Research Article

RP-HPLC Method Development and Validation for Simultaneous Estimation of Lopinavir and Ritonavir in Dosage form and in Plasma

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ABSTRACT

A simple, selective, rapid, precise and economical reverse phase high performance liquid chromatographic (RP- HPLC) method has been developed for the simultaneous estimation of Ritonavir and Lopinavir in pharmaceutical dosage form and in plasma. The method was carried out on reverse-phase C_{18} column, with mixture of methanol: water (85:15v/v) was used as a mobile phase and the p^H was adjusted to 3.5 by using O- phosphoric acid, at 1ml/min flow rate. Different analytical parameters such as linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness were determined according to International Conference on Harmonization (ICH) guidelines. . The linear regression analysis data for the linearity plot showed good linear relationship with correlation coefficient value for Ritonavir and Lopinavir were r^2=0.9998 and r^2=0.9994 in the concentration range of 5-50 µg/ml, 20-200 µg/ ml respectively. Retention times of Ritonavir and Lopinavir were 4.8min and 5.9min.The described HPLC method showed to be sensitive for simultaneous determination of Ritonavir and Lopinavir with regard to the LOD and LOQ values. This method had been extensively validated. These methods allow a number of cost and time saving benefits. The described methods can be readily utilized for the analysis of pharmaceutical preparations.

Keywords: Lopinavir, ritonavir, RP- HPLC determination, validation

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INTRODUCTION

Ritonavir: The chemical name of Ritonavir is 1,3-thiazol-5-ylmethyl N-[(2S, 3S, 5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol 4yl]methyl}) carbamoyl] amino} butanamido]- 1,6 -diphenylhexan-2-yl] carbamate. It has a molecular formula of $C_{37}H_{48}N_6O_5S_2$ and a molecular weight of 720.9 g/mol. It is white crystalline powder and soluble in methanol, slightly soluble in water. Ritonavir is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Protease inhibitors block the part of HIV called protease. Ritonavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs [1].

Lopinavir: The chemical name of Lopinavir is **(**2S)-N-[(2S,4S,5S)-5-[2-(2,6dimethyl-phenoxy) acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]-3- methyl-2-(2-oxo-1,3-diazinanyl) butanamide. It has a molecular formula of $C_{37}H_{48}N_4O_5$ and a molecular weight of 628.8008 g/mol. It is white crystalline powder and soluble in methanol and slightly soluble in water. Lopinavir inhibits the HIV viral protease enzyme by forming an inhibitor-enzyme complex therapy by preventing the cleavage of the gag-pol polyproteins. Immature, non

infectious viral particles are subsequently produced [2].

Lopinavir is an inhibitor of the HIV-1 protease. Ritonavir inhibits the CYP3A-mediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir.

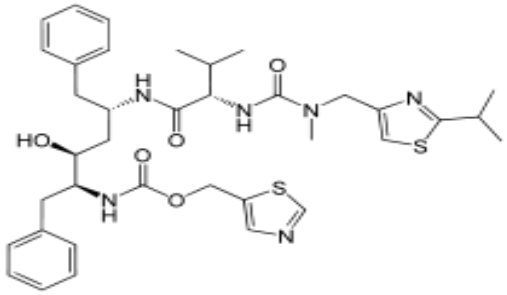

Figure 1: Structure of Ritonavir

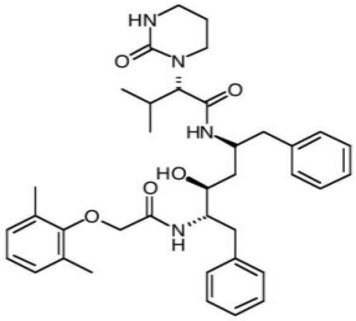

Figure 2: Structure of Lopinavir

A Literature review revealed that very few analytical methods like UV spectroscopy, liquid chromatography appeared in the literature for the determination of simultaneous estimation of Ritonavir and Lopinavir. In view of the need for a suitable RP-HPLC method for routine analysis of Ritonavir and Lopinavir in formulation, an attempt was made to develop simple, precise and accurate analytical method for estimation of Ritonavir and Lopinavir and find out its applicability to its determination in formulation.

