعة عن طريق تحليل الاستشراب السائل عالي الدقة في نفس الوقت لدوائي "لوبي نافير" و"ريتو نافير" مجتمعين في جرعة دواء واحدة.

طرق البحث: تم تطوير التحديد الكمي لاستقرار دوائي "لوبي نافير" و"ريتو نافير" المضادة للفيروسات مجتمعين في جرعة دواء واحدة، بطريقة تحليل الاستشراب السائل عالي الدقة.

النتائج: تم التحقق من صحة الاستقرار الدوائي بهذه الطريقة وثبتت دقتها النوعية، وحساسيتها، وسرعتها، وصمودها وقوتها. لقد تم تعريض دوائي "لوبي نافير" و"ريتو نافير" لمختلف ظروف الإجهاد المتسارعة، من أجل الوصول لنتائج استقرار دوائي دقيقة.

الاستنتاجات: نستنتج من هذه الدراسة أن هذه الطريقة يمكن تطبيقها لمراقبة الجودة الروتينية لدوائي "لوبي نافير" و"ريتو نافير" مجتمعين في جرعة دواء واحدة، وكذلك الدواء السائب. كما يمكن استنتاج أن هذه الطريقة المقترحة ذات حساسية ودقة عاليتين، ويمكن تطبيقها بنجاح لتقييم موثوق به لمعرفة مقدار المواد الدوائية الفعالة الموجودة بالمنتجات التجارية لدوائي "لوبي نافير" و"ريتو نافير".

الكلمات المفتاحية: تدرك؛ تحليل الاستشراب السائل عالي الدقة؛ لوبي نافير؛ التحقق من الطريقة؛ ريتونافير

Abstract

Objectives: A simple, accurate, precise and rapid stability indicating HPLC method for simultaneous determination of Lopinavir and Ritonavir in combined dosage forms.

Methods: A validated stability indicating reversed phase high-performance liquid chromatographic method was developed for the quantitative determination of two antiviral drugs viz. lopinavir (LPV) and ritonavir (RTV) on Agilent TC C18 (2) 250 × 4.6 mm, 5 µ column using mobile phase composition of acetonitrile: 0.05 M phosphoric acid (55: 45, v/v) at a flow rate of 1.2 ml/min.

Results: Quantification was achieved with ultraviolet detection at 240 nm. The retention time obtained for ritonavir was at 4.35 min and for lopinavir was at 6.68 min. The result obtained with the detector response was found to be linear in the concentration range of 8–48 µg/ml for lopinavir and 2–12 µg/ml for ritonavir. This method has been validated and shown to be specific, sensitive, precise, linear, accurate, rugged, robust and fast. LPV and RTV were subjected to different accelerated stress conditions. The degradation products, were well resolved from the pure drug with significantly different retention time values.

Conclusion: It is concluded that this method can be applied for routine quality control of LPV and RTV in tablet dosage forms as well as in bulk drug. Hence the proposed method is highly sensitive, precise and accurate and it successfully applied for the reliable quantification of active pharmaceutical ingredient (API) content in the commercial formulations of lopinavir and ritonavir.

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Keywords: Degradation; High-performance liquid chromatography; Lopinavir; Method validation; Ritonavir

Introduction

Lopinavir, (2*S*) -*N*-[(2*S*, 4*S*, 5*S*) -5-[2- (2, 6-dimethylphenoxy) acetamido]-4-hydroxy-1,6-diphenylhexan -2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl) butanamide.[1] Ritonavir,1,3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[(2*S*)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl] carbamate.[2] Lopinavir (LPV) is a protease inhibitor that has been co-formulated with a low dose of ritonavir (RTV) to improve its pharmacokinetic properties, resulting in substantially increased plasma exposure that maintains high drug levels throughout a 12-h dosing interval.[3–5] The chemical structures of drugs are shown in (Figure 1).

