

ANALYTICAL METHODS FOR THE QUANTIFICATION OF RITONAVIR IN PHARMACEUTICALS, A COMPARATIVE EVALUATION

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(Received June 14, 2022; Revised July 14, 2022; Accepted July 15, 2022)

ABSTRACT. Ritonavir is an antiretroviral medication used to treat AIDS (Acquired Immune Deficiency Syndrome)/HIV (Human Immunodeficiency Virus). This article describes the development and validation of spectrophotometric and liquid chromatographic techniques for ritonavir quantification in pharmaceutical formulations. Liquid chromatographic analyses were carried out using a C18 column and a mobile phase consisted of 20 mM KH_2PO_4 (pH = 3.0) and acetonitrile (45:55, v/v), with a flow rate of 1.2 mL min^{-1} and UV detection at 235 nm. For the spectrophotometric analyses, ethanol was employed as a solvent. The UV spectra of the standard and sample solution were scanned between 200 and 800 nm, and λ_{max} was determined as 235 nm. Analytical techniques have been validated in accordance with the processes outlined in the ICH (International Council for Harmonization) recommendations. The results showed that the analytical procedures were linear, accurate, precise, and robust. The recovery values of the methods are within the standard norms (98–102%). Following that, a statistical comparison of analytical techniques was carried out, and the findings revealed no significant difference. Consequently, the developed analytical techniques might be used quality control analysis of ritonavir in pharmaceuticals.

KEY WORDS: Ritonavir, Analysis, Method, Validation, Spectrophotometer, HPLC

INTRODUCTION

As of March 07, 2022, WHO had documented 5,998,301 deaths and 445,096,612 cases of Covid-19 worldwide [1]. There is presently no antiviral or therapeutic treatment available to treat Covid-19. Covid-19 illness is uncontrolled and has become a severe worldwide concern due to its high fatality rates.

Infected people have been given off-label treatments [2–4]. A large variety of antivirals have been investigated in in vitro research as well as approved clinical trials. The majority of them are protease inhibitors from a biological perspective [5–9]. Protease is a virus-growing enzyme. Protease inhibitors can prevent these enzyme activities. These inhibitors bind selectively to viral proteases and block the proteolytic cleavage of protein precursors required to produce infectious viral particles. As a result, viral replication is prevented. Ritonavir, indinavir, amprenavir, nelfinavir, saquinavir atazanavir drugs are in this class. They are used in highly active anti-retroviral therapy. Among these drugs, ritonavir is used for the treatment of HIV/AIDS. It is often used alone or in combination with other anti-retroviral drug [10]. The chemical properties of ritonavir have been presented in Table 1.

The ritonavir monograph is officially available in both the United States Pharmacopoeia and the British Pharmacopoeia describing the high-pressure liquid chromatography (HPLC) technique for the quantification of ritonavir in bulk powder [11, 12].

According to a literature review, various spectrophotometric methods for determination of ritonavir in pharmaceutical formulations have been developed [13–15]. In the literature, several HPLC methods for determination of ritonavir have been published [16–18]. Some of these are for the simultaneous detection of antiretrovirals in human plasma by reversed phase high performance liquid chromatography methods [19–22], two high performance liquid chromatography methods

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[23] and one high performance thin layer chromatography method [24] for determining ritonavir in pharmaceutical formulations were published in the literature.

However, some of these techniques were quite complex and had limitations such as sample preparation, gradient elution, and long run time. These procedures also require the use of expensive equipment, specialized reagents, and substantial volumes of organic solvents.

The aim of this study is to develop analytical methods that are simple, quick, inexpensive, and well-validated for the quantification of ritonavir in pharmaceutical formulations using LC chromatographic and UV spectrophotometric techniques. In addition, these methods to be developed will be less time-consuming and cheaper than other published methods. The validation of the developed analytical methods was carried out according to the procedures described in ICH guidelines Q2(R1) for the validation of analytical methods [25, 26]. Furthermore, the applicability and reliability and of these methods have been assessed by concentrating on routine quality control analyses. The results obtained from analytical methods were compared statistically using the least-squares method. This is the first study to statistically compare two different analytical methods developed for the quantification of ritonavir in pharmaceutical preparations.

