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Simultaneous Determination And Validation Of Darunavir Ethanolate And Ritonavir In Binary Mixture By Liquid Chromatography

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Abstract: A simple and accurate method to determine Darunavie ethanolate (DRV) and ritonavir (RTV), in binary mixture, was developed and validated using liquid chromatography (LC). The LC separation was achieved on a Phenomenex Luna C18 column (250 mm, 4.6mm i.d., 5µm), in the isocratic mode using acetonitrile: water (60: 40, v/v), pH adjusted to 3.2 ± 0.02 with formic acid at a flow rate of 1 mL/min. The retention times were about 5.02 and 7.60 min for DRV and RTV, respectively. Quantification was achieved with UV-visible detector at 245 nm over the concentration range of 6-60 and 2-20 µg/mL for DRV and RTV respectively, with mean recoveries of 99.54-100.18 % and 99.16-100.20 % for DRV and RTV, respectively. The method was validated, and was found to be simple, specific, accurate, precise and robust. The method was successfully applied for the determination of DRV and RTV in binary mixture without any interference from common excipients.
Key words: Darunavir ethanolate, Ritonavir, Binary Mixture, Liquid Chromatography.

INTRODUCTION

Darunavir (DRV), [(1*S*,2*R*)-3-[[(4-amino phenyl)sulfonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl)propyl]-carbamic acid (3*R*,3a*S*,6a*R*)-hexahydrofuro[2,3-*b*]furan-3-yl ester monoethanolate, a new protease inhibitor (PI), is used to treat human immunodeficiency virus (HIV) type-1 [1]. According to in vitro experiments, DRV was active against HIV-1 with PI resistance mutations and against PI resistant clinical isolates [2-4]. DRV is expected to be effective in antiretroviral treatment-experienced patients, such as those possessing HIV-1 strains which are resistant to more than one PI [5-7]. Chemically, ritonavir (RTV) is (5S,8S,10S,11S)-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-oicacid,5-thiazolylmethyl ester [8]. RTV is a selective, competitive and reversible inhibitor of both HIV-1 and HIV-2 proteases. It is widely used in the treatment against AIDS and particularly to inhibit liver enzyme, viz., cytochrome P450-3A4 (CYP3A) [9].

DRV blocks HIV protease an enzyme which is needed for HIV to multiply. When DRV used with RTV in combination therapy, they may reduce the amount of HIV in blood and increase CD4 (T) cell count, so DRV is always used with 100 mg RTV in combination with other antiretroviral agents [10]. Central Drugs Standard Control Organization of India has been approved DRV for treatment of HIV infection coadministered with 100 mg RTV [11].

Literature survey revealed that different analytical method for determination of DRV have

been reported, which include HPLC with UV detection (HPLC-UV) to determine the concentration of DRV in human plasma [12], HPLC-MS method for the simultaneous determination of DRV and 11 other antiretroviral agents in plasma of HIV infected patients [13] and validation of Plasma DRV Concentrations determined by the HPLC Method for protease Inhibitors [14].

Many reports are available for the estimation of RTV in pure power, formulation and biological samples. Several HPLC methods were published for determination of antiretrovirals including RTV in different formulations [15-18]. HPTLC was used for simultaneous determination of ritonavir and lopinavir in capsules [19]. A few CE and LC–MS methods were reported for analysis of RTV and its metabolites in biological fluids [20-23]. UV-Derivative spectrophotometric method for determination of RTV in capsule [24]. LC–MS/MS studies of ritonavir and its forced degradation products [25]. The present report describes specific, precise, accurate and sensitive HPLC and HPTLC methods for simultaneous estimation of DRV and RTV in Binary mixture.

