Original Article

Development and Validation of RP-HPLC Method for Quantification of Total, Free and Entrapped Ritonavir in Lipid Nanocarriers and Drug content of Film Coated Fixed Dose Formulation

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ABSTRACT

Aim: The current study was aimed to develop and validate a simple isocratic, precise, sensitive, accurate and robust reverse phase HPLC analytical method for the quantification of total, free and entrapped ritonavir in lipid nanocarriers and drug content of the film-coated fixed-dose formulation. **Materials and Methods:** The method was developed by using Inertsil ODS-3V C_{18} column as stationary phase, orthophosphoric acid (OPA) in water (pH 3.0) and acetonitrile as mobile phase. Further, the method was validated following ICH Q2(R1) guidelines. Ritonavir loaded lipid nanocarriers were developed using hot-emulsion and probe-sonication method. A solid phase extraction (SPE) method was developed for the separation of free and entrapped ritonavir. Total ritonavir was extracted from lipid nanocarriers (LNCs) and film-coated tablets using methanol and analyzed with validated method. **Results:** The responses were found to be linear and precise over a range of 0.25 μg/mL to 16 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) of the RP-HPLC method were found to be 14.06 ng/mL and 42.60 ng/mL, respectively which emphasizes the sensitivity of the method. The recovery of ritonavir from LNCs was found to be within 3% of the total drug present in LNCs. Similar, amount of drug recovery was found from assay of marketed formulation i.e., > 97%. **Conclusion:** The SPE method is capable of separating free and entrapped ritonavir from LNCs. The developed HPLC method is sensitive, robust, economical and gives high recovery. The developed HPLC method can also be employed for routine quantitative analysis of ritonavir in bulk formulation.

Key words: HPLC method, Ritonavir, Validation, SPE, NLC, Bulk formulation.

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INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) was first recognized in the year 1981. Human immunodeficiency virus (HIV), a lentivirus belongs to the family Retroviridae is the causative organism for AIDS.[1] HIV causes a steady decline in the population of T-lymphocytes with CD4+ glycoprotein.[2] As the CD4+ T- lymphocyte count decreases the immunodeficiency progresses, immune response decreases and many opportunistic diseases viz. Mycobacterial, Salmonella, Pneumococci, Cytomegalovirus, Herpes, and other infections and neoplasms develop.[3] Protease inhibitors (PIs), are a key component of HAART (highly active antiretroviral therapy) regimen in combination with other class of drugs helps in suppressing the viral load, maximizing survival, reducing morbidity, restoring and maintaining the regular immunological function.[4] Ritonavir, a highly effective protease inhibitor, but the poor bioavailability, frequent dosing and concomitant toxicities limited the use of ritonavir in the first-line therapy. Ritonavir has a profound inhibitory effect

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on CYP3A4 in liver and the intestinal tract. Because of this, the co-administration of ritonavir exhibits a very high potent effect on the absorption and metabolism of other PIs. The use of ritonavir-boosted regimens allows extended dosing intervals for the primary PIs, with associated additional advantage in reducing pill burden and adherence, and improves potency with a decreased likelihood of resistance compared to un-boosted PIs.[5] Ritonavir is a large and peptidomimetic molecule with a molecular weight of 721 g/mol. The neutral form of ritonavir is highly lipophilic with a log D value of 4.3.[6] Ritonavir belongs to the class II as per BCS classification and exhibits a low oral bioavailability due to its poor solubility in the aqueous medium.[7]

Over the past few decades, nanotechnology attracted the attention of researchers in the field of medicine, especially in areas of novel drug delivery through various routes of administration.[8] LNCs such as liposomes, solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), niosomes and ethosomes are being prepared by the researchers for the delivery of drugs belonging to the various classes. SLNs consist of a matrix formed by the solid lipid in which the drug is entrapped. SLNs were mainly prepared to overcome the limitations associated with colloidal carriers such as emulsions, polymeric nanoparticles and liposomes. NLCs are the modified SLNs where the drug is entrapped in a matrix consisting of a combination of solid lipid and liquid lipid.[9] Structural imperfections in the solid lipid resulting from the incorporation of liquid lipid lead to the less ordered crystalline arrangement which helps in the prevention of drug leakage during the storage. Moreover, NLCs offer high drug loading efficiencies in comparison to SLNs apart from the stability.[10] Optimization of nanoformulations is a necessary step for the development of an ideal carrier with the best characteristics that include particle size, polydispersity index (PDI), zeta potential, drug loading and drug entrapment efficiency.[11] Determination of the amount of drug that is entrapped inside the matrix is one of the crucial parameters for the evaluation of formulations during the developmental stage. There are several methods for the separation of entrapped and unentrapped drugs from formulations such as dialysis bag diffusion, ultracentrifugation, gel filtration, micro-dialysis, capillary electrophoresis, hydrodynamic chromatography, size exclusion chromatography, cross-flow filtration and field flow fractionation. Most of these methods cannot be applied in all the laboratories owing to their high cost. The economical methods (for examples dialysis bag diffusion) takes more time for the separation of free drug from the formulations and the obtained results from method are often not so obvious. Few methods (for example ultra-centrifugation method) of separation may results in the breakdown of the nanoformulations leading to the redistribution of free and entrapped drug molecules. These problems can be addressed by the SPE method as it works on the basis of interaction between solid stationary phase and analyte that makes this method more specific.[12,13] Furthermore, once the entrapped and free drug is separated from nanoformulation it is very important to have a sensitive, robust and economical quantitative method for the quantification. Analytical methods for the quantification of ritonavir in pharmaceutical dosage forms (Emletra, Ritocom, Kaletra) by HPLC were reported by various researchers.[14] During the development of nanoformulations such as NLCs, there is a need of analytical method with high sensitivity, as free and entrapped portions of nanoformulations may encounter with lower concentrations. Hence, there is a need to work on the sensitivity of the analytical method. In the current study, a simple, fast, cost-effective, robust and highly sensitive analytical method was developed and validated following ICH guidelines. The analytical method validated in the current study was applied to quantify total, free and entrapped drug content of ritonavir-loaded NLCs. The HPLC method was also employed for the assay of ritonavir in a film-coated commercial pharmaceutical oral formulation.

