Original Research Article

Analytical methods for the quantification of Ribavirin in pharmaceutical preparations, A comparative study

ABSTRACT

An efficient therapeutic drug or antiviral agent to treat Covid-19 is still not available. But, previously licensed pharmaceuticals to treat other virus infections are used on an off-label basis either alone or in combination. Ribavirin is one of them. It is a broad-spectrum antiviral drug and is used to treat the hepatitis C virus. This article explains the development and assessment of high-pressure liquid chromatographic and UV-Vis spectrophotometric methods for the quantification of ribavirin in pharmaceutical preparations. HPLC analyzes were performed using a C18 column and a mobile phase composed of 20 mM KH_2PO_4, with a flow rate of 0.8 mL min-1 and UV detection at 207 nm. For the spectrophotometric analyzes, ultra-pure water was used as a solvent. UV spectrum of standard and sample solution were recorded between 200 and 400 nm and ribavirin was detected using a wavelength of 207 nm. Both methods have been validated according to the procedures described in ICH guidelines Q2(R1) for the validation of analytical methods. The results showed that both spectrophotometric and liquid chromatographic methods were linear, precise, accurate, rugged, and robust with RSD values less than 1.00%, and the recovery percentage was within standard limits (98-102%). Then a statistical comparison of these two analytical methods was performed, and the results of both methods showed no significant difference. It was found that both methods were not statistically significant concerning each other in the 95% confidence interval ($p<0.05$). As a result, the developed analytical methods were determined to be accurate, highly effective, reliable, fast, simple, and could be used for routine quality control analysis of ribavirin in pharmaceutical formulations.

Keywords: Ribavirin, HPLC, UV, analysis, method, validation

1. INTRODUCTION

Globally, as of 24 September 2021, WHO has reported 4.724.876 deaths and 230.418.451 cases of COVID-19 [1]. It, a novel coronavirus-related pneumonia, has become a public health disaster on a global scale [2]. Confirmed antiviral treatment has not been discovered for COVID-19 so far [3,4]. Ribavirin (RBVN) is a nucleoside analog that has been synthesized. The molecular structure of RBVN is presented in Figure 1. It is an antiviral drug that is active against both DNA and RNA viruses [5]. It was used for antiviral therapy, taking into account its broad-spectrum antiviral activity, during the outbreak of severe acute respiratory syndrome in 2003 and the Middle East respiratory syndrome in 2012 [6–8].

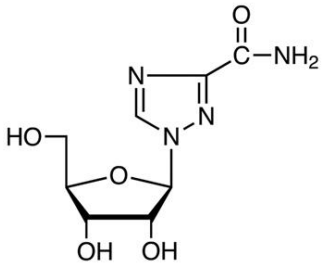

Figure 1: The molecular structure of RBVN

Based on prior experience and justification, RBVN has also been utilized in clinical practice in COVID-19. Clinical trials have been conducted to determine its efficacy in the treatment of individuals with severe COVID-19 pneumonia. RBVN treatment has been shown to decrease mortality [9]. The ribavirin 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 ribavirin in bulk powder and inhalation solutions[10, 11]. According to a literature review, various spectrophotometric methods for the determination of RBVN in pharmaceutical preparations have been developed [12-15]. Other techniques have been described, including capillary electrophoresis (CE) [16], high-performance liquid chromatography (HPLC-UV) [17-20], polarimetry [21], infrared (IR) [22], and flow injection chemiluminescence [23], as well as thin-layer chromatography (TLC), which was put into practice after various stress conditions were applied to the drug[24]. There are several articles on the detection of RBVN in biological fluids; the methodologies used herein include liquid chromatography-tandem mass spectrometry [25], solid-phase extraction and HPLC [26], reverse phase HPLC [27-28], and radioimmunoassay [29]. An LC-MS–MS technique was used to assess RBVN and viramidine in human plasma at the same time [30].
This study aims to develop analytical methods that are simple, quick, inexpensive, and well-validated for the quantification of RBVN in pharmaceutical formulations using LC chromatographic and UV spectrophotometric techniques. 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 [31,32]. The results obtained from these analytical methods were compared statistically using the least-squares method. Furthermore, the applicability and reliability of these methods have been assessed by concentrating on routine quality control analyses.