EXPERIMENTAL

Reagents and materials

DRV and RTV powder with 99.94 and 99.96 % purity, respectively. LC grade acetonitrile (S.D. Fine Chemicals, Ahmedabad, India) methanol and water (Finar chemicals Ltd., Ahmedabad, India), formic acid (Spectrochem Pvt Ltd., Mumbai, India). The nylon 0.45 µm-47 mm membrane filter (Gelman laboratory, Mumbai, India)

Apparatus and chromatographic conditions

A Shimadzu (Columbia, MD) HPLC system (Class vp) equipped with manual injector of 20µL loop, UV-Visible detector and Phenomenex (Torrence, CA) Luna C18 column (250mm x 4.6mm i.d., 5µm) was used. An Acculab ALC-210.4 (India) analytical balance, and an ultra sonic cleaner (Frontline FS 4, Mumbai, India) were used. The LC system was operated isocratically at 25 ± 2 ^{0}C using mobile phase comprised of acetonitrile: water (60: 40, v/v), pH adjusted to 3.2 ± 0.02 with formic acid at a flow rate of 1 mL/min. The mobile phase was filtered through nylon 0.45µm-47mm membrane filter and was degassed before use. The determination was performed at 245 nm using Class vp software. The injection volume was 20 µL and the total run time was 10 min.

Preparation of DRV and RTV Standard solutions

Accurately weighed DRV (300 mg) and RTV (100 mg) were transferred to a 100 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard solution of DRV (3000 µg/mL) and RTV (1000 µg/mL. An aliquot (5.0 mL) of the solution was transferred to a 50 mL volumetric flask, and diluted to the mark with mobile phase to obtain a mixed working standard solution DRV (300 µg/mL) and RTV (100 µg/mL).

Preparation of Sample solution

The binary mixture of DRV and RTV was prepared in the ratio of 3:1. Accurately weighed DRV (300 mg) and RTV (100 mg) was transferred to a 100 mL volumetric flask containing 30 mL methanol. Common excipients, which are used in the tablet formulation, were added in this mixture and the content was sonicated for 15 min. The flask was allowed to stand at room temperature for 5 min, and the volume was made up to the mark with methanol to obtain the sample stock solution (3000 and 1000 µg/mL) of DRV and RTV, respectively. The solution was filtered through 0.45µm membrane filter. An aliquot (5.0 mL) was transferred to a 50 mL volumetric flask, and diluted to the mark with mobile phase to obtain working sample solution (300 and 100 µg/mL) of DRV and RTV, respectively. An aliquot (0.8 mL) was transferred to a 10 mL volumetric flask, and diluted to the mark with mobile phase to obtain the sample solution (24 and 8 µg/mL) for DRV and RTV, respectively.

Method validation

The methods were validated for the following parameters following the International Conference on Harmonization (ICH) guidelines [26].

Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Chromatograms of standard and sample solutions of DRV and RTV were compared in order to provide an indication of specificity of the method.

Linearity (Calibration curve)

Aliquots (0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 mL equivalent to 6, 12, 24, 36, 48 and 60 µg/mL for DRV; and 2, 4, 8, 12, 16 and 20 µg/mL for RTV) were transferred in a series of 10 mL volumetric flasks and diluted to the mark with mobile phase.

An aliquot (20 µL) of each solution was injected under the operating chromatographic conditions as described above. Responses were recorded. Calibration curves were constructed by plotting the peak areas versus the concentrations, and the regression equations were calculated. Each response was average of three determinations.

Accuracy (% Recovery)
The accuracy of the methods was determined by calculating recoveries of DRV and RTV by the standard addition method. Known amount of standard solutions of DRV (12, 24 and 36 µg/mL) and RTV (4, 8 and 12 µg/mL) were added to a pre-analysed sample solution of DRV (24 µg/mL) and RTV (8 µg/mL). Each solution was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves.

Precision
The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 6 different concentrations of DRV(6, 12, 24, 36, 48 and 60 µg/mL) and RTV (2, 4, 8, 12, 16 and 20 µg/mL).

Limit of Detection and Limit of Quantification
The limit of detection (LOD) and the limit of quantification (LOQ) of the DRV and RTV were calculated using the standard deviation of responses and slopes using signal-to-noise ratio.