A literature survey reveals analytical methods like UV spectrophotometric,[6–9] HPTLC,[10–12] HPLC,[13–15] LC-MS for simultaneous determination of lopinavir and ritonavir in pharmaceutical dosage forms and biological fluids[16–19] are reported. However, no references are reported so far for the stability indicating simultaneous determination of said drugs by HPLC method. It is needed to determine the intrinsic stability of a drug substance in formulation and to establish degradation pathways of drug substances and drug products. It is also necessary to understand the chemical properties of drug molecules. So it was planned to develop and validate simple, rapid and precise stability indicating HPLC method for simultaneous estimation of said drugs in combined dosage form. Few reports were published concerning the degradation behavior of RTV and its forced degradation products, of which, recent report used Stability-indicating HPLC and HPTLC method and LC-MS-MS. The current study is comparable to the reported work.[20,21] In addition, the active pharmaceutical ingredient eluted earlier at 4.35 min and 6.68 min in the proposed study as compared to 4.82 and 9.0 min in the reported method. The parent drug stability test guidelines (Q1A) issued by International Conference on Harmonization (ICH) requires that analytical test procedures for stability samples should be fully validated and the assays should be stability indicating.[22–24]

Experimental

Materials and Methods

LPV and RTV were procured as a gift samples by Emcure Pharmaceuticals Ltd., Pune India. All the reagents used were of HPLC grade were purchased from MERCK, India. The commercially available tablets containing a combination of RTV-50 mg and LPV -200 mg were procured from pharmacy. All the solutions for analysis were prepared and analyzed freshly.

Instruments

Agilent technologies 1260 LC system with gradient pump connected to DAD UV detector and Agilent TC C18 (2) 250 × 4.6 mm, 5 µ column, LC-GC AGN204PO balance was used for all weighing.

Method development

Chromatographic conditions

Chromatographic separation was achieved on Agilent TC C18 (2) 250 × 4.6 mm, 5 µ column using mobile phase composition of acetonitrile: 0.05 M phosphoric acid (55: 45, v/v) at a flow rate of 1.2 ml/min with 240 nm UV detection. A typical absorption spectrum of LPV and RTV as shown in Figure 2. The retention time obtained for RTV was at 4.35 min and for LPV was at 6.68 min as shown in Figure 3. Diluent was prepared by mixing 550 ml of acetonitrile, 450 ml of 0.05 M phosphoric acid, filtered through 0.45 µm and degassed before use.

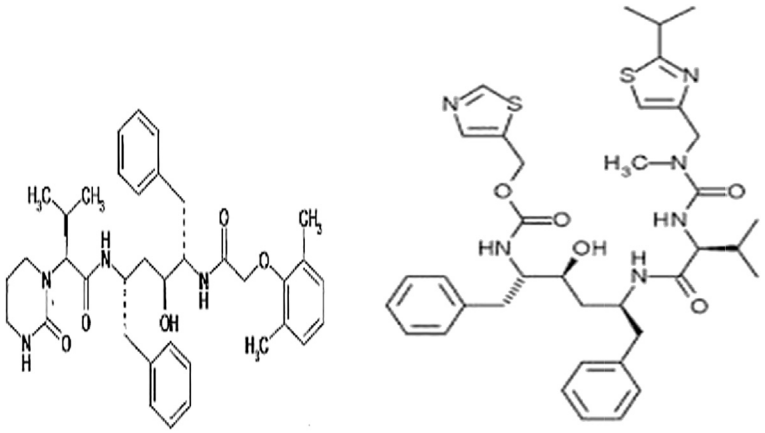

Figure 1: Chemical structure of Lopinavir and Ritonavir.

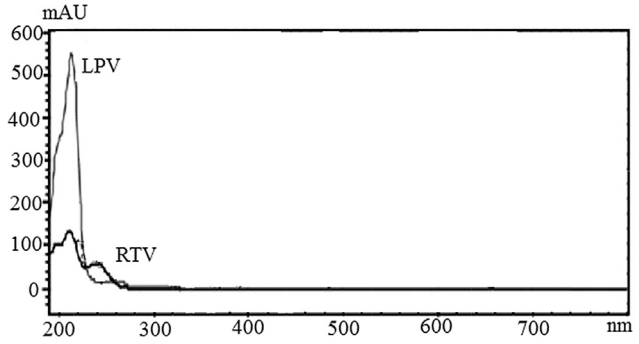

Figure 2: Overlain spectra of Lopinavir and Ritonavir.

Preparation of stock solution

Accurately weighed quantity of LPV and RTV (10 mg) was transferred to 10.0 ml volumetric flask. Then small amount methanol was added and ultrasonicated for 5 min and diluted up to the mark with methanol. (Concentration: 1000 µg/ml of LPV and RTV).

Preparation of standard working solution

From the stock solution pipette out 1 ml into 10 ml volumetric flask and makeup the final volume with methanol (100 µg/ml).