However, no studies on a comparative comparison of spectrophotometric and liquid chromatographic techniques for ritonavir quantification have been published. The purpose of this research is to develop analytical methods that are simple, quick, cheap, and well-validated for the quantification of ritonavir in pharmaceutical formulations using LC chromatographic and UV spectrophotometric techniques. In addition, these methods to be developed will be less time consuming and cheaper than other published methods. This is the first study to statistically compare two different analytical methods developed for the quantification of ritonavir in pharmaceutical preparations.

EXPERIMENTAL

Instruments

An Agilent 1260 HPLC system consisting of Chemstation software, and UV-Vis detector was used during method development and validation in chromatographic analysis. Shimadzu UV-1800 spectrophotometer consisting of UV-Probe software and a double beam using 1.0 cm quartz cells was used during spectrophotometric analysis.

Chemicals

Milli-Q water purification system (Millipore) was used to produce HPLC grade water. Pure ritonavir and Norvir (100 mg) were supplied from Abbvie Pharmaceuticals. (Istanbul, Turkey). All solvents used in the study were of HPLC grade and were obtained from Merck (Germany).

Standard solutions

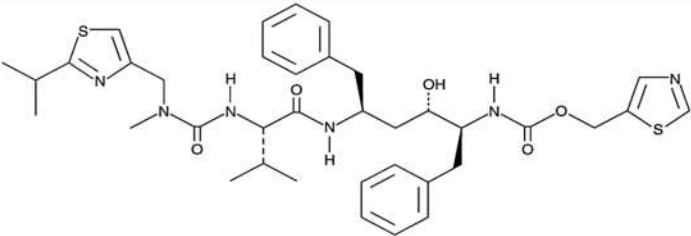
An appropriate amount of ritonavir was dissolved in ethanol and a stock standard solution ($500 \mu\text{g mL}^{-1}$) was prepared. Six working standard solutions in the concentration range of $10\text{--}60 \mu\text{g mL}^{-1}$ were prepared by serial dilutions of the stock standard solution with ethanol. These standard solutions were then filtered using 0.22 micron syringe filters.

Sample solutions

A total of 10 tablets were weighed and ground into fine powder by grinding in a mortar. The powder equivalent to 25 mg of ritonavir was weighed accurately, taken into a 50 mL of volumetric flask. 20 mL of ethanol was added to dissolve ritonavir, the volume was diluted to 50 mL with ethanol when dissolution was complete. Finally, ethanol was added up to the marked line, and

shaked homogenously for 15 min. This supernatant was filtered through membrane filter (0.22 μm pore size). 1 mL of this solution was taken and transferred to a 10 mL of volumetric flask. The volume was made up with ethanol and a ritonavir solution ($50 \mu\text{g mL}^{-1}$) was obtained.

Table 1. Chemical properties of ritonavir.

Molecular formula	$\text{C}_{37}\text{H}_{48}\text{N}_6\text{O}_5\text{S}_2$
Molecular weight	720.9 g mol^{-1}
IUPAC name	1,3-Thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl) methyl] carbamoyl] amino] butanoyl] amino]-1,6-diphenylhexan-2-yl] carbamate
log P	2.9
pK _a	3.8; 6.2
Solubility	It is soluble in methanol, ethanol, and isopropanol, but practically insoluble in water
Chemical structure	

Determination of λ_{max}

Using ethanol, the spectrophotometer was first calibrated to zero. Standard ritonavir solution at a concentration of $30 \mu\text{g mL}^{-1}$ was scanned between 200 and 800 nm wavelengths in a spectrophotometer device to determine the wavelength at which it absorbed the maximum.

Calibration curves

The standard solutions (20 μL) were injected into the liquid chromatographic system. It was determined that the peak areas obtained with the concentrations of the standard solutions were proportional. A calibration curve was created by plotting the concentration against the peak area for the chromatographic method, Slope, intercept and correlation coefficient were calculated from this calibration curve. These standard solutions were scanned in the wavelength range of 200 - 800 nm in a spectrophotometer and λ_{max} was determined as 235 nm. The absorption values of each standard solution at a wavelength of 235 nm were determined. It was determined that the corresponding absorbance values were proportional to the concentrations of the standard solutions. A calibration curve has been developed by plotting the concentration against the absorbance for the spectrophotometric method, Slope, intercept and correlation coefficient were calculated from this calibration curve.

Assay of ritonavir in tablets

Three sample solutions prepared were injected into the HPLC system (20 μL). It was analyzed using the developed method, the peak areas were determined, and the ritonavir contents in the tablet were calculated.

