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Development and validation of a stability indicating HPTLC method for the Simultaneous analysis of lopinavir and ritonavir in fixed-dose Combination tablets

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ABSTRACT

A simple, accurate, precise and rapid HPTLC method for simultaneous determination of Lopinavir (LPV) and Ritonavir (RTV) in combined dosage forms. The method is based on HPTLC separation of the two drugs followed by densitometric measurements of their spots at 266 nm. The separation was carried out on Merck TLC aluminium sheets of silica gel 60F 254 using Benzene: methanol: acetic acid (8: 2:0.4, v/v/v) as a mobile phase. LPV and RTV gave sharp and well defined peak at Rf 0.63 and 0.44, respectively. Calibration curves were linear in range 800-4800 ng/spot and 200-1200 ng/spot for LPV and RTV, respectively. Method was successively applied to tablet formulation. 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 Rf values.The method is successively applied to pharmaceutical formulation; No chromatographic interferences from the tablet excipients were found. The suitability of this HPTLC method for quantitative determination of the compounds is proved by validation in accordance with the requirements of ICH guidelines.

KEY WORDS: *Lopinavir; Ritonavir High-Performance Thin-Layer Chromatography; Method Validation; Degradation.*

INTRODUCTION

Lopinavir [1S-[1R*,(R*),3R*,4R*]]-N-[4[[(2,6dimethyl-phenoxy)acetyl]amino]-3-hydroxy-5-phenyl1(phenylmethyl)pentyl] tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2H)pyrimidine acetamid e. Ritonavir1-2,10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-me thylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2, 4, 7, 12-tetraazatridecan-13-oicacid,5-thiazolylmethylester, [5S- (5R*, 8R*, 10R*, 11R*) [1, 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 (**Fig. 1)**.

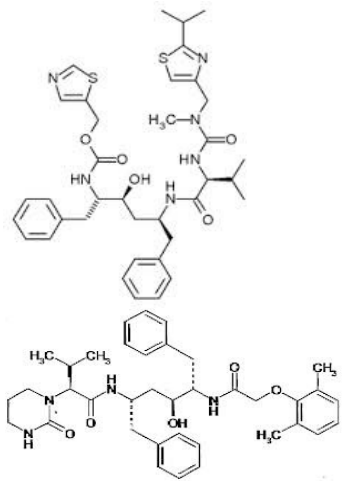

Fig.1: Chemical structure of Lopinavir and Ritonavir

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A literature survey reveals analytical methods like UV spectrophotometric [6-9], HPTLC [10-13], HPLC [14-17], LC-MS for simultaneous determination of lopinavir and ritonavir in pharmaceutical dosage forms and biological fluids [18-21] are reported however, no references are reported so far for the stability indicating simultaneous determination of said drugs by HPTLC method. So it was planned to develop and validate simple, rapid and precise stability indicating TLC densitometry method for simultaneous estimation of said drugs in combined dosage form. 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

1. Materials and Reagents:

LPV and RTV were kindly supplied as a gift sample by Emcure Pharmaceuticals Ltd., Pune India. All the reagents used were of analytical reagent grade (S.D. Fine Chemicals, Mumbai, India) and used without further purification.

2. Instrumentation and chromatographic conditions:

The samples were spotted in the form of bands of width 6 mm with 100 µL sample syringe on precoated silica gel aluminium plate 60 F254 (20 cm×10 cm) with 250 µm thickness; (E MERCK, Darmstadt, Germany) using a Camag Linomat V (Switzerland). The plates were prewashed with methanol and activated at 110^{0}C for 5 min, prior to chromatography. A constant application rate of 150 nl/ sec was employed and space between two bands was 15.4 mm. The slit dimension was kept at 6 mm × 0.45 mm. The mobile phase consists of Benzene: methanol: acetic acid (8: 2:0.4, v/v/v). Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The optimized chamber saturation time for mobile phase was 20 min, at temperature (25^{0}C ± 2); the relative humidity (60% ± 5%); the length of chromatogram run was 8 cm and TLC plates were air dried. Densitometric scanning was performed on Camag TLC Scanner 3 equipped with winCATS software version 1.3.0 at 266 nm. The source of radiation utilized was deuterium lamp. Evaluation was performed using peak area with linear regression.

