ORIGINAL RESEARCH ARTICLES

HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS QUANTITATION OF LOPINAVIR AND RITONAVIR IN TABLET FORMULATION

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ABSTRACT

The present work describes a simple, accurate and precise HPTLC method for simultaneous quantitation of ritonavir (RVR) and lopinavir (LVR) in tablet formulation. Chromatographic separation of both drugs was performed on precoated aluminum plates, silica gel 60 F_{254} as the stationary phase and the solvent system consisted of toluene: ethyl acetate: methanol: glacial acetic acid in the ratio of 6.5:2.5:0.5:0.5 (V/V/V/V). Densitometric evaluation of the separated zones was performed at 266 nm. The two drugs were satisfactorily resolved with R_f values of 0.242 ± 0.03 and 0.413 ± 0.02 for RVR and LVR, respectively. The accuracy and reliability of the method was assessed by evaluation of linearity (400-2000 ng/spot for RVR and 1600-8000 ng/spot for LVR), precision (intra-day RSD 0.16-0.38% and inter-day RSD 0.21-0.60 % for RVR and intra-day RSD 0.35-0.58 % and inter-day RSD 0.26-0.55 % for LVR) and recovery (99.54 ± 0.62 % for RVR and 100.45 ± 0.65 % for LVR), in accordance with ICH guidelines.

Keywords: HPTLC, Lopinavir, Ritonavir, Quantitative analysis and Validation.

INTRODUCTION

The determination of low concentration and poorly absorbing analytes in pharmaceutical associations constitutes a challenging problem in current pharmaceutical analysis.

Lopinavir (LVR), (α,*S*)-*N*-[(1*S*,*3S*,4*S*)-4-[[(2,6 dimethylphenoxy)-acetyl]amino]-3-hydroxyl-5-phenyl-1-(phenylmethyl)pentyl]tetrahydro-α-(1 methylethyl)2-oxo-1-(2*H*)-pyrimidineacetamide) (Fig.1), is a white to almost white powder[1]. It acts as a protease inhibitor used as antiretroviral agent and in the treatment of HIV-AIDS. It is official in Indian Pharmacopoeia[2]. which recommends HPLC method for its analysis.

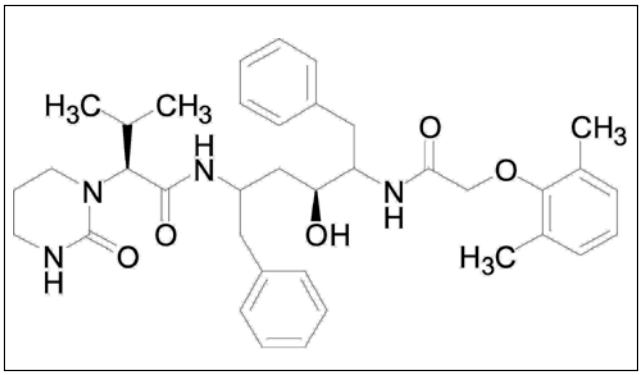

Fig. 1: Chemical Structure of Lopinavir (LVR)

Ritonavir (RVR), (5*S*,8*S*,10*S*,11*S*)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis-(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid-5-thiazolyl methyl ester (Fig. 2), is a white to off white powder[1]. It acts as protease inhibitor used as antiretroviral agent and in the treatment of HIV-AIDS. It is official

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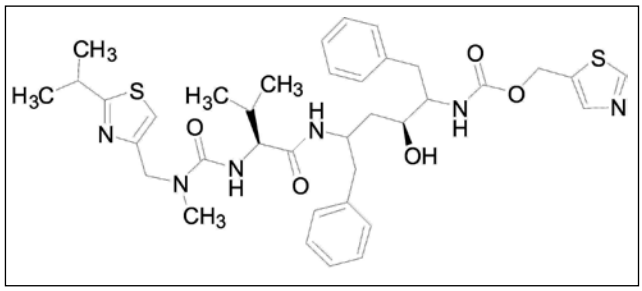

Fig. 2: Chemical Structure of Ritonavir (RVR)

Recently tablet formulations of RVR and LVR have been introduced in the market. Literature review revealed that few methods have been reported for analysis of RVR[3-11] and LVR[12-18] either individually or in combination with other drugs. Some HPLC[19-32] methods were reported for simultaneous estimation of both drugs in combination. However, to date there are apparently no published reports about the simultaneous quantitation of RVR and LVR by HPTLC in tablet formulation. The present study reports on the simultaneous quantitation of RVR and LVR by HPTLC in tablet formulation. The proposed method is validated as per ICH guidelines[33].