Analysis method conditions

An Agilent brand 1260 model HPLC apparatus with a UV-Vis detector was used for chromatographic analyses. The mobile phase was a 20 mM KH₂PO₄ solution (pH 3.0, with orthophosphoric acid) at a flow rate of 1.2 mL min⁻¹. The mobile phase was filtered by passing through a 0.22 µm membrane filter and degassed in an ultrasonic bath before use. The column was ODS (4.6 mm x 250 mm, 3.0 µm particle size) and stored at 25 °C. Ritonavir detection was performed at 235 nm with a UV detector. The run time was 15 minutes under these conditions. All absorbance measurements were taken with a dual-beam spectrophotometer at 235 nm.

Analytical method validation

Analytical techniques were validated according to the procedures specified in the ICH guideline Q2(R1) [25,26]. All studies such as accuracy, linearity, sensitivity, precision, robustness, specificity, system suitability were investigated as validation parameters. Six working standard solutions (10 - 60 g mL⁻¹) were prepared by serial dilutions of the stock solution with ethanol. These standard solutions were then filtered using 0.22 micron syringe filters. Then, 20 µl of each standard solution was injected into the liquid chromatographic system three times for validation of the chromatographic method. Ritonavir's peak areas and retention times were determined and recorded. The calibration curve was created by graphing the peak area against the concentration, and its linearity was assessed using regression analysis. The correlation coefficient, slope, and intercept were all calculated. Each analysis was carried out in triplicate. The absorbances of standard solutions at a wavelength of 235 nm were measured on a spectrophotometer. Analyzes were performed in triplicate.

The standard addition method was used to determine the methods's accuracy. A known amount of standard was added to the previously tested sample solution at three different levels (80, 100, and 120 %). The developed methods were used to examine all of the samples. The RSD and recovery for each concentration were determined. Absorbance values was measured directly for UV spectrophotometric technique by comparing the estimated and observed concentrations, the recovery percent was computed.

The precision of the analytical methods was evaluated by intra-day repeatability and inter-day repeatability. Standard solutions at the concentrations of 40, 50, and 60 µg mL⁻¹ were examined three times in the same day in intraday precision studies and standard solutions of the same concentration were evaluated three times on three consecutive days for inter-day precision experiments. The results of precision studies were presented as a percentage of RSD.

The sensitivity of analytical techniques was evaluated using the limits of detection (LOD) and the limits of quantitation (LOQ). To calculate the LOD and LOQ from the calibration curve, we utilized the following formulas.

$$\text{LOD} = 3.3 \times \sigma \text{ S}^{-1} \qquad \text{LOQ} = 10 \times \sigma \text{ S}^{-1}$$

where, σ indicates the standard deviation of the calibration curve's intercept and S indicates the calibration curve's slope.

Peak purity was determined by comparing the chromatograms at the peak's beginning, pinnacle, and end for specificity in the HPLC method.

The analytical techniques' robustness was evaluated by looking at the effects of tiny but purposeful of parameter modifications on results. The same sample was examined under varied circumstances, such as variations in flow rate of the mobile phase (0.1 mL min⁻¹) and detector wavelengths (2 nm), to test the robustness of the chromatographic method, and the influence has been detected on the system suitability characteristics. Standard solution (30 µg mL⁻¹) was injected six times for system suitability testing. The peak area, theoretical plate number, tailing factor, capacity factor, and asymmetry factor were all determined.

The standard solution was kept at room temperature for up to two days and refrigerated (4 °C) for up to five days for stability experiments. The developed methods were then used to analyze these solutions.

Comparative analysis

Analytical techniques were found to be adequate for determining the quantity of ritonavir in pharmaceutical formulations after validation. Statistically, the recovery percentages of analytical techniques used on commercial medications were compared. For comparison, the F- and t-tests were utilized.

RESULTS AND DISCUSSION

The validation of analytical methods was carried out in accordance with the methods outlined in the ICH guidelines.