2. MATERIAL AND METHODS

Milli-Q (Merck KGaA, Darmstadt, Germany) water treatment system was used to obtain ultra-pure water. RBVN pure grade and commercial pharmaceutical Viron (200 mg per tablet) were supplied from Arven Pharmaceutıcal Industry and Trade Inc. (Istanbul, Turkey). All solvents used in the study were purchased from Merck (Darmstadt, Germany) and were of HPLC grade. Potassium dihydrogen phosphate (99.99%, anhydrous basis, suprapur) and Phosphoric acid (85%, suitable for HPLC, LiChropur) were purchased from Merck (Darmstadt, Germany). Membrane filters (0.45 µm pore size) used for filtration were obtained from Millipore (Massachusetts, USA).

Instrumentation specifications
A Shimadzu 1800 Double beam UV-Vis spectrophotometer, with UV-Probe software and 1.0-cm quartz cells, was used for UV spectrophotometric analyses. The quantification of RBVN was carried out at the wavelength of 207 nm and the measurements were taken against ultrapure water as a blank.

An Agilent 1260 LC system composed of a quaternary pump, autosampler, UV detector, and Chemstation software, was used for HPLC analysis. The mobile phase consisted of 20 mM KH_2PO_4 solution with a pH of 7.5 at a flow rate of 0.8 mL min-1 and the Agilent C18 (250 mm × 4.6 mm i.d., 5 µm particle size) column was used. RBVN detection was performed at the wavelength of 207 nm.

Standard solutions preparation
To prepare a stock-standard solution, 50 mg of pure grade drug was weighed precisely and transferred to a 100 mL volumetric flask. 80 mL of ultrapure water was added and sonicated for 5-10 minutes. Finally, the volume was made up of ultrapure water and filtered through membrane filters (0.45 µm pore size). Six working standard solutions were prepared at the concentration range of 10-60 µg mL-1 by using ultrapure water to dilute the stock standard solution. These working standard solutions were scanned in the range of 200-400 nm on the UV spectrophotometer to determine the value of λmax. The absorbance values of the standard solution series at the wavelength λmax were recorded and it was shown that the absorbance values were proportional to the concentration of standard solutions. The same standard solutions were used for HPLC analyses. 20 µL of each standard solution were injected into the HPLC system. Regression analysis was performed using the least-squares technique with the data obtained after the calibration curve was drawn with the peak area versus their standard concentration.

Sample solution preparation
A total of 20 tablets were weighed and finely powdered. The powder equivalent to 50 mg of RBVN was weighed accurately, taken into a 1000 mL of volumetric flask. Finally, ultra-pure water was added up to the marked line and shaked homogenously for 15 minutes. This supernatant was filtered through a membrane filter (0.45 µm pore size). This solution contains RBVN of 50 µg mL-1 and is ready to analyze by UV spectrophotometer or HPLC.

Method validation
Both analytical methods have been validated according to the procedures described in ICH guidelines Q2(R1) [31,32]. Validation parameters (Linearity, accuracy, precision, sensitivity, specificity, robustness, system suitability tests, stability studies) have been investigated.