Preparation of mobile phase

The mobile phase was prepared by mixing acetonitrile: 0.05 M phosphoric acid (55: 45, v/v). The mobile phase was filtered through 0.45 µm and degassed before use.

Preparation of working sample solution

Twenty tablets of LOPIMUNE (containing 200 mg of LPV and 50 mg of RTV) were weighed and powdered. The average weight of tablet was determined (0.6248 gm). The powder equivalent to 40 mg of LPV was transferred to 100 ml standard flask and small amount methanol was added. The solution was sonicated for 15 min, and the final volume was made with same to obtain solution (400 µg/ml). The mixture was then filtered through a nylon 0.45 mm membrane filter. The above solution was suitably diluted with mobile phase to obtain final dilution of LPV and RTV (24 µg/ml and 6 µg/ml).

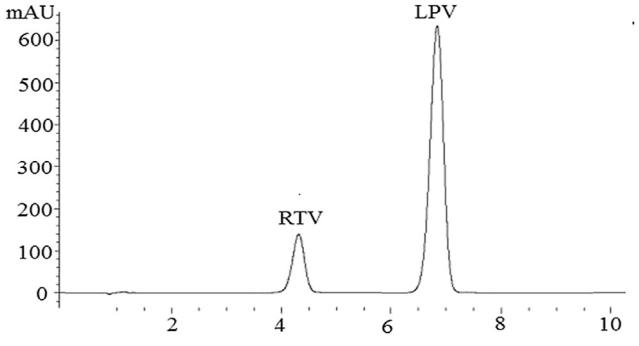

Figure 3: Chromatogram of standards of Lopinavir(LPV) and Ritonavir(RTV).

Method validation

The method was validated for its linearity range, accuracy, precision, sensitivity and specificity. Method validation was carried out as per ICH guidelines.

Linearity

Calibration curve was constructed by plotting peak area V^s concentration of LPV and RTV solutions, and the regression equation was calculated. The calibration curve was plotted over the concentration range 8–48 µg/ml. for LPV and 2–12 µg/ml for RTV. Accurately measured standard working solutions of LPV (0.8, 1.6, 2.4, 3.2, 4.0, 4.8 ml) were transferred to a series of 10 ml volumetric flasks and diluted up to the mark with mobile phase. Standard working solutions of RTV (0.2,0.4,0.6,0.8,1.0,1.2 ml) were transferred to a series of 10 ml volumetric flasks and diluted up to the mark with mobile phase. Aliquots (20 µl) of each solution were injected under the operating chromatographic condition described above.

Accuracy

Recovery studies were carried out by adding known quantities of standards at different levels to the pre-analyzed sample. The pre-analyzed samples were spiked with extra 80, 100 and 120% of the standard LPV and RTV. The solutions were prepared in triplicates and the accuracy was indicated by % recovery.

Precision

Precision of the method was studied as intra-day and inter-day variations and also as repeatability. Intra − day precision was determined by analyzing, the three different concentrations 16 µg/mL, 24 µg/mL and 32 µg/mL of LPV and 4 µg/mL, 6 µg/mL and 8 µg/mL of RTV respectively, for three times in the same day. Day − to Day variability was assessed using above mentioned three concentrations analyzed on three different days, over a period of one week. This result shows reproducibility of the assay. Repeatability was performed by multiple injections of a homogenous sample of 24 µg/mL of LPV and 6 µg/mL of RTV, respectively that indicates the performance of the HPLC instrument under chromatographic conditions.

Robustness

The robustness of developed method was assessed by varying two parameters, which includes variation of flow rate, change in mobile phase composition using 24 µg/mL of LPV and 6 µg/mL of RTV solutions respectively.

Sensitivity

Sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). LOD = 3.3 × ASD/S and LOQ = 10 × ASD/S, where ASD is the average standard deviation and S is the slope of the line. In order to determine detection and quantification limit, concentrations in the lower part of the linear range of the calibration curve were used. The stock standard solutions were injected in conc. range of 8–16 µg/mL (LPV) and 2–4 µg/mL (RTV).

Specificity and selectivity

Specificity is a procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix.

Ruggedness

Appropriate volume of sample solution, 24 μg/mL of LPV and 6 μg/mL of RTV were prepared and analyzed by two different analysts using similar operational and environmental conditions. Peak area was measured for same concentration solutions, six times.