3. Preparation of standard solutions and calibration graphs:

Combined standard stock solution containing 2400 µg /ml of LPV and 600 µg/ ml of RTV was prepared in methanol .Calibration was done by Hamilton syringe with the help of automatic sample applicator Linomat V on TLC plate that gave concentration 800-4800 ng/spot of LPV and 200-1200 ng/spot of RTV, respectively. Each concentration was spotted six times on the TLC plates. The plates were developed using previously described mobile phase. The calibration graph was plotted as peak areas *versus* corresponding concentrations.

4. Method validation:

4.1. Precision:

Repeatability of sample application was assessed by spotting 2400 ng/spot for LPV and 600 ng/spot for RTV of drug solution six times on a TLC, followed by development of plate. The intra-day precision (%RSD) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day. Inter-day precision (%RSD) was assessed by analyzing drug solutions within the calibration range on three different days over a period of a week.

4.2. Limit of detection and limit of quantification:

In order to determine detection and quantification limit, concentrations in the lower part of the linear range of the calibration curve were used. Stock solution of LPV and RTV was prepared and different volume of stock solution in the range 800 to 1000 ng for LPV and 200 to 400 ng for RTV were spotted in triplicate. The amount of both the drugs by spot *versus* average response (peak area) was graphed and the equation for this was determined. The standard deviations (S.D.) of responses were calculated. The average of standard deviations was calculated (A.S.D.). Detection limit was calculated by (3.3×A.S.D.)/*b* and quantification limit was calculated by (10×A.S.D.)/*b*, where "*b*" corresponds to the slope obtained in the linearity study of method.

4.3. Specificity:

Specificity of the method was ascertained by analysing standard drug and sample. The mobile phase resolved both the drugs very efficiently, as shown in **Fig 2**. The spot for LPV and RTV was confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of LPV and RTV was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot.

4.4. Accuracy:

Recovery study was carried out by over spotting 80%, 100% and 120% of the standard drug solution of LPV and RTV and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check the recovery of the drug at different levels in formulation.

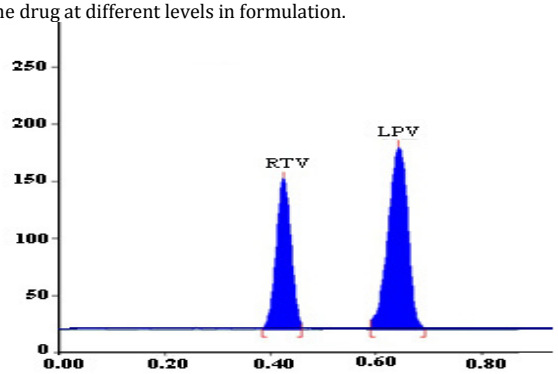

Fig. 2: Densitogram of RTV (R_f 0.44 ± 0.03) and LPV (R_f 0.63 ± 0.03) drug solutions in Benzene: methanol: acetic acid (8: 2: 0.4 *v/v/v*).

4.5. Robustness:

Robustness was studied in six replicate at the concentration level of 2400 ng/spot for LPV and 600 ng/spot for RTV. In this study, seven parameters (mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography and chromatography to spotting) were studied and the effects on the results were examined.