Selectivity
For the UV method, standard and blank solutions were scanned at the wavelength of 200-400 nm. The blank solution was ultrapure water. The wavelength at which RBVN absorbs maximum was determined. It was determined that absorbance values at λmax changed linearly with standard solution concentrations. For the HPLC method, according to the requirements of the selectivity parameter, the operational conditions are selected as follows:
Theoretical plate number (N) >2000
Tailing factor (RBVN peak) ≤ 2.
Selectivity requirements were met by changing operating conditions such as mobile phase composition, concentration, and flow rate. The concentration of the KH_2PO_4 solution in the mobile phase (15-25 µM), the mobile phase flow rate (0.7-0.9) mL min-1, and the pH of the mobile phase (7-8) were changed at the specified ranges. RBVN standard solution was injected 6 times under the same operating condition to determine the precision of the

Comment [SR1]: 190 nm

instrument. The value of the relative standard deviation of the percentage of the RBVN for the peak area and the retention time was calculated. These values should not be more than 2.0% [2].

Linearity

Stock standard solutions (500 µg mL-1) were prepared in triplicate. Each of these stock solutions was diluted with the same solvent to obtain six standard solutions at the concentration range of 10-60 µg mL-1 for both methods. The linearity was examined by analyzing six standard solutions (n=3) at the range of 10-60 µg mL-1 for both methods. Calibration curves were plotted with concentration versus peak area for the HPLC method, and absorbance versus concentration for the UV spectrophotometric method. Regression analysis was performed using the least-squares method with the data obtained from both analytical methods.

Precision

The repeatability of both methods was assessed by analyzing the sample solution six times in the same day. Similarly, intra-day and inter-day precision were assessed by analyzing sample solutions on the same day and three consecutive days, respectively. RBVN contents and (R.S.D.%) values were computed.

Accuracy

Analytical recovery tests were carried out using the standard addition method to check the accuracy of the developed methods and to investigate the effects of formulation additives. A reference standard solution of RBVN at three different concentration levels was added to the sample solutions. Sample solutions were prepared at every three levels in triplicate and analyzed by both analytical methods to determine the average recovery % and R.S.D.% values.

Sensitivity

The detection limit (LOD) and quantitation limit (LOQ) were used to evaluate the sensitivity of chromatographic and spectrophotometric methods. They were calculated separately depending on the standard deviation of the slope and intercept of the calibration curve by using the equations (1) and (2), respectively.

$LOD = 3.3x\sigma/S$ (1)

$LOQ = 10x\sigma/S$ (2)

Where σ: standard deviation of y-intercept and S: the slope of the calibration curve

Specificity

The sample solution was prepared freshly and injected into the LC chromatographic system. The chromatogram was examined, and it was evaluated whether there were interference peaks. The same sample solution was scanned at the wavelength range of 200-400 nm to assess the presence of possible interfering bands on the UV spectrophotometer instrument.

Ruggedness

The robustness of the proposed methods was assessed by analyzing the sample solution on different days and by different analysts. RBVN content and relative standard deviation (R.S.D.%) values were calculated.

Analysis of pharmaceutical formulations

The freshly prepared sample solution was filtered using a filter (0.45 µm pore size) and then analyzed by both methods.

Comparative analysis

Both analytical methods were found to be appropriate for the quantification of RBVN in pharmaceuticals after validation. When both analytical methods were used on commercial pharmaceuticals, the recovery percentages were compared statistically. The F-test and t-test were used for this purpose.

Stability of solutions

Over 24 hours, the reference standard solutions were evaluated for stability. During the stability research, standard solutions were kept at room temperature (25 °C) and shielded from light.

3. RESULTS AND DISCUSSION

Method development

RBVN has been described as "freely soluble in water" in several pharmacopeias [10,11]. As a result, ultrapure water was selected as the solvent to obtain UV spectrum at the wavelength range of 200-400 nm [Figure 2]. After evaluating the spectrum, because of the appropriate molar absorbtivity of RBVN in this region, a wavelength of 207 nm was selected for measurements.