MATERIALS AND METHODS

Reagents and Chemicals

The bulk drugs of ritonavir (RVR) and lopinavir (LVR) were procured from Sri Krishna Pharmaceuticals Ltd. Shamshabad, Andhra Pradesh, India; it was used without further purification. Fixed dose combination tablets (EMLETRA, EMCURE Pharmaceuticals Pvt. Ltd) containing 50 mg of RVR and 200 mg of LVR were procured from market. All chemicals and reagents were of analytical grade and purchased from Merck Chemicals, Mumbai, India.

Instrumentation

The samples were spotted in the form of bands (6 mm) with a CAMAG 100 µL sample (Hamilton, Bonaduz, Switzerland) syringe on silica gel precoated aluminum 60 F_{254} plates, (10 cm x 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany) using a CAMAG LINOMAT-V (Switzerland) sample applicator. The plates were prewashed with methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 0.1µL/s was used and the space between two bands was 5 mm. The slit dimension was kept at 5 mm x 0.45 mm and the scanning speed was 10 mm/s. The monochromator bandwidth was set at 20 nm, each track was scanned three times and baseline correction was used. Linear ascending development was carried out in a 20 cm x 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25°C ± 2), at relative humidity of 60 % ± 5. The length of each chromatogram run was 8 cm. Following the development the TLC plates were dried in current air, by an air dryer in a wooden chamber with adequate ventilation. The flow rate in the laboratory was maintained unidirectional (laminar flow, towards the exhaust). Densitometric scanning was performed using a CAMAG TLC scanner III in the reflectance absorbance mode and operated by WinCATS software (V 3.15, CAMAG). The source of radiation used was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from peak areas.

Preparation of Standard Stock Solutions

Standard stock solutions at a concentration of 200 µg/mL of RVR and 800 µg/mL of LVR were prepared separately using methanol. From the standard stock solution, the mixed standard solution was prepared using methanol to contain 200 ng/µL of RVR and 800 ng/µL of LVR. The stock solution was stored at 2-8°C and protected from light.

Optimization of the TLC Method

The TLC procedure was optimized with a view to develop a simultaneous assay method for RVR and LVR, respectively. The mixed standard stock solution (200 ng/µL of RVR and 800 ng/µL of LVR) were spotted onto TLC plates and run in different solvent

systems with wavelength set to 266 nm, where both drugs showed considerable absorbance. Initially, toluene, acetone and methanol were tried in different ratios. Toluene was used to impart the necessary non-polarity to mobile phase and to obtain a suitable R_f value. Initially, toluene, acetone and methanol in the ratio of 5:6:2 (V/V/V) was selected but tailing and fronting with peaks was observed. Then acetone was completely removed from mobile phase and the volume of toluene in the mobile phase was increased from 5 to 6 mL to decrease R_f value, ethyl acetate (2 mL) and glacial acetic acid (0.5 mL) were added to reduce tailing and fronting effects of peaks. Finally, the optimum mobile phase consisted of toluene: ethyl acetate: methanol: glacial acetic acid in the ratio of 6.5:2.5:0.5:0.5 (V/V/V/V). In order to reduce the neck less effect the TLC chamber was saturated for 20 min using saturation pads. The mobile phase was run up to a distance of 8 cm; which takes approximately 20 min for complete development of the TLC plate.

Validation of the Method

Validation of the optimized TLC method was carried out with respect to the parameters like linearity, detection limit, quantification limit, precision, accuracy and robustness. Validation was performed in compliance with international standards, using adequate statistics estimates[34].

Linearity and Range

From the mixed standard stock solution 200 ng/µL of RVR and 800 ng/µL of LVR, solutions were spotted on the TLC plate to obtain a final concentration of 400-2000 ng/spot for RVR and 1600-8000 ng/spot for LVR. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations (400, 800 and 1200 ng/spot for RVR and 1600, 3200 and 4800 ng/spot for LVR) of the drugs six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of Detection and Limit of Quantitation

LOD/LOQ parameters are not a requirement for drug assay; however it is always useful to demonstrate the analyses are being conducted in the region which is above the LOQ value. The limits of detection (LOD) were established from the standard deviation of response (SD) and the slope (*b*) of calibration curves prepared with reference sample solutions having concentrations in the vicinity of LOD, calculated by formula LOD = 3.3 (SD/*b*) and assuming that the response-concentration relation is linear in the range from the maximum possible concentration of the analyzed compounds down to zero.

The limits of quantitation (LOQ) were established by the formula LOQ = 10(SD/*b*).

Robustness of the Method

Following the introduction of small changes in the mobile phase composition (±2 %), the chamber saturation period (± 10 %), development distance (± 10 %), the time from spotting to chromatography and from chromatography to scanning was varied from ±10 min., the effects on the results were examined. The plates were prewashed with methanol and activated at 60°C for 2, 5 and 7 min respectively prior to chromatography. One factor at a time was changed at a concentration level of 800ng/band and 3200 ng/band for RVR and LVR, respectively.