Robustness
The robustness was studied by analysing the same samples of DRV and RTV by deliberate variation in the method parameters. The change in the responses of DRV and RTV were noted. Robustness of the method was studied by changing the extraction time of DRV and RTV from binary mixture by ± 2 min, composition of mobile phase by ± 2 % of organic solvent, flow rate by ± 0.2 mL/min.

System-Suitability Test
System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were asymmetry of the chromatographic peak, peak resolution and repeatability, as RSD of peak area for replicate injections. The precision of the instruments was checked by repeatedly injecting (n = 6) DRV (24 µg/mL) and RTV (8 µg/mL).

Determination of DRV and RTV in binary mixture
The responses of sample solutions were measured at 245 nm for quantitation of DRV and RTV by the proposed method. The amount of DRV and RTV present in the sample solutions were determined by fitting the responses into the regression equations of the calibration curve for DRV and RTV, respectively.

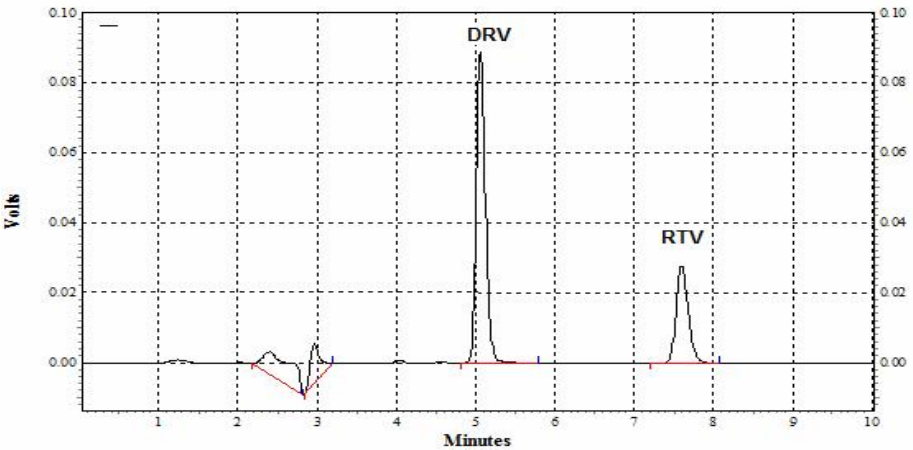

Figure 1: Chromatogram of DRV (24 µg/mL) and RTV (8 µg/mL) from binary mixture with retention time of 5.02 and 7.60 min, respectively

Table 1: Regression analysis of the calibration curves for DRV and RTV (*n*=3)

Parameter	LC	
	DRV	**RTV**
Linearity range	6-60 µg/mL	2-20 µg/mL
Slope	43052	41526
Standard deviation of slope	52.78573	43.1895
Intercept	12505	-34412
Standard deviation of intercept	131.4648	176.936
Correlation coefficient, *r*	0.9998	0.9998

n = Number of determinations

Table 2: Results of recovery study for DRV and RTV (*n*=3)

Drug	Amount taken	Amount added	Amount found	Recovery, ± SD, %	RSD[a], %
DRV µg/mL	24	0	24.04	100.15 ± 0.53	0.53
	24	12	35.84	99.54 ± 0.39	0.39
	24	24	48.01	100.03 ± 0.31	0.31
	24	36	60.11	100.18 ± 0.54	0.54
RTV µg/mL	8	0	7.93	99.16 ± 0.62	0.62
	8	4	12.00	99.97 ± 0.34	0.34
	8	8	16.03	100.20 ± 0.50	0.50
	8	16	20.03	100.14 ± 0.57	0.57

aRSD = Relative Standard deviation, *n* = Number of determinations

Table 3: Result of robustness study for DRV and RTV (*n*=3)