MATERIALS AND METHODS

Chemicals and reagents

Reference standard of ritonavir was obtained as a gift sample from Dr. Reddy's Laboratories, Hyderabad, India. Ritonavir tablets with a label claim of 100 mg (Ritomune) were purchased from a local Pharmacy store. Strata ™ X (10 mg/mL) cartridges for solid phase extraction were purchased from Phenomenex, Bangalore, India. Glyceryl monostearate (GMS) was purchased from Fine Organics, Mumbai, India. Stearic acid was purchased from Finar, Ahmedabad, India. Oleic acid was purchased from Sigma Aldrich, Bangalore, India. Tween 20 was purchased from Merck, Mumbai, India. Sodium Lauryl Sulphate (SLS) was purchased from Loba Chemie Pvt Ltd. HPLC grade acetonitrile and methanol were purchased from SD Fine Chemicals, Mumbai. OPA was purchased from Spectrochem Private Limited, Mumbai. Type-I water (ultrapure water) was produced in house using Millipore water purifier system.

Instrumentation

Zeta sizer (NanoZS, Malvern Instruments, UK) was used for the determination of particle size, PDI and zeta

potential. A Shimadzu HPLC (LC-2010CHT model, Shimadzu Corporation, Kyoto, Japan) with a variable wavelength programmable UV-VIS detector was employed for the development and validation of the reverse phased HPLC method. The chromatograms were recorded with a computer connected to the instrument, with Lab Solutions v.5.57. Inertsil ODS-3V C_{18} column (250 × 4.6 mm, 5 µm, 100 Å) was used for the chromatographic separation of ritonavir. Ultrapure water was obtained from water purification system (Millipore Direct-Q3, Millipore Corporation). pH of the mobile phase was adjusted using digital pH meter (Eutech Instruments pH 510). Ultrasonic bath (Medica Instrument Mfg. Co.) was used for sonication of the mobile phase.

Preparation of mobile phase

Ultrapure water (1000 mL) was taken in a reagent bottle and pH was adjusted to 3.0 with OPA (20 %v/v). The prepared aqueous phase was filtered through cellulose nitrate filter paper with a pore size of 0.22 µm and 47 mm diameter using Millipore filtration unit and sonicated for 5 min in ultrasonic bath.

Preparation of stock solution

Ritonavir (10 mg) was weighed and dissolved in 1.0 mL of methanol in a volumetric flask with 5 min vortex. The final concentration of ritonavir was adjusted to 1000 µg/mL by adding methanol as the diluent.

RP-HPLC method development

The development of HPLC method is a complex procedure involving various factors like the type of instrument used, variety of stationary phase (different column chemistries and dimensions of columns), mobile phase, and other operational parameters. The nature of the analyte plays a key role in the selection of suitable parameter during the method development. The mobile phase composition and its pH significantly affect the retention time (R*t*) of the analyte whereas the column oven temperature would have only a minor effect. Reverse phase HPLC is the most common approach for the separation of small molecules whose molecular weight is less than 2000 Da. A normal C_{18} or Octadecylsilane column is the usual choice for the development of the method. In the current study, different buffers such as OPA, formic acid and acetic acid were studied at different pH values. The selection of organic solvent and the ratio of aqueous to organic solvent also plays a major role in the RT and separation of analyte. The effect of acetonitrile and methanol as the organic solvent and also the different ratios of aqueous to the organic solvent were studied. A reverse phase HPLC column of C_{18} with octadecylsilyl groups (Inertsil ODS-3V C_{18}) was used as the stationary phase. The Inertsil ODS-3V C_{18} column, a flow rate (1.2 mL/min), UV detection wavelength (242 nm), column temperature (25°), and injection volume (20 µL) were kept constant while taking trials for the mobile phase optimization. RT, number of theoretical plates (NTP) and tailing factor were considered as the influential factors for the optimization of mobile phase and other parameters.

RP-HPLC method validation

The developed RP HPLC method for ritonavir was validated with the aspects of system suitability, specificity, accuracy, precision, linearity, the limit of detection, the limit of quantification, and robustness according to the guidelines of ICH Q2 (R1).