System suitability test

According to USP, system suitability tests are integral part of liquid chromatographic methods. They are used to verify the suitability for the resolution and reproducibility of the chromatographic system. Solutions of LPV 24 μg/mL and RTV 6 μg/mL for chromatographic conditions were tested for system suitability testing.

Forced degradation of LPV and RTV

Acid and base induced degradation

LPV and RTV solutions were treated with 1 mL of 1 M methanolic HCl and 1 M methanolic NaOH. The solutions were kept at room temperature for 8 h. These solutions were diluted with the mobile phase to reach a final concentration of 10 μg/mL of LPV and 10 μg/mL of RTV.

Hydrogen peroxide − induced degradation

LPV and RTV solutions were treated with 1 mL of hydrogen peroxide 30%. These solutions were kept at room temperature for 8hr and were protected from the light. After the specified time intervals, the solutions were diluted with the mobile phase to reach a final concentration of 10 μg/mL of LPV and 10 μg/mL of RTV. After the previous treatments, the solutions were filtered with a 0.45-μm filtration disc prior to injection to the column.

Dry heat degradation product

LPV 10 mg and RTV 10 mg were stored at 55 °C for 3 h in oven separately. They were transferred to 10 ml volumetric flask containing methanol and volume was made up to the mark with methanol. The solutions were diluted with the mobile phase to reach a final concentration of 10 μg/mL of LPV and 10 μg/mL of RTV. The chromatograms were run by injecting the sample in the column.

Light heat degradation products

The 10 mg of LPV and RTV were dissolved in 10 ml of methanol separately. The solutions were kept in the sun light for 8 h. The 1 ml of above solutions were taken and diluted up to 10 ml with methanol. The solutions were diluted with the mobile phase to reach a final concentration of 10 μg/mL of LPV and 10 μg/mL of RTV. The chromatograms were run by injecting the sample in the column.

Results

Optimization of HPLC method

Several mobile phases were tried using various proportions of different aqueous phases and organic modifiers. The best chromatogram was obtained using the above mentioned mobile phase. Decreasing the acetonitrile content in the mobile phase led to longer retention times and excessive peak tailing. Methanol was tried as an organic modifier and different phosphoric acid strengths were tried (0.025 and 0.075 M). Phosphoric acid was substituted by acetic acid in some trials. In all these trials, the chromatograms showed broad asymmetric peaks and/or increased retention times and, consequently, fewer theoretical plates for the eluted peaks. LPV and RTV exhibits UV absorption mainly at 240 nm. The previously described chromatographic conditions showed symmetrical peaks at 4.35 ± 0.002 min for RTV and 6.68 ± 0.002 min for LPV, with retention factor (K′) of 1.86 and 1.97 respectively. The resolution of standard RTV and LPV in the presence of degradation products was satisfactory. Ultimately, a mobile phase consisting of acetonitrile: 0.05 M phosphoric acid (55: 45, v/v) was selected for validation purposes and stability studies.

Linearity

Linearity responses for LPV and RTV were assessed in the concentration ranges 8−48 μg/ml for and 2−12 μg/ml, respectively. The linear equations for the calibration plots were $Y = 26468X - 49911$ and $Y = 26041X - 5698$, with correlation coefficient (r) being 0.999 and 0.999 for LPV and RTV, respectively. Range was established with five replicate readings of each concentration.

Validation of the method

Accuracy

To the pre analyzed sample a known amount of standard solution of pure drug (LPV and RTV) was applied at three different levels. These solutions were subjected to re-analysis by the proposed method and results of the same are shown in Table 1.

Precision

Precision of the method was determined in terms of intra-day and inter-day variation (%RSD). Intra-day precision (% RSD) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day.

Table 1: Recovery studies.

Initial amount [μg/mL]		Concentration of excess drug added to analyte [μg/mL]		% Recovery [n = 3]		% RSD	
LPV	RTV	LPV	RTV	LPV	RTV	LPV	RTV
24	6	19.2	4.8	100	99.49	0.57	1.19
24	6	24	6	100.79	99.43	0.90	0.92
24	6	28.8	7.2	99.35	99.96	0.16	1.25

Table 2: Result of precision study.

Drug	Amount applied ug/ml	Intraday precision [%RSD, n = 3]	Inter-day precision [%RSD, n = 3]
LPV	16	0.967	1.136
	24	1.217	0.300
	32	0.790	0.674
RTV	4	1.081	1.371
	6	0.708	0.511
	8	0.572	0.904

Inter-day precision (%RSD) was assessed by analyzing the drug solutions within the calibration range on three different days over a period of a week. The results are shown in Table 2.