Accuracy

Accuracy of proposed method was ascertained on the basis of recovery study performed by standard addition method. Accuracy of the method was carried out by applying the method to drug sample (RVR and LVR combination tablets) to which known amounts of RVR and LVR standard drugs corresponding to 50, 100 and 150% of label claim had been added (standard addition method) and analyzed by running

chromatograms in optimized mobile phase.

Analysis of a Marketed Formulation

To determine the content of RVR and LVR in conventional tablets (brand name: EMLETRA, Label claim: 50 mg rotinavir and 200 mg lopinavir, per tablet), 20 tablets were weighed, their mean weight determined and finely powdered. The weight of the tablet triturate equivalent to 20 mg of RVR was transferred into a 10 mL volumetric flask containing 5 mL methanol, sonicated for 30 min and diluted to 10 mL with methanol. The resulting solution was centrifuged at 3,000 rpm for 5 min. Then the above filtered solution was diluted to produce a concentration of 200ng/µL and 800ng/µL for RVR and LVR, respectively and 4 µL of this solution (800 and 3200 ng/spot for RVR and LVR, respectively) was applied to a TLC plate which was developed in optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined.

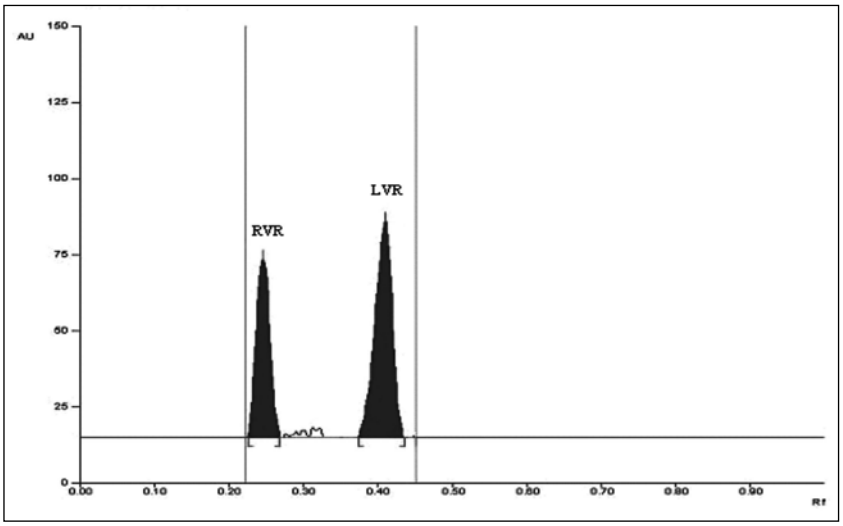

Fig. 3 : Densitogram of Ritonavir (Peak-1(RVR): 800 ng/band, R_f = 0.24 ± 0.021) and Lopinavir (Peak-2 (LVR): 3200 ng/band, R_f = 0.41 ± 0.015) in laboratory mixture

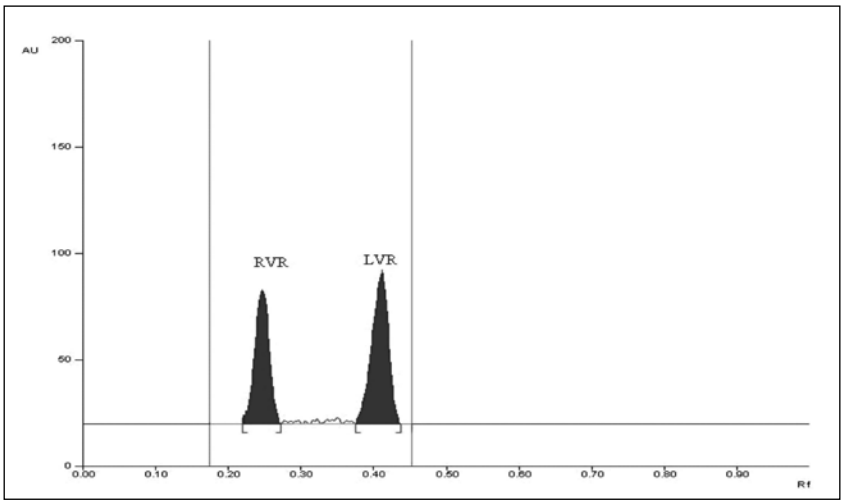

Fig. 4: Densitogram of Ritonavir (Peak-1(RVR): 800 ng/band, R_f = 0.242 ± 0.03) and Lopinavir (Peak-2 (LVR): 3200 ng/band, R_f = 0.413 ± 0.02) of formulation (EMLETRA) showing no interference of excipients in analysis

RESULTS AND DISCUSSION

The results of validation studies on simultaneous quantitation method developed for RVR and LVR in the current study involving toluene: ethyl acetate: methanol: glacial acetic acid in the ratio of 6.5:2.5:0.5:0.5 (V/V/V/V) as the mobile phase for TLC are given below.