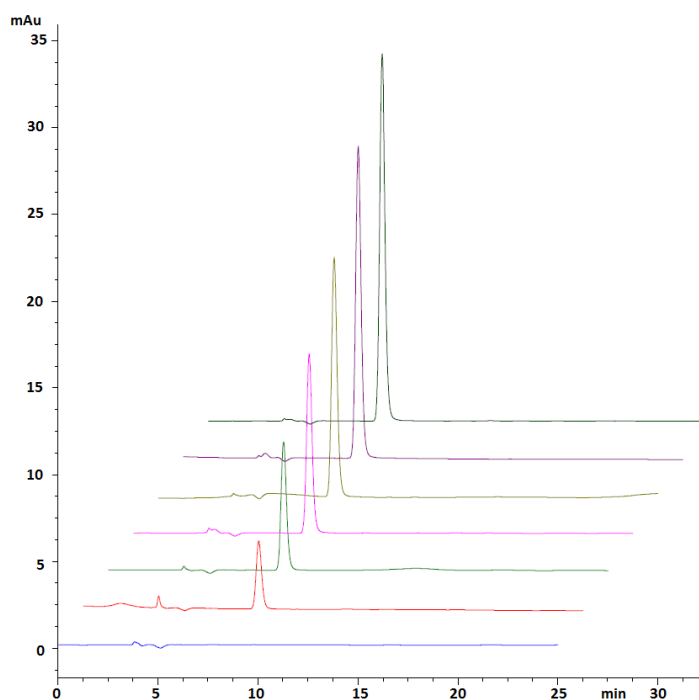


Figure 1. Overlapping chromatograms of standard solutions for liquid chromatographic method.

HPLC method

Using the peak shape and analysis time, the chemical composition of the mobile phase and flow rate were determined. Many mobile phases were tried. Finally, it was established that 20 mM KH_2PO_4 (pH: 3) was the optimal mobile phase for short run duration and symmetrical peak point. Figure 1 depicts the overlap chromatograms of the standard solutions, whereas Figure 2 depicts the chromatographic method's linearity plot. Ritonavir had a retention time of 10.00 min. The

developed methods have been validated following ICH guidelines for accuracy, linearity, precision, specificity, system suitability, robustness, and sensitivity (LOD and LOQ). Since the peak purity index of ritonavir was determined as 0.9998, the method was determined to be specific. According to linear regression analysis, peak area and analyte concentration show a very good correlation

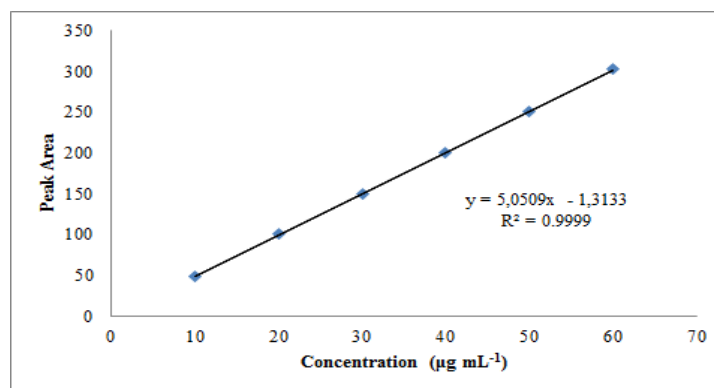


Figure 2. Linearity graph for liquid chromatographic method.

The calibration curve had a correlation coefficient (r^2) of 0.9998 ± 0.0006 and was linear over the concentration range of 10 to $60 \mu\text{g mL}^{-1}$. Linear regression equation was $y = 5.0509x - 1.3133$. The detection and quantification limits were determined as $0.90 \mu\text{g mL}^{-1}$ and $2.70 \mu\text{g mL}^{-1}$, respectively, and these values indicated the sensitivity of the method.

The results and statistical evaluations presented in Table 1 show the high accuracy and precision of the method. Low relative standard deviation (RSD) values were obtained from the recovery experiments and showed good accuracy. The RSD for intraday and interday repeatability studies as recommended in ICH guidelines. Values were determined as < 2 . The RSD values for intraday repeatability and interday repeatability ranged from 0.0940 to 0.1515 and 0.1840 to 0.2615 , respectively. This showed that the developed liquid chromatographic method is precise. Regression and validation parameters for analytical methods are presented in Table 2.

Table 2. The parameters for validation and regression ($n = 6$).