Repeatability

Repeatability of sample application was assessed by multiple injections of a homogenous sample of 24 µg/mL of LPV and 6 µg/mL of RTV respectively that indicates the performance of the HPLC instrument under chromatographic conditions. The % R.S.D. of LPV and RTV were found to be 0.89 and 0.31, respectively.

Robustness

The standard deviation of peak areas were calculated for each parameter and % R.S.D. was found to be less than 2%. The low % R.S.D. values as shown in Table 3 indicated robustness of the method.

Sensitivity

LOD was found to be 0.30 and 0.10 for LPV and RTV respectively. LOQ was found to be 0.91 and 0.30 for LPV and RTV respectively. This indicates the sensitivity of the developed method.

Specificity and selectivity

Both the drugs eluted forming well shaped, symmetrical single peaks, well separated from the solvent front. The method is quite selective. There was no other interfering peak around the retention time of LPV and RTV; also the base line did not show any significant noise.

Table 3: Robustness study.

Chromatographic conditions	RTV	LPV
	t_R	t_R
Flow Rate (mL/min)		
0.90	4.89	6.98
1.00	**4.35**	**6.68**
1.10	4.18	6.45
Mean ± SD	**4.473 ± 0.35**	**6.703 ± 0.42**
Mobile phase composition (v/v)		
52:48	4.65	6.84
55:45	**4.35**	**6.68**
58:42	4.19	6.45
Mean ± SD	**4.396 ± 0.19**	**6.656 ± 0.157**

Table 4: System suitability test.

System suitability parameters	Proposed method	
	LPV	RTV
Retention time (t_R)	2.24	7.26
Capacity factor (K')	21	67
Theoretical plate (N)	7169	5891
Tailing factor (T)	0.90	1.08

Ruggedness

The ruggedness of the proposed method was evaluated by two different analysts. The results for LPV and RTV were found to be 99.68%, 99.50% and 100.69%, 100.89%, respectively.

System suitability test

Chromatographic condition such as Retention Time (R_t), Capacity Factor (K'), Theoretical Plate (N), Tailing Factor (T) were tested and results are given in Table 4. The summary of validation parameters were listed in Table 5.

Stability-indicating property

The chromatogram of samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure LPV and RTV as well as some additional peaks at different retention time. It was successfully separated from all the degradation products as confirmed by the resolution values calculated for each chromatogram. The number of degradation products with their retention time values, content of LPV and RTV remained, and percentage recovery were calculated and listed in Table 6.

Discussion

Stability-indicating HPLC method was developed for the simultaneous estimation of LPV and RTV in commercial tablet dosage form. Quantification was achieved with ultraviolet detection at 240 nm. The separation was carried out on

Table 5: Summary of validation parameter.

Parameter	LPV	RTV
Linearity range [ug/ml]	8–48	2–12
Regression equation [Y = mX + C]	Y = 26468X − 49911	and Y = 26041X − 5698
Correlation coefficient	0.999	0.999
Limit of detection [ng]	0.30	0.10
Limit of quantitation [ng]	0.91	0.30
% Recovery [n = 3]	99.35–100.79	99.43–99.96
Ruggedness [%]		
Analyst I [n = 3]	99.68	99.50
Analyst II [n = 3]	100.69	100.89
Precision [% RSD]		
Repeatability [n = 6]	0.89	0.31
Inter-day [n = 3]	0.790–1.217	0.300–1.136
Intra-day [n = 3]	0.572–1.081	0.511–1.371
Robustness	Robust	Robust
Specificity	Specific	Specific

Table 6: Forced degradation of LPV and RTV.

Sample exposure condition	Number of degradation products [R_t values]		Recovery [%]	
	LPV	RTV	LPV	RTV
1 M HCl, 8 h,RT[a]	2 (0.48,0.51)	2 (0.25,0.38)	90.86	89.69
1M NaOH,8 h, RT[a]	3 (0.29,0.49,0.53)	2 (0.18,0.31)	85.24	85.52
30%H_2O_2,8 h,RT[a]	1 (0.78)	1 (0.25)	95.21	91.48
Heat, 3H, 55 °C	2 (0.56,0.61)	1 (0.41)	97.58	99.02
Photo, 8 h	1 (0.46)	1 (0.26)	99.86	100.35

[a] RT = Room temperature.