Linearity and Range

The standard curves were linear over the concentration range of 400-2000 ng/spot (400, 800, 1200, 1600 and 2000 ng/spot) for RVR and 1600-8000 ng/spot (1600, 3200, 4800, 6400 and 8000 ng/spot) for LVR. Each solution was spotted three times, densitogram of RVR and LVR in laboratory mixture, shown in Fig. 3.

The calibration curve for RVR had a correlation coefficient of 0.9993 and for LVR had a correlation coefficient of 0.9926, indicating a linear relationship between the concentrations of both drugs and peak area over the range investigated. The slope of the curves was 1.221 with the intercept of 95.7 for RVR and 0.168 with the intercept of 469.5 for LVR.

Table I: Precision Studies

Concentration (ng/spot)	Repeatability (n = 6)		Intermediate precision (n = 6)	
	Measured Concentration ± SD	% RSD	Measured Concentration ± SD	% RSD
RVR				
400	398.25± 1.5	0.38	397.87± 2.4	0.60
800	796.36± 2.03	0.25	797.12 ± 1.9	0.24
1200	1194.87± 1.9	0.16	1196.16 ± 2.5	0.21
LVR				
1600	1597.54± 6.4	0.40	1598.45 ± 4.3	0.26
3200	3196.9± 18.5	0.58	3198.58 ± 17.6	0.55
4800	4798.82± 16.6	0.35	4795.39 ± 19.8	0.41

Table II: Robustness Study

Parameter	RVR (% RSD)	LVR (% RSD)
Mobile Phase Composition (± 2 %)	1.34	0.98
Chamber Saturation Period (± 10%)	1.58	1.25
Development Distance (± 10%)	0.78	0.45
Time from Spotting to Chromatography (± 10 min.)	1.23	0.98
Time from Chromatography to Scanning (± 10 min.)	1.27	0.89

Table III: Recovery Studies

Drug	Amount Taken (ng/band)	Amount Added (ng/band)	Total Amount Recovered (ng/band)	% Recovery ± % RSD
RVR	800	400	1195.441	99.62 ± 0.653
	800	800	1600.846	100.05 ± 0.338
	800	1200	1979.225	98.96 ± 0.855
LVR	3200	1600	4798.611	99.97 ± 0.500
	3200	3200	6499.008	101.55 ± 0.549
	3200	4800	7987.103	99.84 ± 0.905

LOD and LOQ

The LOD were 122.43 ng/spot and 491.04 ng/spot for RVR and LVR, respectively. The LOQ were 371 ng/spot and 1488 ng/spot for RVR and LVR, respectively, determined by using the equations: LOD = 3.3 SD/*b*; LOQ = 10 SD/*b*, where SD is the standard deviation of the response and "*b*" corresponds to the slope obtained from the linearity study of the method.

LOD and LOQ obtained are adequate values for the detection and quantification of RVR and LVR in tablets.

Precision

The results of the repeatability and intermediate precision experiments are shown in Table I. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2%, respectively as recommended by ICH guidelines.

Robustness

The robustness was evaluated by an intentional minor modification in the experimental conditions of the proposed method. The % RSD of the peak areas was calculated for each parameter and values were found to be less than 2%. The low values of the % RSD, as shown in Table II, indicated that the optimized method is robust.

Accuracy

Accuracy of the method was investigated by applying the method to drug sample (RVR and LVR combination tablets) to which known amounts of RVR and LVR standard drugs corresponding to 50, 100 and 150% of label claim had been added (standard addition method), and analyzed by running chromatograms in optimized mobile phase. The results of recovery studies are shown in Table III.

Analysis of Marketed Formulation

Experimental results of the amount of RVR and LVR in tablets, expressed as a percentage of drug recovery related to label claims, were found to be in good agreement with the label claims, thereby suggesting that there is no interference from any of the excipients which are normally present in tablets (Fig. 4). The drug content was found to be 99.73% ± 0.246 for RVR and 98.90%± 0.279 for LVR, respectively.

CONCLUSION

Introducing HPTLC into pharmaceutical analysis represented as a major step in terms of quality assurance. Today HPTLC is rapidly becoming a routine analytical technique due to its advantages of high sample throughput and the need for minimum sample preparation. It's major advantage is that several samples can be run simultaneously using a small quantity of mobile phase-unlike LC, thus reducing the analysis time and cost per analysis. The developed HPTLC technique is precise, specific and accurate. Statistical analysis proves that the method is that suitable for analyzing of RVR and LVR in tablet formulation without any interference from excipients. It may be extended to study the degradation kinetics of RVR and LVR and also for its estimation in plasma and other biological fluids. The proposed HPTLC method is simpler, rapid and more flexible than LC.

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