Parameters (validation and regression)	Liquid chromatographic technique	UV-Vis spectrophotometric technique
Range of concentrations ($\mu\text{g mL}^{-1}$)	10–60	10–60
Detection and quantification limits ($\mu\text{g mL}^{-1}$)	0.90/2.70	1.0/3.0
Slope/standard error of slope	$0.0509 \pm 0.0006 / 0.5638$	$0.0121 \pm 0.0005 / 0.0015$
Intercept/standard error of intercept	$0.3133 \pm 0.0042 / 0.5430$	$0.0073 \pm 0.0004 / 0.0015$
Correlation coefficient	0.9999 ± 0.0006	0.9995 ± 0.0009
Standard deviation (residuals)	0.37	1.27
Recovery (%)	100.06 ± 0.37	100.22 ± 0.21
Precision	Intra-day (RSD)	$0.1021 - 0.1638$
	Inter-day (RSD)	$0.1935 - 0.22724$

Minor modifications made intentionally in the experimental conditions did not make a statistically significant difference on the system suitability parameters. RSD values for each method parameter were calculated and determined to be < 2 .

System suitability tests demonstrated the suitability of the proposed liquid chromatographic method for routine quality control analysis. The capacity factor was determined as 9.85. The tailing factor was set at 1.155, which indicates strong peak symmetry. The theoretical plate number was determined as 6542, this value indicates high level of column efficiency. The results of the system suitability tests show that the devised technique fits these requirements within acceptable limits. Standard solutions are stable at room temperature, according to solution stability studies.

UV-Vis spectrophotometric method

The spectrophotometric technique was validated in accordance with ICH standards. Standard solutions were scanned in the spectrophotometer in the wavelength range of 200–800 nm. It was observed that ritonavir had maximum absorption at 260 nm wavelength. The overlapping spectrum of the standard solutions is presented in Figure 3. A graph of absorbance values against concentration values of standard solutions was created. The linearity graph of the standard solutions is presented in Figure 4. Standard solutions demonstrated linear relationship in the range of 10 to 60 $\mu\text{g mL}^{-1}$. Correlation coefficient (r^2) was 0.9995 ± 0.0009 . The regression equation was found to be $y = 0.0122x - 0.0043$. In the recovery studies, the standard addition technique was used for the three concentrations and acceptable RSD, ranging from 99.20% to 100.98%. Detection and quantification limits were determined as $1.00 \mu\text{g mL}^{-1}$ and $3.00 \mu\text{g mL}^{-1}$, respectively.

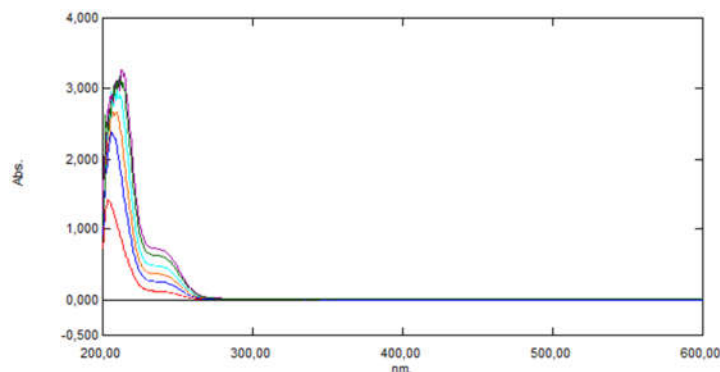


Figure 3. Overlap spectrums for UV-Vis spectrophotometric method.

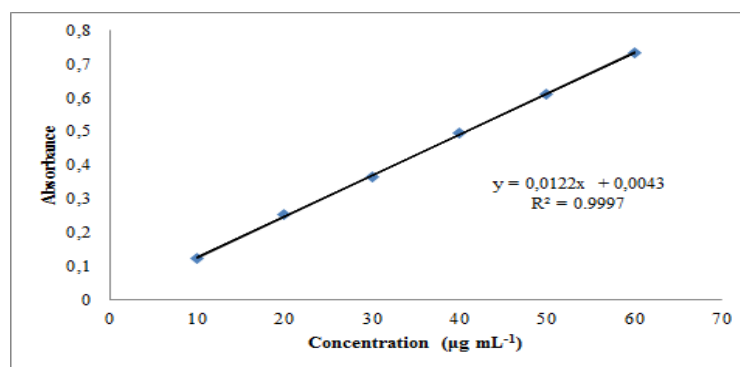


Figure 4. Linearity graphs for spectrophotometric method.