System suitability

To perform the system suitability experiment six consecutive injections of ritonavir at a concentration of 16 µg/mL were injected on to HPLC and analyzed. For all the six samples, peak area, NTP and tailing factor of the analyte were recorded, average and percent relative standard deviation (% RSD) were calculated.

Specificity

For specificity, one blank sample and one ritonavir sample of lowest concentration (250 ng/mL) were injected. RT, area of the analyte peak were recorded and the percentage of interference at analyte RT in the blank sample was calculated.

Linearity

For the construction of calibration curve, a series of eight concentrations covering a range of 0.25 to 16.00 µg/mL were analyzed in triplicates with the developed RP-HPLC method. The peak areas of each concentration was recorded and a calibration curve was constructed by plotting mean peak area versus concentration. The linear regression, slope, intercept and coefficient of determination (r^2) were calculated from the curve.

Accuracy

The drug recovery technique was used to determine the accuracy test. Accuracy experiment was performed at three concentration levels (75%, 100%, and 125%). Samples were prepared by mixing 3, 6, and 9 µg/mL of ritonavir with equal volumes of 6 µg/mL sample to get a final concentration of 4.5, 6, and 7.5 µg/mL, which represents the test samples with a concentration level of 75%, 100%, and 125%, respectively. Three different samples (as references) with concentrations of 4.5, 6,

and 7.5 µg/mL were also prepared directly from the stock solution (1000 µg/mL) and these samples were considered as the reference samples. All the samples were injected to HPLC with the developed method and peak areas were recorded. Percentage recovery was calculated by using the below given formula.

$$\text{Percentage recovery} = \frac{\text{Peak area of the test}}{\text{Peak area of the reference}} \times 100$$

Precision

Intraday and inter-day precision studies were performed with the developed RP-HPLC method at three different concentrations lower (0.3 µg/mL), middle (7 µg/mL) and higher (14 µg/mL) levels. For intraday precision, six replicates of all the three levels (lower, middle, and higher) were analyzed in the morning (day 1) and evening (day 1). For interday precision, samples were analyzed by injecting six injections at all three levels on day 1 morning and day 2 morning. Peak areas of all the samples were recorded and % RSD of peak area was calculated at each concentration level.

LOD and LOQ

LOD and LOQ values were calculated using the standard deviation of the response and slope of the calibration curve by using the following formulae:

$$\text{LOD} = 3.3 \times \frac{SD_a}{m}$$

$$\text{LOQ} = 10 \times \frac{SD_a}{m}$$

Where 'SD_a' is the standard deviation of the y- intercept and 'm' is the slope of the calibration curve.[15,16]

Robustness

The optimized parameters for the developed RP-HPLC method were subjected to slight changes to evaluate the robustness of the method. Small changes were made in method parameters such as flow rate, aqueous to organic phase ratio, pH of buffer, injection volume, temperature of column oven, and wavelength of UV detector. With each parameter, sample with a concentration of 7 µg/mL was analyzed in triplicate. Peak area, NTP and tailing factor were recorded and the % RSD was calculated.

Application of the method

Preparation and characterization of ritonavir loaded NLCs

Ritonavir-loaded NLCs consisting of GMS, stearic acid, oleic acid, Tween 20, and SLS were prepared using hot emulsion and ultra-probe sonication method. Two different batches were prepared, batch I with GMS as solid lipid and oleic acid as liquid lipid whereas batch II was prepared using a combination of GMS and stearic acid as solid lipids and oleic acid as liquid lipid. The nanoparticles were characterized for particle size, PDI and zeta potential, where formulation samples were diluted appropriately with ultrapure water before analyzing. For total drug content samples, formulation (0.1 mL) was dissolved in methanol (0.9 mL) and sonicated in bath sonicator for 15 min. Further, dilutions were made with methanol and analyzed using a validated HPLC analytical method.

Separation of the free and entrapped drug from ritonavir loaded NLCs

The free and entrapped ritonavir from NLCs were separated using the SPE method using Strata X cartridges. The cartridges were conditioned with methanol (1 mL) followed by equilibration with ultrapure water (1 mL). Formulation (1 mL, diluted with ultrapure water in a ratio of 1:1, % v/v) was passed through these equilibrated cartridges. The eluent was collected for the estimation of the entrapped drug, followed by two times washing with 1 mL of ultrapure water. Finally, free drug bound to the sorbent was eluted by passing 1 mL of methanol. The eluent collected for the entrapped drug estimation was diluted with methanol and sonicated for 15 min on bath sonicator to extract the drug entrapped inside the matrix and injected onto HPLC after appropriate dilution with methanol. The eluent collected for the free drug quantification was diluted appropriately with methanol and injected onto HPLC using the validated method. The encapsulation efficiency was calculated using the formula given in literature.[17]

Accuracy of ritonavir in NLCs

To the blank formulation, different concentration of ritonavir standard drug was added to get final concentrations of 75 %, 100 % and 125 % of 4500 µg/mL and mixed properly. The drug added was recovered by dissolving the lipid contents in methanol and further appropriate dilutions were made with the same. Finally, samples were analyzed using HPLC to determine the recovered amount of drug added to the blank formulation.