Agilent TC C18 (2) 250 × 4.6 mm, 5 µ column using mobile phase composition of acetonitrile: 0.05 M phosphoric acid (55: 45, v/v) at a flow rate of 1.2 ml/min. The retention time obtained for ritonavir was at 4.35 min and for lopinavir was at 6.68 min. The results obtained with the detector response were found to be linear in the concentration range of 8–48 µg/ml for lopinavir and 2–12 µg/ml for ritonavir. The value of correlation coefficients greater than 0.999 indicate good linearity response in the above mentioned range. The sensitivity of the method was assessed by determining LOD and LOQ. For LPV, LOD and LOQ were found to be 0.30 and 0.91 respectively and for RTV, LOD and LOQ were found to be 0.10 and 0.30 respectively. The proposed method was applied for pharmaceutical formulation and % label claim for LPV and RTV were found to be 99.89 and 100.34 respectively. The amount of drugs estimated by proposed method was in good agreement with the label claim. The recovery studies were carried out at 80, 100, 120% level. The % recovery for LPV and RTV were found to be 99.35–100.79 and 99.43–99.96, respectively; % RSD values less than 2 indicative of accuracy of the method. The method was found to be precise as indicated by the inter-day, intra-day and repeatability analysis; % RSD less than 2. In robustness study, two parameters (flow rate, change in mobile phase composition) were studied and the effects on the results were examined. Low values of % RSD proved method to be robust.

Stability of LPV and RTV was carried out by forced degradation study. The chromatograms of samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure LPV and RTV as well as some additional peaks at different R_t values. The chromatogram of LPV showed additional peak at R_t value 0.48, 0.51 in the acid degraded samples and 0.29, 0.49, 0.53 in base degraded samples. The sample degraded with hydrogen peroxide showed additional peak at R_t value of 0.78. The sample degraded in dry heat showed additional peak at Rt 0.56, 0.61 and in photo light at Rt 0.46. The chromatograms of RTV showed additional peak at R_t value 0.25,0.38 in the acid degraded sample and 0.18,0.31in base degraded sample. The sample degraded with hydrogen peroxide showed additional peak at R_t value of 0.25. The sample degraded in dry heat showed additional peak at Rt 0.41 and in photo light at Rt 0.26. The method is successively applied to pharmaceutical formulation; No chromatographic interferences from the tablet excipients were found. The suitability of this HPLC method for quantitative determination of the compounds is proved by validation in accordance with the requirements of ICH guidelines. Statistical data showed that RP-HPLC methods are robust, rugged, sensitive and accurate as compared to existing analytical methods. RP-HPLC methods are costly and time consuming but produce more accurate and precise results.

Conclusion

The developed stability indicating RP-HPLC method provides simple, accurate, economic, precise, rapid and reproducible quantitative analysis for simultaneous determination of LPV and RTV in tablets. A new mobile phase was found during the method development process. The method was validated as per ICH guidelines and can be employed for routine analysis.

Conflicts of interest

All authors have none to declare.

Authors' contributions

All authors have made substantial contribution to all of the following; 1. Conceptualization of the study design, data acquisition, data analysis and interpretation; 2. drafting of the article and revising it critically and 3. final approval of the version submitted.

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References

1. www.drugbank.ca/drugs/DB01601.
2. Budavari S, Neil MJO, Smith A, Heckelman P, Kinneary J. *The Merck Index*. 12th ed. USA: Merck; 1996p.1418. Monograph No. 8402.
3. Barragan P, Podzamczer D. Lopinavir/ritonavir: a protease inhibitor for HIV-1 treatment. **Expert Opin Pharmacother 2008**; 9: 2363–2375.
4. Cvetkovic RS, Goa KL. Lopinavir/ritonavir: a review of its use in the management of HIV infection. **Drugs 2003**; 63: 769–802.
5. Kaplan SS, Hicks CB. Lopinavir/ritonavir in the treatment of human immunodeficiency virus infection. **Expert Opin Pharmacother 2005**; 6: 1573–1585.
6. Nagulwar VP, Bhusari KP. Simultaneous estimation of ritonavir and lopinavir by vierodt.s uv Spectrophotometric method in combined tablet dosage form. **Int J Pharm Sci 2010**; 2(2): 533–536.
7. Nagulwar VP, Bhusari KP. Development of uvspectrophotometric first order derivative method for the simultaneous estimation of ritonavir and lopinavir in combined tablet dosage form. **Int J Pharm Sci Res 2012**; 3(7): 2317–2320.
8. Nagulwar VP, Bhusari KP. Simultaneous estimation of ritonavir and lopinavir by absorption ratio (Q-analysis) UV spectrophotometric method in combined tablet dosage form. **Der Pharm Lett 2010**; 2(1): 196–200.