The spectrophotometric approach devised allowed for the quick measurement of ritonavir in pharmaceutical formulations. Ethanol was chosen as the solvent due to its excellent solubility and stability. This solvent also has good spectrum characteristics. Low RSD results imply that the procedure is precise. It was observed that the method was highly accurate and the average recovery values approached 100%. In addition, the small values of LOD and LOQ show that the method is quite sensitive.

Analysis of pharmaceutical formulations by analytical methods developed

Analytical methodologies established were used to commercial pharmaceutical formulations. Table 3 shows the findings of the examination of ritonavir-containing pills. The study yielded values that were extremely close to the levels stated on the labels.

Table 3. Analysis results of pharmaceutical formulations.

Commercial formulation	Label claim (mg/tablet)	Spectrophotometric technique		Chromatographic technique	
		Found (mg in tablet)	Assay % \pm SD	Found Ritonavir (mg in tablet)	Assay % \pm SD
Norvir	100	99.97	99.97 \pm 1.27	100.04	100.04 \pm 0.33

Statistical analysis

The F-test and the t-test were used to compare analytical methods statistically. The statistical tests revealed that the experimental data obtained from the analytical methods were not statistically different. It was determined that the calculated F and t values were lower than the table values of the analytical methods with a 95% confidence interval. The analytical methods presented in this study proved to be amenable to the accurate measurement of ritonavir in pharmaceutical formulations. Table 4 displays data on the statistical comparison outcomes of analytical methodologies.

In this investigation, two independent analytical techniques were devised to determine the amount of ritonavir in pharmaceuticals: chromatographic and spectrophotometric methods. The conditions of the developed analytical methods have been optimized and validated. Several analytical methods have been reported in the literature to determine the amount of ritonavir in pharmaceutical products.

Table 4. Statistical comparison results of analytical methods.

Statistical parameter, A = 0.05, Confidence interval: 95%, n = 6	Liquid chromatographic method	UV-Vis spectrophotometric method
Average Assay	100.04	99.97
SD	0.33	1.27
RSD	0.33	1.27
Standard error	0.5638	0.0038
F-test: $F_{\text{calculation}}/F_{\text{table}}$	0.17/0.23	
t-test: $t_{\text{calculation}}/t_{\text{table}}$	1.92/2.71	

Some of these techniques are quite complex, requiring long analysis times, expensive apparatus, special reagents, and large amounts of organic solvents. In addition, the spectrofluorimetric and spectrophotometric methods that have been published in the literature require complex and time-consuming sample preparation. No research has yet been conducted in

which two different analytical methods are created and compared statistically in any of these studies. Therefore, our study is the first study in this direction. The characteristics of the previously reported HPLC methods and the proposed method are presented in Table 5.

Table 5. Characteristics of previously reported HPLC methods and the proposed method.

Mobile phase	Column	Concentration range ($\mu\text{M L}^{-1}$)	Correlation coefficient (R^2)	Precision (RSD)	LOD/LOQ in mg mL^{-1}	Retention time (min)	Application	Ref
A mobile phase consisting of methanol and water (67:33, v/v)	A reversed-phase C ₈ (125 × 4.0 mm i.d., 5 μm particle size)	100–300	0.9990	Intraday: 0.64 Interday: 0.86	0.64 0.95	7.80	Bulk and pharmaceutical formulations	[16]
The mobile phases consisted of acetonitrile, phosphate buffer pH 4.0 and water: A (35:28:37 v/v/v) and B (70:28:2 v/v/v)	A Hypersil BDS C18 column (25 cm × 4.6 mm, 5 μm)	0.05–125	0.9990	Intraday: 1.93 Interday: 2.96	0.394 1.194	21.00	Bulk and pharmaceutical dosage forms	[17]
A mixture of acetonitrile and water (41:59, v/v)	Xterra C8 (150 × 3.9 mm, 5 μm) column	0.187–10.0	0.9989	Intraday: 12.50 Interday: 16.50	0.10 0.19	14.07	Human plasma	[18]
A mobile phase consisted of 20 mM KH ₂ PO ₄ (pH: 3.0) and acetonitrile (45:55, v/v)	ODS C ₁₈ (250 mm - 4.6 mm, particle size 5 μm)	10–60	0.9998	Intraday: 0.10 Interday: 0.19	0.90 2.70	10.00	Bulk and pharmaceutical dosage forms	Proposed method