Comment [SR2]: The method used for measurement is simple and old

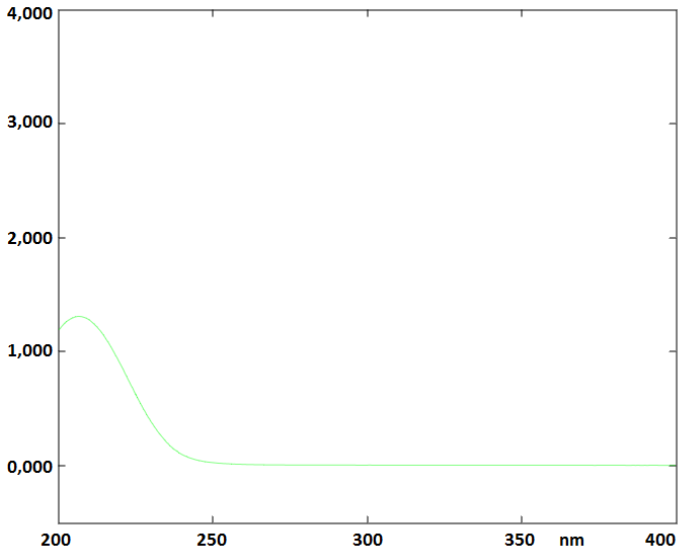

Figure 2. UV spectrum of standard solution (30 µg mL-1)

Comment [SR3]: The spectrum should be clear from the beginning 190 nm

By changing the column, mobile phase composition, pH, and flow rate, LC chromatographic method was optimized. Finally, we decided to use a mobile phase consisting of 20 mM KH2PO4 with a pH of 7.5 at a flow rate of 0.8 mL min-1 and a C18 column. Detection was carried out at a wavelength of 207 nm. As seen in the chromatogram, a short run time and sufficient peak symmetry (tailing factor: 1.44) were obtained [Figure 3]. Table 1 shows the system suitability parameters.

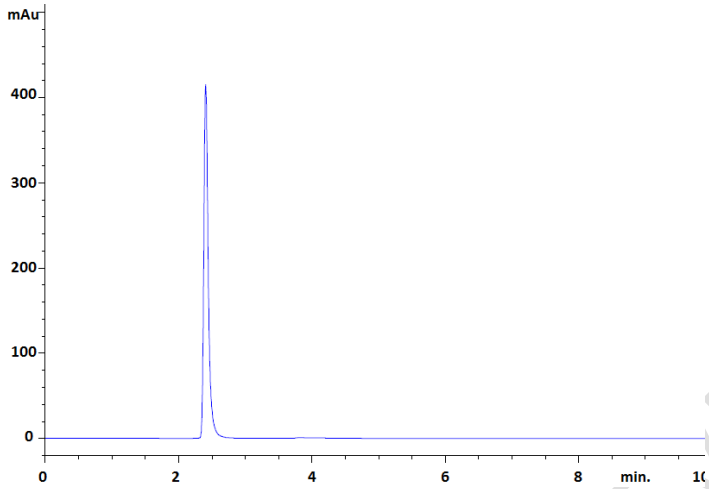

Figure 3. Chromatogram of the standard solution of RBVN (30 µg mL-1)

Table 1. Results of system suitability tests

Parameters (n=5)	Values
Retention time, min	2.404
Tailing factor	1.440
Theoretical plates, N	6955
Capasity factor	1.22

Linearity

It was determined that there was a linear connection between the concentration of the standard solution and the response for both methods. Table 2 displays the results of the regression analysis. The calculated correlation coefficients (r2) were higher than 0.999 indicating that these methods were linear. Linearity graphs and overlap chromatograms of both methods are presented in Figure 2.

Table 2. Validation parameters

Parameters	HPLC method	UV metod
Concentration range	10-60	
Correlation coefficient (r^2)	0.9999	0.9997
Slope	63.671	0.0413
Intercept	-4.0000	-0.0015
Precision (n=6) R.S.D. %	0.2811	0.3305
Recovery (n=9) R.S.D. %	0.1703-0.2150	0.2456-0.3085
LOD/LOQ	0.70/2.00	1.10/3.30