Variable	Optimized value	Range	DRV	RTV
Extraction time, min	15	13	99.55	99.54
		15	99.97	100.12
		17	100.11	99.92
Acetonitrile, %	60	58	100.03	99.97
		60	99.98	100.12
		62	99.94	100.08
Mobile phase pH	3.2	3.0	99.86	99.94
		3.2	99.95	100.11
		3.4	100.08	100.04
wavelength	245	244	100.09	99.96
		245	99.98	100.04
		246	99.80	100.09
Flow rate, mL/min	1.0	0.8	99.59	100.07
		1.0	99.96	100.04
		1.2	99.62	99. 84

Table 4: System suitability test parameters for DRV and RTV

Parameter	DRV ± % RSD[a]	RTV ± % RSD[a]
Retention time, min	5.02 ± 0.10	7.59 ± 0.11
Tailing factor	1.12 ± 0.67	1.24 ± 0.72
Asymmetry	1.14 ± 0.45	1.18 ± 0.44
Theoretical plates	8821 ± 0.80	10832 ± 0.66

RSD[a] = Relative Standard deviation.

Table 5: Analysis results for DRV and RTV binary mixture (*n*=5)

DRV			RTV		
Labelled amount (mg)	Amount found (mg)	DRV ± SD[a], %	Labelled amount (mg)	Amount found (mg)	RTV ± SD[a], %
300	299.76	99.92 ± 0.43	100	100.05	100.05 ± 0.36

SD [a] = Standard deviation, *n* = Number of determinations

RESULT AND DISCUSSION

The mobile phase consisting of acetonitrile: water (60: 40, v/v), pH adjusted to 3.2 ± 0.02 with formic acid, at a flow rate of 1 mL/min, was found to be satisfactory to obtain good peak symmetry, better reproducibility and repeatability for DRV and RTV. Quantification was achieved with UV-visible detector at 245 nm based on peak area. The retention times were about 5.02 and 7.60 min for DRV and RTV, respectively (Figure 1).

Method Validation

The method was found to be specific as no significant change in the responses of DRV and RTV was observed after 24 h. The excipients present in binary mixture didn't interfere with the chromatographic responses of DRV and RTV. It indicates that the proposed method is specific. Linear correlation was obtained between peak area and concentration for 6-60 µg/mL DRV and 2-20 µg/mL RTV. The linearity of the calibration curves were validated by the value of correlation coefficient of the regression (*r*). The regression analysis of the calibration curves is shown in Table 1. The recovery study was carried out by the standard addition method. The percent mean recoveries obtained for DRV and RTV were 99.54-100.18 % and 99.16-100.20 %, which were satisfactory (Table 2). The values of % RSD for intraday and interday variations were found to be in range of 0.43-0.74 and 0.72-1.19 % for DRV, and 0.60-0.82 and 0.85-1.05 %;for RTV, respectively. The % RSD values indicate the proposed method is precise. The LOD and LOQ were found to be 0.1575 and 0.4773 µg/mL for DRV, 0.0828 and 0.2510 µg/mL for RTV.

The methods are found to be robust as the results were not significantly affected by slight variation in the chromatographic conditions such as extraction time by ±2 min, composition of mobile phase by ±2%, pH of mobile phase by ± 0.05, wavelength by ±1nm and flow rate of the mobile phase by ±0.2mL (Table 3). % RSD for repeatability was found to be 0.71 and 0.68 for DRV and RTV, respectively. System suitability test parameters are listed in (Table 4).

Determination of DRV and RTV in binary mixture

The proposed Liquid chromatography was successfully applied for determination of DRV and RTV in binary mixture. The results obtained for DRV and RTV were comparable with the corresponding claim percentage (Table 5).

CONCLUSION

Liquid Chromatographic method was developed for determination of DRV and RTV in combination. The method was validated and found to be simple, sensitive, specific, accurate, precise and robust. Statistical findings of the assay for DRV and RTV in binary mixture indicated satisfactory results. Hence, the method can be used successfully for the routine analysis of combined forms of DRV and RTV.

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