Recovery of ritonavir from NLCs

To the pre-analyzed NLCs formulation (strength of the formulation was 2.59 mg/mL for the batch I and 2.64 mg/mL for batch II), ritonavir standard dilution (100 µg/mL) was added mixed properly. The total drug present in this mixture was extracted by dissolving in

methanol. Further, appropriate dilutions were made with methanol and analyzed using the HPLC method.

Extraction of ritonavir from a film-coated commercial pharmaceutical oral formulation

Ten film-coated tablets each containing 100 mg of ritonavir were weighed and ground into a fine powder. A quantity of powder equivalent to 10 mg was weighed and transferred to a 10 mL volumetric flask. In that 2 mL of methanol was added and vortexed to dissolve the contents. The volume was made up to the mark with methanol. Further, the sample was diluted with mobile phase to get a final concentration of 6 µg/mL. The prepared sample was analyzed with validated HPLC analytical method.

Ethical statement

The current study does not involve any experiments on human beings or animal. Hence, the study did not require any clearance from any ethics committee or review board.

Statistical analysis

Results are expressed as average ± SD. All statistical calculations were performed using Microsoft Office Excel® 2016.

RESULTS AND DISCUSSION

RP HPLC method development

During the initial trials of method development, various types of stationary phases with C_{18} alkyl chains were tried for the reverse phase chromatographic separation of ritonavir. Among the all tested columns Inertsil ODS-3V C_{18} column was found with adequate separation and resolution. Previously, Ahmed *et al.*, and Javana *et al.*, reported HPLC methods for the quantification of ritonavir in analytical samples using C_{18} columns.[18,19] In the current study, the impact of different mobile phases on separation, response, and resolution of peak was studied. Acetonitrile was selected as the organic solvent over methanol considering the advantages of acetonitrile in achieving sharp peaks and higher NTP. Acidified water was used as the aqueous phase. The phosphoric acid, formic acid and acetic acid were tried for acidification of aqueous mobile phase and phosphoric acid with pH of 3.0 has given better results (less tailing factor) in comparison to others. Previously, Al-Zoman *et al.*, developed a method using phosphate buffer with pH 3.0 for the estimation of ritonavir and other compounds. As small column was used in this study, shorter RT was achieved but the sensitivity of the method was low (linearity range is of 1.7 to 40 µg/mL).[20] As a mixture of acetonitrile and acidified water was used as mobile phase in the current study, that would help in saving column life because of the absence of salts. Moreover, post analysis column wash and maintenance are very much simple when this type of mobile phase is used for the analysis. The ratio of aqueous to organic mobile phase is crucial in achieving a good peak shape and also plays a key role in retaining the analyte on column. Different ratios of aqueous to organic solvents were tried. A ratio of 40:60 %v/v of aqueous to organic mobile phase gave a good resolution with optimum RT, NTP and peak area.

RP HPLC method validation

System suitability

System suitability is performed to ensure that all the analytical operations, sample preparation procedures and equipment are integral part of the system for the intended use.[21,22] In the current study, system suitability performed for the method was found to be within limits with the % RSD for the RT and peak area less than 1%. The data is represented in Table 1.

Specificity

The RT time of the ritonavir was found to be 8.66 min. There was no interference observed for blank sample at the RT of ritonavir which evidences the specificity of the method. The chromatograms of blank and ritonavir are presented in Figure 1.

Linearity

Linearity of the method explains the relation between the amount of analyte present in the sample and the instrument response.[21,22] The calibration curve constructed for ritonavir was found to be linear in the range of 0.25 µg /mL to 16.0 µg/mL. The regression analysis equation was found to be y = 13.58x - 10.326 with a coefficient of determination (R^2) value of 0.9999. The obtained R^2 value indicates that the curve is linear. The results of study are presented in Figure 2.

Table 1: System suitability of ritonavir.

S. No	Parameter	Average ± SD (*n*=6)	% RSD
1	Analyte peak area (mV-min)	212935 ± 420.17	0.20
2	RT (min)	8.661 ± 0.01	0.07
3	NTP (per column length)	7379 ± 26.86	0.36
4	Tailing factor (5%)	1.046 ± 0.00	0.16
5	Tailing factor (10%)	10.045 ± 0.00	0.13

min: minutes; mV: milli volts; NTP: number of theoretical plated; RSD: relative standard deviation; RT: retention time; SD: standard deviation

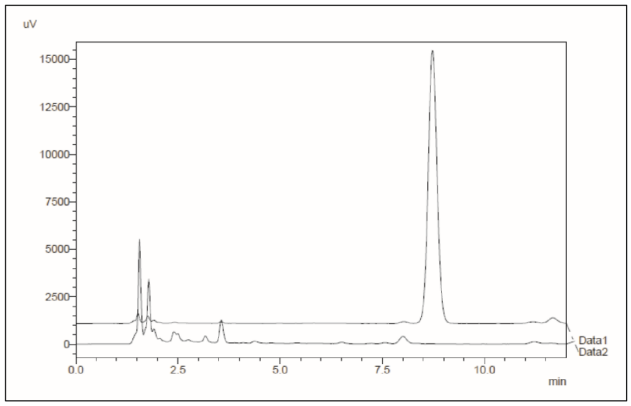

Figure 1: Chromatograms of mobile phase (data 1) and ritonavir (data 2).