MATERIALS AND METHODS

Chemicals: Ritonavir and Lopinavir as pure standard reference drugs were obtained from Hetero labs, Hyderabad, India. Methanol and HPLC grade water were purchased from Merck (India) Ltd, Mumbai, India. The 0.45µm and 0.2µm nylon filters were purchased from Advanced Micro Devices Pvt. Ltd. Kaletra (200mg of Lopinavir and 50mg of Ritonavir combination tablets) tablets were purchased from local market.

Equipments: Analysis was performed on a chromatographic system of Agilent LC 1200 gradient HPLC equipped with a manual rheodyne injector (20µl) and Diode array detector (DAD). The chromatographic separation was achieved on Qualisigold C_{18} (250mmX4.6mm, 5µm) analytical column.

Selection of Wavelength

Appropriate dilution was prepared using standard stock solution of 20µg/ml of Ritonavir and 20µg/ml Lopinavir in methanol. Both the solutions were scanned over range of 200-400 nm, using medium scan speed. From the overlain spectra, 225nm has been selected as detection wavelength for HPLC method. The overline spectra are shown in the (**Figure 3**).

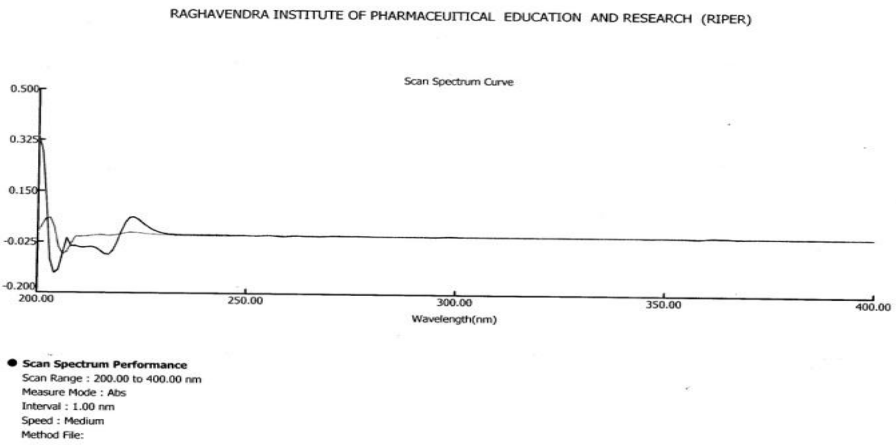

Figure 3: Overline Spectra of Ritonavir and Lopinavir

Optimization of chromatographic conditions: Drug solubilities were examined in literature and found to be soluble in methanol and water. Drugs pKa were known from literature as 13.98 & 14.23 for LPV & RTV, so the pH of the mobile phase adjusted to 3.5.

The detection wavelength was optimized in the double beam spectrophotometer, by scanning sample in the range of 200-400nm. From the overlaid spectrum of LPV & RTV, 225nm was selected for the simultaneous quantification in HPLC method. A different mobile phase ratio of methanol and water starting from pure methanol to gradual increment of aqueous phase in steps was performed in C_{18} (150X4.6mm, 5µ) column and the results were showed in table 1 and (**Figure 4**).

Table 1: Optimization of Mobile Phase for RTV & LPV

S. No	Mobile phase composition (methanol : water) 1 ml/min	Retention times(min)		Asymmetry	
		RTV	LPV	RTV	LPV
1	90:10	4.4	4.8	1.04	1.05
2	**85:15**	**4.8**	**5.9**	**1.04**	**1.05**
3	80:20	5.6	7.1	1.2	1.0