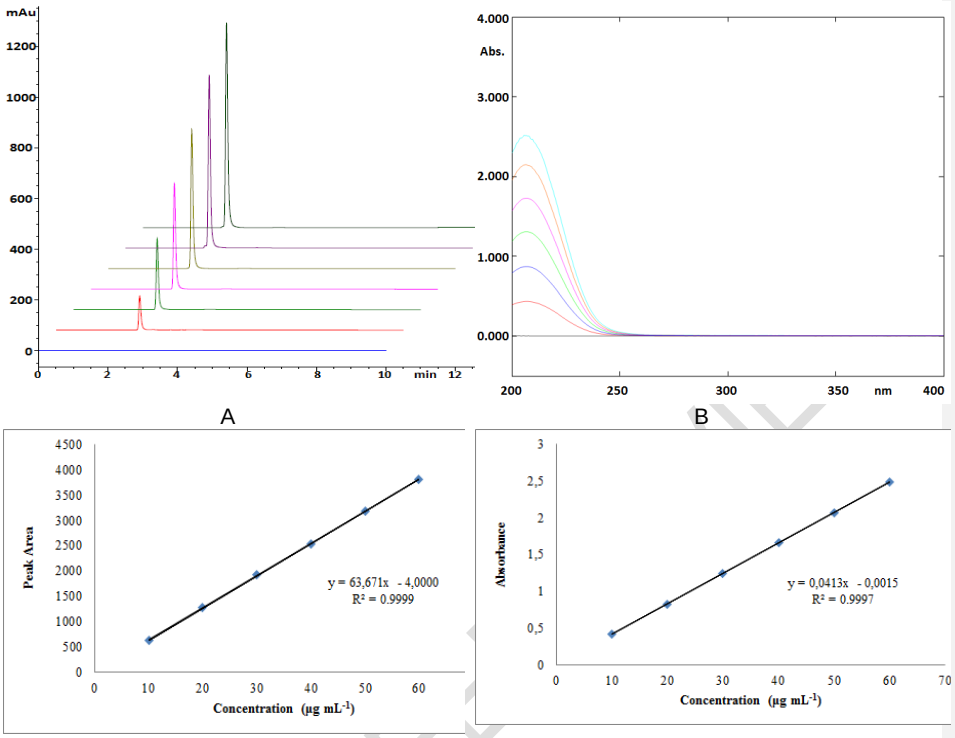

Figure 4. A. Overlap chromatogram of the LC chromatographic method (10-60 µg mL^{-1})
B. Overlap chromatogram of the UV spectrophotometric method (10-60 µg mL^{-1})
C: Linearity graph of the LC chromatogrpaphic method (10-60 µg mL^{-1})
D. Linearity graph of the UV spectrophotometric method (10-60 µg mL^{-1})

Precision

Precision data of both analytical methods were presented in Table 2. Both analytical methods provided good precision with R.S.D.% values less than 2.0, but the LC chromatographic method is more precise than the UV spectrophotometric method.

Accuracy

The accuracy of both analytical methods was evaluated by performing recovery studies. The analyte recovery has been determined by adding various amounts of standard (120%, 100%, and 80%) to the sample solution and analyzing them by the developed methods. Both analytical methods demonstrated average recoveries of close to 100%. Table 3 shows average recovery % and R.S.D.% values.

Table 3. Recovery tests results

Methods	Level %	Amount spiked	Amount recovered	Average recovery %	R.S.D. (%)*
Spectrophotometric method	80	16	15.95	99.69	0.256
	100	20	19.96	99.80	0.203
	120	24	24.03	100.13	0.260
Chromatographic method	80	16	16.03	100.19	0.125
	100	20	19.97	99.85	0.160
	120	24	24.02	100.08	0.110

*(n=3), R.S.D.%: Percentage Relative Standard Deviation

Ruggedness

The relative standard deviation values reported as less than 2% indicate the robustness of both analytical methods. Table 3 shows the results of the ruggedness test.