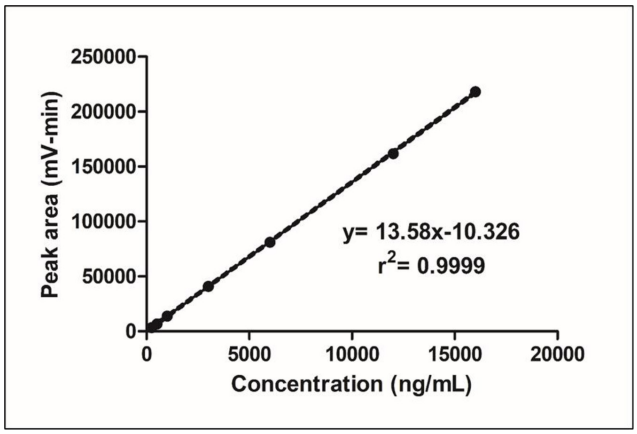

Figure 2: Linearity plot of ritonavir with RP-HPLC method.

Table 2: Accuracy data of ritonavir.

S. No	% Level	Spiked Conc. (ng/mL)	Peak area (mV-min)	Recovered concentration (ng/mL)	Percentage Recovery
			Average ± SD (*n*=3)	Average ± SD (*n*=3)	Average ± SD (*n*=3)
1	75	4500	62310 ± 36.12	4589.15 ± 2.66	101.98 ± 0.06
2	100	6000	79904 ± 56.51	5884.71 ± 4.16	98.08 ± 0.07
3	125	7500	101432 ± 118.82	7470.00 ± 8.75	99.60 ± 0.12

min: minutes; mL: milliliter; mV: milli volts; ng: nano gram; SD: standard deviation

Accuracy

Accuracy is the measure of closeness of the experimental value to the true value. Recovery is the weight/ amount of compound or analyte of interest that is recovered from the sample during the sample preparation procedure. Recovery is expressed as the percentage quantity of the analyte of interest analyzed to the theoretical quantity of the analyte present in the sample. In the current study, accuracy was expressed in terms of recovery at three different concentration levels.[21,22] The percentage recovery was found to be within ± 3% for all the three levels (viz. 75%, 100% and 125%) and data is presented in Table 2.

Precision

Precision experiment determines the closeness of agreement (degree of scatter) between the repeated measures of the same samples and analyzed under same analytical conditions.[21,22] The % RSD values of peak area obtained from intra-day precision samples was found to be less than 1% for lower, middle and higher concentrations whereas it was found to less than 2% for inter-day precision. The % RSD for both intra-day and inter-day precision are within acceptable limits of ICH Q2(R1) guidelines. This indicates that the method developed for the quantification of ritonavir is precise. The precision data from the study are shown in Table 3.

LOD and LOQ

LOD is the least concentration that can be detected with the method but not necessarily quantified. LOQ is the least amount of analyte of interest that can be quantifies with acceptable precision and accuracy.[21,22] In the current study, LOD and LOQ were calculated as 14.06 and 42.60 ng/mL respectively. The lower LOD and LOQ values indicate that the method is sensitive to detect and quantify ritonavir at lower concentrations with the developed RP HPLC method.

Robustness

Robustness of a method is its ability to remain unaffected by small and deliberate changes that are made in method parameters.[21,22] The % RSD was found to be less than 1% for peak area, NTP and tailing factor of the sample that was analyzed by optimized method with small and deliberate changes. The data evident that method is robust for the slight variations in the method parameters. The obtained results are depicted in Table 4.

Table 3: Intraday and interday precision data of ritonavir.

S No	Intraday precision			S. No	Interday precision		
	Peak Area (mV-min)				Peak Area (mV-min)		
	300 ng/mL	7000 ng/mL	14000 ng/mL		300 ng/mL	7000 ng/mL	14000 ng/mL
1	3980	93077	185913	1	4087	93197	187020
2	3923	93123	185742	2	3973	92691	184812
3	3996	93022	186028	3	3902	92577	187187
4	3896	93116	186005	4	3904	92613	186997
5	3894	92998	186063	5	3990	92039	187536
6	3895	93044	185948	6	3996	92410	187227
7	3928	92683	184997	7	3928	92683	184997
8	3924	92870	184993	8	3924	92870	184993
9	3856	92901	185066	9	3856	92901	185066
10	3899	92870	185066	10	3899	92870	185066
11	3885	92830	185059	11	3885	92830	185059
12	3924	92623	185051	12	3924	92623	185051
Intraday average ± SD	3917 ± 39.30	92930 ± 163.16	185418± 483.58	Interday average ± SD	3939 ± 62.87	92692 ± 287.84	185850 ± 1135.74
Intraday % RSD	1.00	0.18	0.26	Interday % RSD	1.60	0.31	0.61

min: minutes; mL: milliliter; mV: milli volts; ng: nano gram; RSD: relative standard deviation; SD: standard deviation

Table 4: Robustness data of ritonavir.