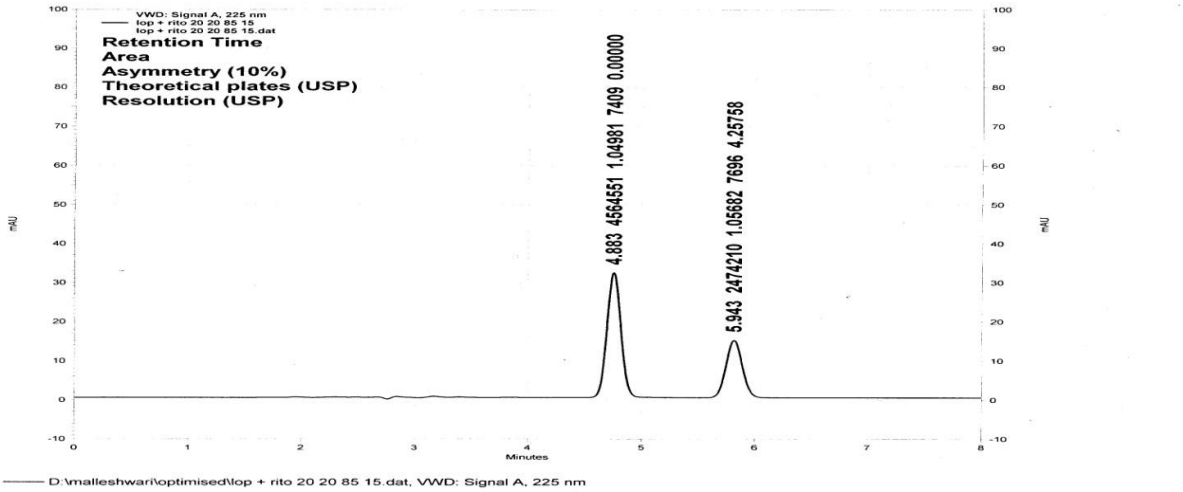

Figure 4: Methanol: Water (85: 15) Optimized Chromatogram

Chromatographic conditions: The mobile phase consisted of methanol, water in the ratio of 85:15. The mobile phase was filtered through a 0.45µ membrane and degassed for 8min. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1ml/min and the injection volume was 20µl. The column temperature was maintained at 23±1^{0}C. The eluents were monitored at 225nm. The optimized conditions were shown in (**Table 2**). **Sample preparation:** Plasma was collected from Rural Development Trust (RDT) hospital, Anantapuramu. Four clean tubes were taken and to each 0.5 ml of plasma and 0.5 ml of 200 mg/ml drug solution was added. For extracting drug four different types of solutions were used such as 1% tri chloro acetic acid, ACN, 1% H_2SO_4 and diethyl ether. Drugs were extracted by centrifugation process. Clear supernatant solution separated and filtered through 0.2mm filter and 20ml of solution was injected to the column and chromatograms were recorded.

Table 2: Optimized Chromatographic Conditions

S. No	Parameters	Conditions
1	Mobile Phase Optimized	Methanol : Water - 85:15
2	Stationary Phase	C_{18} (250 × 4.6 mm i.d.,5 µm)
3	Flow Rate	1 ml / min
4	Run Time	8 min
5	Column Temperature	23°C
6	Volume of Injection	20 µL
7	Detection Wavelength	225 nm
8	Retention time of Drug (min)	RTV - 4.8, LPV - 5.9

RESULTS AND DISCUSSION

Validation of developed method [3-5]

Specificity: The specificity of the method was evaluated with regard to interference due to presence of any other excipients. The (**Figures 5 & 6**) shows that drug were clearly separated from its excipients. Thus, the HPLC method presented in this study is selective.

Accuracy (Recovery): Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 80%, 100%, 120%. The recovery studies were carried out three times and the percentage recovery and percentage relative standard deviation of the recovery were calculated for both drugs and shown in the (**Table 3**).

Precision: To assess the precision of the method, intra-day and inter-day measurements of drugs were completed with computation of the coefficient of variations (C.V) for replicate sample (n = 3) using concentrations of 10 (LQC), 20 (MQC), 30 (HQC) for RTV and 40 (LQC), 80 (MQC), 120 (HQC) for LPV. Both intra-day and inter-day samples of both drugs were calibrated with standard curve concurrently prepared on the day of analysis [6-7]. The results were shown in the (**Table 4**).