CONCLUSION

Chemicals that are detrimental to the environment and human health are avoided as much as feasible in the spectrophotometric and chromatographic procedures described in this study. The gadgets employed in the new procedures are no more expensive than those used in published investigations. Both analytical approaches are cost-effective. The results show that these methods are appropriate for estimating the quantity of ritonavir in pharmaceutical formulations. The devised spectrophotometric approach has no complicated processes. It is less expensive and requires less time. These are the benefits of the spectrophotometric method we created above the chromatographic approach we just devised. When statistically contrasted, the chromatographic approach is more accurate and exact than the spectrophotometric method. Excipients in pharmaceutical formulations had no effect on the results of either technique of analysis. Finally, because the proposed procedures are simple, specific, quick, accurate, and precise, they may be successfully used for ritonavir measurement in pharmaceutical formulations.

ACKNOWLEDGEMENTS

The author would like to thank Abbvie Pharmaceuticals for providing the ritonavir reference compounds and Norvir tablet.

REFERENCES

1. World Health Organization (WHO), Coronavirus Disease (COVID-19) Dashboard: Situation Report, 24 September; **2021**.
2. Madrid, P.B.; Panchal, R.G.; Warren, T.K.; Shurtleff, A.C.; Endsley, A.N.; Green, C.E.; Kolokoltsov, A.; Davey, R.; Manger, I.D.; Gilfillan, L.; Bavari, S.; Tanga, M.J. Evaluation of Ebola virus inhibitors for drug repurposing. *ACS Infect. Dis.* **2016**, *1*, 317–326.
3. Tanenbaum, L.; Tuffanelli, D.L. Antimalarial agents: Chloroquine, hydroxychloroquine, and quinacrine. *Arch. Dermatol.* **1980**, *116*, 587–591.
4. Markham, A. Baricitinib: First global approval. *Drugs* **2017**, *77*, 697–704.
5. Cao, B.; Wang, Y.; Wen, D.; Liu, W.; Wang, J.; Fan, G.; Ruan, L.; Song, B.; Cai, Y.; Wei, M.; Li, X.; Xia, J.; Chen, N.; Xiang, J.; Yu, T.; Bai, T.; Xie, X.; Zhang, L.; Li, C.; Yuan, Y.; Chen, H.; Li, H.; Huang, H.; Tu, S.; Gong, F.; Liu, Y.; Wei, Y.; Dong, C.; Zhou, F.; Gu, X.; Xu, J.; Liu, Z.; Zhang, Y.; Li, H.; Shang, L.; Wang, K.; Li, K.; Zhou, X.; Dong, X.; Qu, Z.; Lu, S.; Hu, X.; Ruan, S.; Luo, S.; Wu, J.; Peng, L.; Cheng, F.; Pan, L.; Zou, J.; Jia, C.; Wang, J.; Liu, X.; Wang, S.; Wu, X.; Ge, Q.; He, J.; Zhan, H.; Qiu, F.; Guo, L.; Huang, C.; Jia, T.; Hayden, F.G.; Horby, P.W.; Zhang, D.; Wang, C. A trial of lopinavir-ritonavir in adults hospitalized with severe Covid-19. *New Engl. J. Med.* **2020**, *382*, 1787–1799.
6. Ford, N.; Vitoria, M.; Rangaraj, A.; Norris, S.L.; Calmy, A.; Doherty, M. Systematic review of the efficacy and safety of antiretroviral drugs against SARS, MERS or COVID-19: Initial assessment. *J. Int. AIDS Soc.* **2020**, *23*, 1–9.
7. Costanzo, M.; De Giglio, M.A.; Roviello, G.N. SARS CoV-2: Recent reports on antiviral therapies based on lopinavir/ritonavir, darunavir/umifenovir, hydroxychloroquine, remdesivir, favipiravir and other drugs for the treatment of the new coronavirus. *Curr. Med. Chem.* **2020**, *27*, 1–6.
8. Agrawal, S.; Goel, A.D.; Gupta, N. Emerging prophylaxis strategies against COVID-19. *Monaldi Arch. Chest Dis.* **2020**, *90*, 169–172.
9. Farag, A.B.; Wang, P.; Ahmed, M.S.; Sadek, H.A.; Haaham, S. Identification of FDA approved drugs targeting COVID-19 virus by structure-based drug repositioning. *Chemrxiv. Org.* **2020**, 1061–1069.
10. Nalawade, D.; Godge, R.K.; Magar, S.D. Analytical method development and validation of ritonavir: A review. *Res. J. Sci. Technol.* **2020**, *12*, 157–162.
11. Rockville, M.D. *The United States Pharmacopeia*, 31th ed., The official monograph: The United States Pharmacopeial Convention; **2011**; pp. 1186–1187.
12. *The British Pharmacopoeia*, Her Majesty's Stationery Office: London; **2010**; pp. 46–48.
13. Nagulwar, V.P.; Bhusari, K.P. Development of UV spectrophotometric first order derivative method for the simultaneous estimation of ritonavir and lopinavir in combined tablet dosage form. *Int. J. Pharm. Sci. Res.* **2012**, *3*, 2317–2320.
14. Nagulwar, V.P.; Bhusari, K.P. Simultaneous estimation of ritonavir and lopinavir by absorption ratio (Q-analysis) UV spectrophotometric method in combined tablet dosage form. *Der. Pharm. Lett.* **2010**, *2*, 196–200.
15. Dias, C.L.; Bergold, A.M.; Eduardo, P. UV-derivative spectrophotometric determination of ritonavir capsules and comparison with LC method. *Anal. Lett.* **2009**, *42*, 1900–1910.
16. Yekkala, R.S.; Ashenafi, D.; Mariën, I.; Xin, H.; Haghedooren, E.; Hoogmartens, J.; Adams, E. Evaluation of an international pharmacopeia method for the analysis of ritonavir by liquid chromatography. *J. Pharm. Biomed. Anal.* **2008**, *48*, 1050–1054.