4.6. Ruggedness:

The ruggedness of the proposed method was evaluated by two different analysts

5. Analysis of LPV and RTV in marketed formulation:

To determine the content of LPV and RTV simultaneously in conventional tablets (label claim 200 mg LPV and 50 mg RTV); twenty tablets were accurately weighed, average weight determined and ground to a fine powder. A quantity of powder equivalent to 200 mg LPV and 50 mg of RTV was transferred into 100 ml volumetric flask containing 50 ml methanol, sonicated for 30 min and diluted to mark with same solvent. The resulting solution was filtered using 0.45 µm filter (Millifilter, MA). 0.4µL of the above solution applied on TLC plate followed by development and scanning as described in section 2.2.The analysis was repeated for six times. LPV and RTV gave sharp and well defined peaks at Rf 0.63 and 0.44, respectively, when scanned at 266 nm. The results are shown in **Table 1** indicate that there was no interferences from the excipients commonly present in the tablets.

Table No. 1: Assay of tablet formulation

Component	Label Claim [mg]	Amount Found ± SD [ng]	% Label Claim	% RSD [n=6]
LPV	200	2380.84± 25.18	99.20	1.05
RTV	50	593.36 ±1.25	98.89	1.25

*mean of six estimations

6. Forced degradation of LPV and RTV:

6.1. Acid and base induced degradation:

The 10 mg of LPV and RTV were separately dissolved in 10 ml methanolic solution of 1 M HCl and 1 M NaOH. These solutions were kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. The 1 ml of above solutions were taken, neutralized and diluted up to 10 ml with methanol. The resultant solution were applied on TLC plates in triplicates (10 µl each, i.e. 1000 ng/spot). The chromatograms were run as described in section 2.2.

6.2. Hydrogen peroxide – induced degradation:

The 10 mg of LPV and RTV were separately dissolved in 10 ml of methanolic solution of hydrogen peroxide (10%, *v/v*). The solutions were kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. The 1 ml of above solution were taken and diluted up to 10 ml with methanol. The resultant solution were applied on TLC plate in triplicate (10 µl each, i.e. 1000 ng/ spot). The chromatograms were run as described in section 2.2.

6.3. Dry heat degradation products:

LPV 10 mg and RTV 10 mg were stored at 55^0 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. 1.0 µl (1000 ng / spot) was applied on TLC plate in triplicate and chromatogram was run as described in section 2.2.

6.4. 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 resultant solutions were applied on TLC plate in triplicate (10 µl each, i.e. 1000 ng / spot). The chromatograms were run as described in section 2.2.

RESULT AND DISCUSSION

1. Optimization of HPTLC method:

Initially, Benzene : methanol in the ratio 7:3 (*v/v*) was tried for both drugs simultaneously. The spots were not developed properly and dragging was observed. Then, Benzene : methanol in the ratio of 8:2 (*v/v*) was tried. The developed spots were diffused. To the above mobile phase, 0.2 ml acetic acid was added. Both the peaks were symmetrical in nature and tailing was observed. To improve resolution, the volume of acetic acid was increased to 0.4 ml. Finally, mobile phase consisting of Benzene : methanol: acetic acid (8: 2: 0.4, *v/v/v*) gave good resolution. Both the peaks were

symmetrical in nature and no tailing was observed when plate was scanned at 266 nm. The chamber was saturated with the mobile phase for 20 min at room temperature and plates were activated at 110^0 C for 5 min to obtain well-defined spots.

2. Linearity:

Linearity responses for LPV and RTV were assessed in the concentration ranges 800-4800 ng /spot and 200-1200 ng /spot, respectively. The linear equations for the calibration plots were Y = Y = 2.052x +1397and Y = 5.253x + 868.4, with correlation coefficient (r) being 0.997and 0.997 for LPV and RTV, respectively. Range was established with five replicate readings of each concentration.

3. Validation of the method

3.1. Precision:

Precision of the method was determined in the 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.

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

3.2. Limit of detection and limit of quantitation:

Detection limit and quantification limit was calculated by the method as described in section 2.4.2 The LOQ and LOD for LPV were 24.84 ng and 75.30ng. For RTV, LOQ and LOD were found to be 15.83 ng and 14.97 ng, respectively. This indicates that adequate sensitivity of the method.