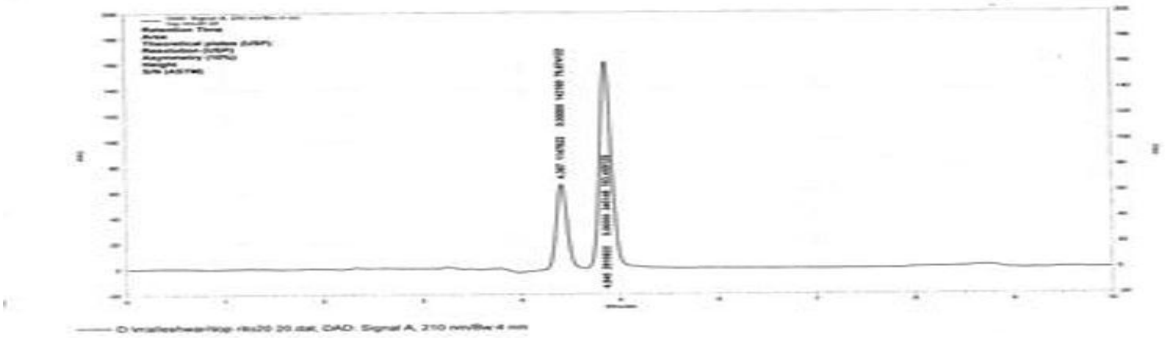

Figure 5: Methanol: Water (90: 10) Chromatogram

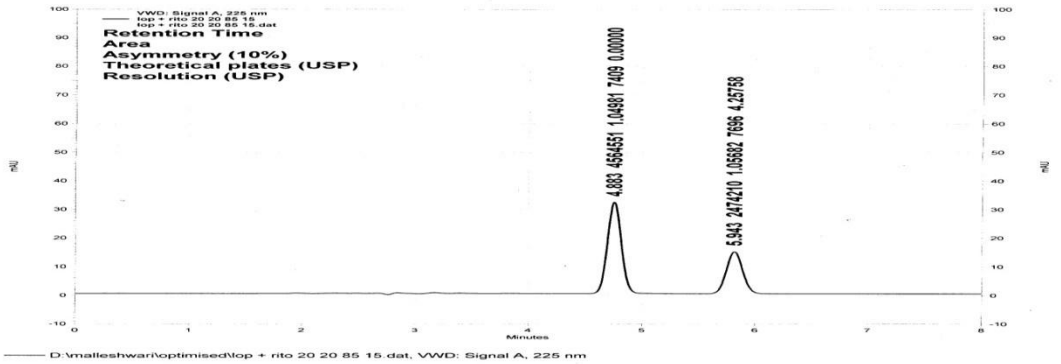

Figure 6: Methanol: Water (85: 15) Optimized Chromatogram

Table 3: Recovery Studies

S. No	Name of the drug	Pre analysed sample (µg/ml)	Recovery level	Amount of drug added(µg/ml)	Total amount found	% Recovery	%RSD
1	RTV	20	80% 100% 120%	16 20 24	35.36 40.70 45.29	98.2 101.75 102.94	0.6 0.8 1.4
2	LPV	80	80% 100% 120%	64 80 96	141.42 162.95 178.64	98.2 101.8 101.5	1.2 1.4 1.5

Table 4: Precision Studies

Precision	RTV		LPV		
	Conc (µg/ml)	%RSD	Conc (µg/ml)	%RSD	Acceptance limit criteria
Intra day	10(LQC) 20(MQC) 30(HQC)	0.4 0.2 0.5	40(LQC) 80(MQC) 120(HQC)	0.5 0.6 0.6	%RSD <2
Inter day	10(LQC) 20(MQC) 30(HQC)	1.1 1.3 1.6	40(LQC) 80(MQC) 120(HQC)	0.8 1.4 1.8	%RSD <2

Linearity: The linearity of calibration curves (peak area Vs concentration) in pure solution was checked over the concentration ranges of about 10-50µg/ml & 40-200µg/ml for RTV & LPV. The total eluting time was 10min. The regression line relating standard concentrations of drug using regression analysis, the calibration curves were linear in the studied range and equations of the regression analysis were obtained**:**