S. No	Parameter	Level	Peak area (mV-min)		NTP (per column length)		Tailing Factor (5%)	
			Average ± SD (n=3)	% RSD	Average ± SD (n=3)	% RSD	Average ± SD (n=3)	% RSD
1	Flow rate (mL)	1.10	103093 ± 254.05	0.25	8073 ± 27.63	0.34	1.038 ± 0.02	0.19
		1.30	87470 ± 270.86	0.31	7303 ± 27.10	0.37	1.032 ± 0.001	0.10
2	MP ratio (ACN: OPA, pH 3.0)	62:38	94066 ± 295.47	0.31	7464 ± 12.66	0.17	1.038 ± 0.002	0.15
		58:42	94514 ± 100.13	0.11	7830 ± 16.62	0.21	1.035 ± 0.003	0.30
3	pH of mobile phase	2.8	94157 ± 94.73	0.10	7539 ± 3.52	0.05	1.038 ± 0.001	0.10
		3.2	94346 ± 75.30	0.08	7389 ± 26.35	0.36	1.012 ± 0.001	0.11
4	Wavelength (nm)	238	92060 ± 142.48	0.15	7538 ± 23.63	0.31	1.053 ± 0.001	0.05
		242	91027 ± 228.66	0.25	7617 ± 23.03	0.30	1.027 ± 0.002	0.22
5	Injection volume (µL)	18	83379 ± 67.18	0.08	7616 ± 16.17	0.21	1.051 ± 0.001	0.1
		22	102135 ± 27.87	0.03	7538 ± 19.86	0.26	1.053 ± 0.001	0.05
6	Column oven temperature (°)	22	85772 ± 89.79	0.10	7524 ± 19.00	0.25	1.049 ± 0.002	0.15
		28	85958 ± 63.80	0.07	6886 ± 25.01	0.36	1.052 ± 0.001	0.05

°: degree centigrade; µL: microliter; ACN: acetonitrile; min: minutes; mL: milliliter; MP: mobile phase; mV: millivolts; nm: nanometer; NTP: number of theoretical plates; OPA: ortho phosphoric acid; RSD: relative standard deviation; SD: standard deviation

Application of the method

Preparation and characterization of ritonavir loaded NLCs

Hot emulsion- ultra-sonication method involves the melting of solid lipids to which a liquid lipid is added to form a matrix that encapsulates the drug. The imperfections resulting from the mixture of solid lipid and liquid lipid help in enhancing the drug loading capacity, stability and also prevent leaching of the drug during the storage period of the formulation. Ultra-sonication is an intense dispersion technique that relies

on hydrodynamic stress caused by collapsing cavitation bubbles. Ultra-sonication of the emulsion leads to the disintegration and dispersion of submicron agglomerates that helps in the formation of homogenous distribution of particles.[23] In this study, the hot emulsion and ultrasonication method resulted in the formation of NLCs with an average particle size of < 300 nm.

PDI is a parameter that describes the degree of uniformity of the particle size distribution in sample. Preparation of a homogenous population of nanoformulation of a certain size is necessary for the development of a successful nanoformulation with stability, safety and efficiency. The excipients used in the preparation of nanoformulation play a key role in the development of homogenous nanocarriers. The PDI values varies between 0 to 1. Values less than 0.5 are considered as highly monodisperse, whereas PDI of more than 0.5 are considered as poly-disperse in nature.[24] In the current study, the average PDI for the batch I was found to be 0.547 ± 0.10 and for batch II it was found to be 0.460 ± 0.04.

Zeta potential reflects the electric charge on the surface of the particle and the colloidal stability of the formulation. Particles with a largely positive or negative charge (more positive than + 30 mV or more negative than -30 mV) will repel with each other resulting in the prevention of aggregates formation in the formulation. In the current study both the batches (batch I and batch II) were found to have zeta potential less than – 50 mV. The total drug content was found to be more than 85 % in both the batches. Particle size, PDI and zeta potential results are presented in Table 5 and distribution of size and zeta potential is presented in Figure 3.

Estimation of free and entrapped drug content in ritonavir loaded NLCs

Determination of amount of drug entrapped inside the lipid matrix is one of the very important parameters in the characterization of lipid based nanoformulations. Total drug content is the routine parameter evaluated in characterization of conventional formulations such as tablets, capsules. And the method for determination of total drug content is usually a simple procedure which involves extraction of compound of interest from the formulation followed by analysis using a suitable analytical technique. Whereas, entrapment efficiency determination involves separation of free and entrapped portions from the nanoformulations which is a critical and complex procedure in comparison to extraction. There are few indirect methods (ultracentrifugation, molecular weight cut-off filtration combined with centrifugation and dialysis) employed by the researchers for the determination of entrapment efficiency. These methods include determination of total drug content along with either free or entrapped portion of the drug from the formulation. But, in all these procedures all the three portions i.e., free drug, entrapped drug and total drug were not analyzed. Either free or entrapped portion was quantified for the calculation of entrapment efficiency.[25-28]

Lv *et al*., evaluated various methods such as centrifugation, ultrafiltration and gel permeation chromatography for determination of entrapment efficiency. It was reported that, centrifugation method was poor in separation of free and entrapped portions in case of nanoformulations with very low size (100 nm). Whereas, particle size with 200 nm it was effective but at a consumption of enormous energy. Similarly, Sephadex gel permeation chromatography was associated with low recoveries that can potentially affect the accuracy of the entrapment measurements. Ultrafiltration method was reported as comparatively better with minor leakage of nanocarriers during the separation of free and entrapped portion of the formulation.[29] Previously Ran *et al*., studied different methods of liposomal sample preparation for the entrapment efficiency determination. These

Table 5: Particle size, PDI and zeta potential results of ritonavir loaded NLCs.