9. Dias CL, Bergold AM, Eduardo P. UV- derivative spectrophotometric determination of ritonavir capsules and comparison with LC method. **Anal Lett 2009**; 42(12): 1900−1910.
10. Patel GF, Vekariya NR, Dholkiya RB, Bhatt HS. Application of TLC-densitometry method for simultaneous determination of lopinavir and ritonavir in capsule dosage form. **Orient J Chem 2009**; 25(3): 727−730.
11. Sulebhavikar AV, Pawar UD, Mangoankar KV, Prabhu-Navelkar ND. HPTLC method for simultaneous determination of lopinavir and ritonavir in capsule dosage form. **E-Journal Chemistry 2008**; 5(4): 706−712.
12. Patwari AH, Patel KA, Dabhi MJ, Desai UH, Ezhava SB, Rathod IS, et al. Simultaneous determination of ritonavir and lopinavir in combined tablet dosage form by HPTLC method. **J Pharm Bioanal Sci 2012**; 1(2): 56−61.
13. Jagadeeswarani M, Gopal N, Pavankumar K, Sivakumar T. Quantitavive estimation of Lopinavir and ritonavir in tablet dosage form by RP-HPLC method. **Am J Pharmatech Res 2012**; 2(2): 576−583.
14. Phechkrajang CM, Thin EE, Sratthaphut L, Nacapricha D, Wilairat P. Quantitative determination of lopinavir and ritonavir in syrup preparation by liquid chromatography. **Mahidol Univ J Pharm Sci 2009**; 36(1−4): 1−12.
15. Madhukar A, Jagadeeshwar K, Naresh K, Armstrong VR, Jayapaul B, Naazneen S. Simple and sensitive analytical method development and validation of lopinavir bulk drug by RP-HPLC. **Der Pharma Chem 2011**; 3(6): 494−499.
16. Kou H, Ye M, Fu Q, Han Y, Du X, Xie J, et al. Simultaneous quantification of lopinavir and ritonavir in human plasma by high performance liquid chromatography coupled with UV detection. **Sci China Life Sci 2012**; 55(4): 321−327.
17. Shrivastav PS, Yadav M, Rao R, Kurani H, Singhal P, Goswami S. Application of a rapid and selective method for the simultaneous determination of protease inhibitors, lopinavir and ritonavir in human plasma by UPLC−ESI-MS/MS for bioequivalence study in Indian subjects. **J Pharm Biomed Anal 2009**; 49: 1115−1122.
18. Temghare GA, Shetye SS, Joshi SS. Rapid and sensitive method for quantitative determination of lopinavir and ritonavir in human plasma by liquid chromatographytandem mass spectrometry. **E-Journal Chem 2009**; 6: 223−230.
19. Vats R, Murthy AN, Ravi PR. Simple, rapid and validated LC determination of lopinavir in rat plasma and its application in pharmacokinetic studies. **Sci Pharm 2011**; 79(4): 849−863.
20. Mohammad H, Azza A, Gazy A, Shaalan RA, Ashour HK. Validated stability-indicating HPLC and HPTLC methods for the determination of ritonavir in bulk powder and in Capsules. **J Food Drug Analysis 2012**; 20(4): 963−973.
21. Rao NR, Ramachandra B, Vali MR, Satyanarayana R. R,LC-MS/MS studies of ritonavir and its forced degradation products. **J Pharm Biomed Anal 2010**; 53: 833−842.
22. Q1A (R2) *International Conference on Harmonisation (ICH) of technical requirements for the registration of pharmaceutical for human use stability testing of new drugs substance and products*; 2003. pp. 1−18.
23. ICH. *Validation of analytical procedures: text and methodology*. Q2(R1); 2005.
24. Sethi PD. *HPTLC: quantitative analysis of pharmaceutical formulation*. New Delhi: CBS Publications; 1996p.162−p.165.