Table 5: Linearity of RTV& LPV

S. No	Ritonavir			Lopinavir		
	Conc (µg/ml)	Peak area Mean±SD	%RSD	Conc (µg/ml)	Peak area Mean±SD	%RSD
1	5	1642618±154.3	0.9	20	2583567±161.9	0.6
2	10	2902847±108.3	0.3	40	399083567±105.18	0.8
3	15	4231894±68.64	1.6	60	6683876±2642.35	0.9
4	20	5703577±998	1.2	80	8365465±10047.54	0.3
5	25	6851666±666	1.4	100	12470110±52915.5	0.4
6	30	8123717±680	0.8	120	14477651±66602.74	0.6
7	35	9620840±132	1.3	140	17220437±89416.87	1.2
8	40	10909098±56.3	0.5	160	18240337±121654.6	0.6
9	45	12503592±59.3	0.4	180	19734981±3999.5	1.4
10	50	13986160±51.3	0.3	200	21293957±40925.7	1.3

$Y = 27237x + 15736$, $r^2 = 0.9998$ for RTV
$Y = 10439x + 15868$, $r^2 = 0.9997$ for LPV.
These linearity curves were shown in (**Figures 7 and 8**). The mean ± standard deviation (SD) for the slope, intercept and correlation coefficient of standard curves (n=3) were calculated [8-11]. The represented data was shown in (**Table 5**).

Limit of detection (LOD), Limit of quantitation (LOQ): The LOD and LOQ for RTV and LPV were determined at a signal to noise ratio 3:1 and 10:1 respectively by injecting a series of dilutions with known concentrations. The LOD, LOQ for RTV were 0.07 µg, 0.23µg & for LPV 0.14µg, 0.44µg respectively for 20µL injection volume [12]. The represented data was shown in (**Table 6**).

Robustness: The optimum HPLC conditions set for this method have been slightly modified for samples of RTV and LPV dissolved in the drug matrix as a means to evaluate the method ruggedness [13]. The small changes include: the flow rate, the mobile phase ratio, the wavelength and the data was shown in (**Table 7**).

System suitability: It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development [14-15]. For this, parameters like plate number (N), resolution (R), relative retention time (α), HETP, peak asymmetry of samples were measured, and shown in (**Table 8**).