17. Dias, C.L.; Rossi, R.C.; Donato, E.M.; Bergold, A.M.; Fröhlich, P. E. LC Determination of ritonavir, a HIV protease inhibitor, in soft gelatin capsules. *Chromatographia* **2005**, *62*, 589–593.
18. Faux, J.; Venisse, N.; Olivier, J.C.; Bouquet, S. Rapid high-performance liquid chromatography determination of lopinavir, a novel HIV-1 protease inhibitor, in human plasma. *Chromatographia* **2002**, *54*, 469–473.
19. Wang, P.G.; Wei, J.S.; Kim, G.; Chang, M.; El-Shourbagy, T. Validation and application of a high-performance liquid chromatography-tandem mass spectrometric method for simultaneous quantification of lopinavir and ritonavir in human plasma using semi-automated 96-well liquid-liquid extraction. *J. Chromatogr. A* **2006**, *1130*, 302–307.
20. Rentsch, K.M. Sensitive and specific determination of eight antiretroviral agents in plasma by high-performance liquid chromatography-mass spectrometry. *J. Chromatogr. B* **2003**, *788*, 339–350.
21. Gangl, E.; Utkin, I.; Gerber, N.; Vouros, P. Structural elucidation of metabolites of ritonavir and indinavir by liquid chromatography-mass spectrometry. Structural elucidation of metabolites of ritonavir and indinavir by liquid chromatography-mass spectrometry. *J. Chromatogr. A* **2002**, *974*, 91–101.
22. Usami, Y.; Oki, T.; Nakai, M.; Sagisaka, M.; Kaneda, T. A simple HPLC method for simultaneous determination of lopinavir, ritonavir and efavirenz in plasma. *Chem. Pharm. Bull.* **2003**, *51*, 715–718.
23. Donato, E.M.; Dias, C.L.; Rossi, R.C.; Valente, R.S.; Fröhlich, P.E.; Bergold, A.M. LC method for studies on the stability of lopinavir and ritonavir in soft gelatin capsules. *Chromatographia* **2006**, *63*, 437–443.
24. Sulebhavikar, A.V.; Pawar, U.D.; Mangoankar, K.V.; Prabhu-Navelkar, N.D. HPTLC method for simultaneous determination of lopinavir and ritonavir in capsule dosage form. *E-J. Chem.* **2008**, *5*, 706–712.
25. Guideline, ICH Harmonised Tripartite. Validation of analytical procedures: Text and methodology. Q2 (R1) 1.20. **2005**, Q2 (R1), 1(20), 05.
26. FDA, Guidance R. Validation of chromatographic methods. Center for drug evaluation and research (CDER). *Food and Drug Administration*, **1994**, 2.