S. No	Batch I			Batch II		
	Particle Size (nm)	PDI	Zeta Potential (mV)	Particle Size (nm)	PDI	Zeta Potential (mV)
1	226.9	0.453	-51.8	244.2	0.447	-61.0
2	233.5	0.484	-54	227.7	0.414	-57.2
3	233.2	0.478	-52	231.4	0.428	-55.7
4	308.4	0.676	-60.8	345.7	0.494	-51.8
5	295	0.664	-61.2	357.3	0.514	-53.6
6	305.8	0.526	-61.3	365.4	0.464	-52.1
Average ± SD	267.13 ± 39.69	0.547 ± 0.10	-56.85 ± 4.72	295.28 ± 67.17	0.460 ± 0.04	-55.23 ± 3.51

mV: millivolts; nm: nanometer; PDI: polydispersity index; SD: standard deviation

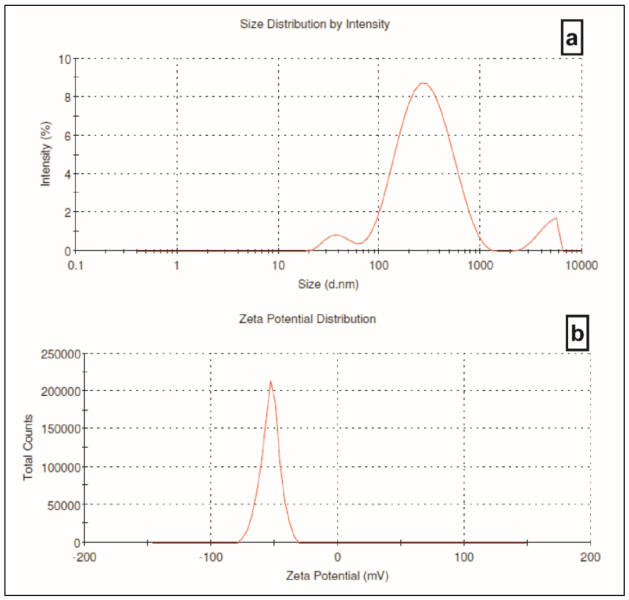

Figure 3: Particle size distribution (a) and zeta potential (b) graphs of ritonavir loaded NLCs.

methods include size exclusion chromatography using Sephadex, SPE, centrifugation ultrafiltration and hollow fiber centrifugal ultrafiltration.[30] Even though high entrapment efficiency (> 90 %) was achieved by Sephadex chromatography method, preparation of Sephadex column that involve 24 hr of swelling is a bit tedious and time consuming. Whereas, SPE cartridges are readily available with a variety of chemistries and there is a flexibility of sample volume also. Moreover, sample preparation is very fast with simple steps. After considering respective pros and cons of various methods, in the current study, SPE method was used for the separation of free and entrapped drug from NLCs. This method was conceptualized, based on the interaction between analyte of interest and the stationary phase present in the cartridge. Hence, selection of cartridge for the analysis depends on the physicochemical properties of compound/ analyte of interest. It is well known that ritonavir is a hydrophobic compound, therefore a reverse-phased cartridge is used for the analysis. The Van der Waals interaction that exists between the hydrophobic analyte and reverse-phase solid stationary phase. It was reported that Strata X cartridges are suitable for the extraction of ritonavir from human plasma. Considering the selectivity of ritonavir to this stationary phase Strata X was used in the current study for the separation of free and entrapped drugs from formulation.[31] During loading of formulation on SPE cartridge, free drug retains on sorbent because of this hydrophobic interaction. Whereas entrapped drug elutes out from the cartridge because of lack of interaction with the sorbent. Washing with water helps in the removal of unwanted matrix components that present on the bed. In the last step, elution of free drug was facilitated by passing methanol through the cartridge as a more lipophilic mobile phase. In this current study, drug entrapment was found to be more than 30 % of the total drug content. The data of percentage free and entrapped is presented in Table 6. From the data it is clear that the sum of free and entrapped portions of the formulation is close to the total drug content which evidences the reliability of the method for the determination of entrapment efficiency.

Accuracy and recovery of ritonavir in NLCs

The amount of ritonavir added to the blank NLCs was extracted using methanol. The accuracy of the samples was found to be more than 98 % at all the three levels. Ritonavir added to the pre-analyzed formulations was extracted using methanol and the percent recovery was found to be more than 90 % for both batch-I and batch-II. The data of accuracy and recovery is presented in Table 7.