3.4. Accuracy:

To the preanalysed sample a known amount of standard solution of pure drug (LPV and RTV) was over spotted at three different levels. These solutions were subjected to re-analysis by the proposed method and results of the same are shown in **Table 2**.

Table No. 2: Results of recovery studies

Components	Initial Amount [ng/band]	Amount added (%)	Amount recovered ± S.D.[ng/band] [n=3]	% Recovered	% RSD
LPV	1600	80	1273	99.52	1.05
	1600	100	1606	100.41	1.28
	1600	120	1906	99.30	0.75
RTV	400	80	322.81	100.87	1.38
	400	100	399.34	99.83	1.45
	400	120	477.89	99.56	0.64

*** mean of three estimations at each level**

3.5. Robustness:

The standard deviation of peak areas was 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.

Table No. 3: Results of Robustness Studies

Parameters	LPV		RTV	
	± SD of peak area [n = 6]	% RSD	± SD of peak area [n = 6]	%RSD
Mobile phase volume	39.25	1.65	9.31	1.56
Mobile phase composition	41.61	1.75	6.78	1.13
Development distance	34.60	1.46	10.51	1.75
Duration of saturation	16.62	0.70	4.38	0.73

3.6. Ruggedness:

The ruggedness of the proposed method was evaluated by two different analysts. The results for LPV and RTV were found to be 99.42 %, 99.62 % and 99.26 %, 99.48 %, respectively.

3.7. Repeatability:

Repeatability of sample application was assessed by spotting (2400 ng/spot of LPV and 600 ng/spot of RTV) of drug solution seven times on a TLC, followed by development of plate and recording the peak area for seven spots. The % R.S.D. for peak area values of LPV and RTV was found to be 1.47 and 1.31, respectively.

The summery of validation parameters were listed in **Table 4**.

Table No. 4: Summary of validation parameter

Parameter	LPV	RTV
Linearity range (ng spot $^{-1}$)	800-4800	200-1200
Correlation coefficient	0.997	0.997
Limit of detection (ng spot $^{-1}$)	24.84 ng	15.83 ng
Limit of quantitation (ng spot $^{-1}$)	75.30ng	14.97 ng
% Recovery (n = 9)	1.026	1.156
Ruggedness (% R.S.D.)		
Analyst I	1.50	1.73
Analyst II	0.90	1.40
Precision (%R.S.D.)		
Repeatability of application (n = 6)	1.47	1.31
Inter-day (n = 3)	0.202-0.399	0.814-1.261
Intra-day (n = 3)	0.250-0.474	0.822-1.214
Robustness	Robust	Robust
Specificity	Specific	Specific

4. 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 R_f values. The spots of degraded product were well resolved from the drug spot. The number of degradation product with their R_f values, content of LPV and RTV remained, and percentage recovery were calculated and listed in **Table 5.**

Table No. 5: Forced degradation of LPV and RTV

Sample exposure condition	Number of degradation products (R_f values)		Drug remained (1000 ng/spot)		Recovery (%)	
	LPV	RTV	LPV	RTV	LPV	RTV
1 M HCl, 8h,RT	2 (0.48,0.51)	2 (0.25,0.38)	956.52	946.89	95.65	94.68
1M NaOH,8h, RT	3 (0.29, 0.56)	2 (0.28,0.31)	945.23	945.23	94.52	94.52
10%H_2O_2,8h,RT	1(0.58)	1 (0.25)	959.12	975.65	95.91	97.56
Photo, 8 h	No degradation	No degradation	999.12	997.42	99.91	99.74
Heat, 3H, 55^{0}C	No degradation	No degradation	996.23	991.28	99.62	99.12

CONCLUSION

The proposed HPTLC method provides simple, accurate and reproducible quantitative analysis for simultaneous determination of LPV and RTV in tablets. The method was validated as per ICH guidelines.

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