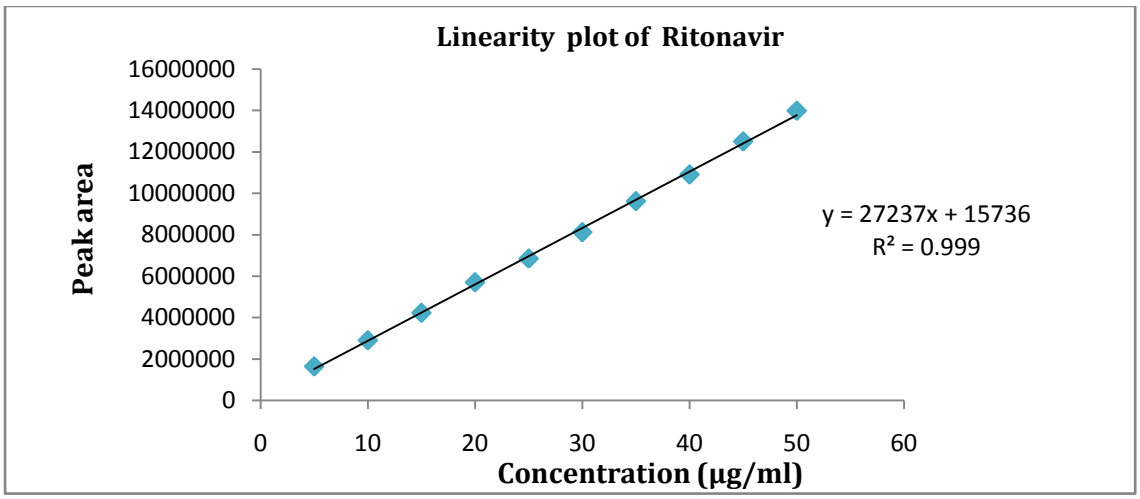

Figure 7: Calibration Plot of Ritonavir

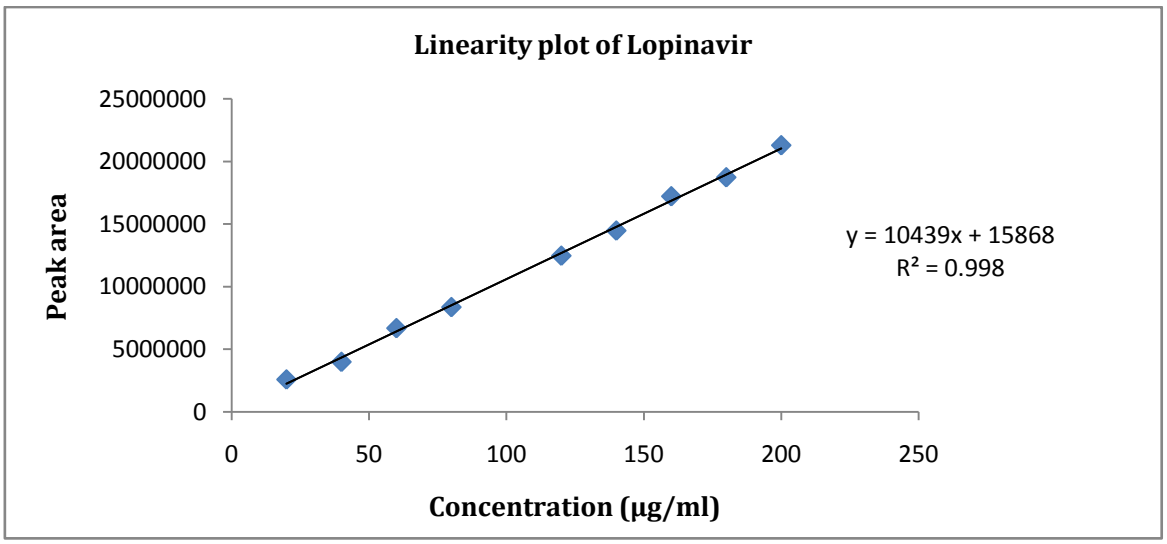

Figure 8: Calibration Plot of Lopinavir

Table 6: LOD and LOQ Values of Ritonavir and Lopinavir

S. No	Parameter	RTV	LPV
1	LOD(µg/ml)	0.07	0.14
2	LOQ(µg/ml)	0.23	0.44

Table 7: Robustness Studies

S. No	Parameter	Standard	Modification	Retention time(min)		Asymmetry	
				RTV	LPV	RTV	LPV
1	Flow rate	1ml/min	0.8ml/min	6.1	7.5	1.05	1.05
			1.2ml/min	4.0	4.9	1.05	1.05
2	Wavelength	225nm	223nm	4.9	6.0	1.0	1.04
			227nm	4.9	6.0	1.05	1.04
3	Mobile phase	85:15	83:17	5.8	7.5	1.03	1.05
			87:13	4.6	5.6	1.01	1.02

Table 8: System Suitability Parameters for Ritonavir and Lopinavir

Parameter	Values obtained (n = 6) Mean ± SD		Acceptance Criteria
	RTV	LPV	
Plate Count	7409± 63.18	7696± 65.36	>2000
Tailing Factor	1.04± 0.025	1.05 ± 0.031	≤ 2.0
Capacity factor	0.64	2.24	< 2
HETP	0.00293	0.00266	----
R_t (min)	4.8	5.9	----

CONCLUSION

New RP- HPLC method had been developed for simultaneous estimation of Ritonavir and Lopinavir in tablet formulation. It was shown that the method was linear, accurate, precise, reproducible, economical, selective and specific providing the reliability of the method. It produces symmetric peak shapes, good resolution, and reasonable retention times for both the drugs. The method was fully validated and showing satisfactory data for all the method validation parameters tested. The recoveries achieved are good by the method. Hence, this method can be applicable for the simultaneous estimation of Ritonavir and Lopinavir in quality control studies for routine analysis. The recovery of drug in plasma was proved to be good. Hence this method can be applicable for the biological analysis.

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