Table 6: Total, free and entrapped drug content results of ritonavir loaded NLCs.

S. No	Trial No	Total drug content (%) (Average ± SD) (n=3)	Entrapped Drug (%) (Average ± SD) # (n=3)	Free drug (%) (Average ± SD) # (n=3)	Free + Entrapped drug (%) (Average ± SD) (n=3)
1	Trial 01	86.44 ± 5.76	31.05 ± 4.98	70.19 ± 8.33	101.24 ± 3.35
2	Trial 02	87.93 ± 3.12	30.19 ± 12.22	72.39 ± 5.54	102.58 ± 6.69

Calculated concerning total drug content; SD: standard deviation

Table 7: Accuracy and recovery results of ritonavir-loaded NLCs.

S. No	Batch No	Accuracy (%) (Average ± SD) (n=3)			Recovery (%) (Average ± SD) (n=3)
		75% (3375 µg/mL)	100 % (4500 µg/mL)	125 % (5625 µg/mL)	
01	Batch I	99.59 ± 0.33	98.68 ± 0.31	100.65 ± 0.11	93.46 ± 1.31
02	Batch II	106.90 ± 0.42	100.11 ± 0.19	104.75 ± 0.17	99.24 ± 1.77

µg; microgram; mL: milliliter; SD: standard deviation

Table 8: Recovery data of ritonavir from film-coated commercial oral formulations.

S. No	Trial Number	Percentage Recovery
1	Trial 1	98.40
2	Trial 2	97.53
3	Trial 3	97.28
4	Trial 4	97.28
5	Trial 5	97.65
6	Trial 6	97.33
Average ± SD		97.44 ± 0.15
% RSD		0.16

RSD: relative standard deviation; SD: standard deviation

Estimation of ritonavir from film-coated commercial pharmaceutical oral formulations

The recovery of ritonavir from film-coated commercial pharmaceutical oral formulations was found to be more than 97 % with a % RSD value of less than 1%. The results (presented in Table 8) were found within the acceptable limits of Indian Pharmacopeia for ritonavir.

CONCLUSION

A simple isocratic, accurate, sensitive, precise and robust reverse phased HPLC method was developed for the quantification of free, entrapped and total ritonavir in NLCs and drug content of the film-coated commercial formulation. The validation of the newly developed method was performed according to the guidelines of ICH Q2(R1). The results of validation parameters evidence that the method is sensitive for detection and quantification of ritonavir at low concentrations. The validation data also confirms that the method is linear (for a range of 0.25 to 16 µg/mL) and the % RSD, less than 2% indicates that the method is precise and robust. Ritonavir-loaded NLCs were prepared by hot emulsion-ultra centrifugation method and, a simple SPE method was developed for the separation of the free and entrapped drugs from NLCs. The total, free and entrapped drug contents of ritonavir-loaded NLCs were quantified using the validated HPLC method. Further, the percentage recovery (more than 97%) of ritonavir from film-coated commercial pharmaceutical formulations analyzed with the help of the validated RP-HPLC method indicates that the method can be used for the quantification of ritonavir from NLCs and also from commercial formulations. The developed RP-HPLC method will be helpful for researchers for the better routine qualitative and quantitative analysis of ritonavir in nano-based formulations and marketed formulations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

%: percentage; %; **°C:** degree Celsius; **µg:** microgram; **µL:** microliter; **µm:** micrometer; **ACN:** Acetonitrile; **AUC:** **BCS:** Biopharmaceutical classification system; **CD4+:** Cluster differentiation 4 positive; **Da:** Dalton; **HPLC:** High-performance liquid chromatography; **ICH:** International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; **L:** Liter; **mg:** milligram; **min:** minutes; **mL:** milliliter; **mm:** millimeter; **MP:** Mobile phase; **ng:** nanogram; **NTP:** Number of theoretical plates; **OPA:** Orthophosphoric acid; **RP HPLC:** reverse Phase High-performance liquid chromatography; **RT:** Retention time; **SD:** Standard deviation; **UV:** Ultraviolet; **v/v:** volume / volume.

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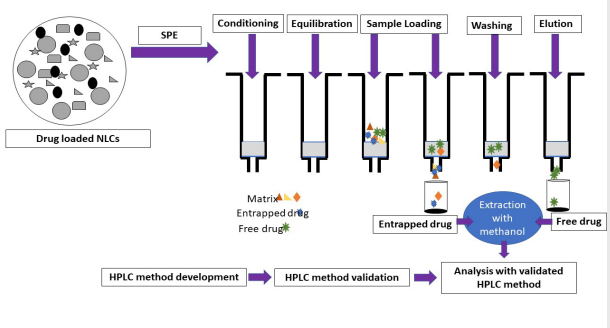
PICTORIAL ABSTRACT



SUMMARY

In the current study, HPLC analytical method was developed for the quantification of ritonavir in LNCs and also film-coated fixed-dose formulation available in the market. The analytical method was further validated according to the ICH guidelines for various parameters. A SPE method was developed for the separation of free and entrapped portions of ritonavir from LNCs. Finally, a developed method was applied for the quantification of ritonavir in LNCs and marketed film-coated formulation.

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