Specificity

The chromatogram obtained from the sample solution containing excipients in its composition was examined, and no interference peaks were observed during the retention period of RBVN for the LC chromatographic method. The spectrum obtained from the sample solution containing excipients in its composition was examined, and no interfering absorption bands were observed at 207 nm for the UV spectrophotometric method,

The LOD and LOQ values for LC chromatographic method were determined as 0.70 µg mL-1 and 2.20 µg mL-1, respectively. The LOD and LOQ values for the UV spectrophotometric method were determined as 1.10 µg mL-1 and 3.30 µg mL-1, respectively.

Application to marketed formulations

RBVN quantification in pharmaceutical formulations has been successfully applied using both developed and validated methods. Table 4 shows the results of the analysis for the tablet containing RBVN sold in pharmacies.

Table 4. Analysis results of the marketed pharmaceutical formulation

Label claim (mg/tablet)	Spectrophotometric method	Chromatographic method
	Found RBVN (mg/tablet)	Found RBVN (mg/tablet)
200	199.56	199.74

Comment [SR4]: It is preferable to use more than one pharmaceutical preparation for the drug
The calculation is also done for more than one concentration

Statistical comparison of methods

Both methods were statistically compared using the F-test and the t-test. Statistical analyzes have shown that there is no significant difference between the values obtained from the analyzes performed by both methods.

F-value and t-value were calculated and these values were found to be lower than the table values of both methods at the 95% confidence interval. Both of the proposed methods are applicable for the quantification of RBVN in pharmaceutical formulations. Table 5 shows the statistical comparison results of both methods.

Table 5. Statistical **comparison** (α=0.05, 95% confidence interval, n=6)

Statistical values	LC chromatographic Method	UV spectrophotometric Method
Average value	99.87	99.78
Standard deviation (S.D.)	0.26	0.51
Relative standard deviation (R.S.D.%)	0.26	0.51
Standard error	0.23	0.76
F-testi	0.25/0.42	
$F_{calculation}/F_{table}$		
t-testi	1.53/2.70	
$t_{calculation}/t_{table}$		

Stability of standard solutions

Throughout 24 hours, the stability of the reference standard solutions was examined. For this purpose, standard solutions were injected into the HPLC system with 8-hour periods and the retention time and peak area were recorded. Table 6 shows the results of the stability study. The R.S.D.% was determined as 0.159 for peak area and 0.087% for retention time. No significant changes in the concentration of the active pharmaceutical ingredient in the standard solution were observed.

Table 6. Standard solution stability (n=3, 50 µg mL^{-1})

Time period hours	Peak area	Average P.A.	S.D.	R.S.D. (%)	Retention time min.	Average R.T. min.	S.D.	R.S.D. (%)
	3182.8				2.402			
8	3191.5	3188.7	5.1	0.159	2.402	2.403	0.001	0.048
	3191.7				2.404			
	3187.1				2.408			
16	3182.8	3183.8	2.9	0.092	2.404	2.406	0.002	0.087
	3181.5				2.405			
	3182.2				2.406			
24	3185.1	3184.8	2.5	0.077	2.403	2.405	0.002	0.064
	3187.1				2.405			

4. CONCLUSION

The UV spectrophotometric method has advantages over the LC chromatographic method because the UV spectrophotometric method generally does not require detailed processes and procedures as in the LC chromatographic method. UV spectrophotometric method is more economical and consumes less time than the LC chromatographic method. However, the statistical comparison of both methods shows that the LC chromatographic method is more precise and accurate than the UV spectrophotometric method. The results show that the LC chromatographic and UV spectrometric methods are sufficient methods for the

quantification of RBVN in pharmaceutical formulations. No interfering peaks were observed during the retention time of RBVN in the chromatographic method and no interfering absorption bands were observed at 207 nm in the spectrophotometric method. Because these analytical methods are rapid, simple, precise, specific, and accurate, they can be applied successfully for routine quality control analysis of RBVN in pharmaceutical preparations.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

Comment [SR5]: References should be written more accurately

1. World Health Organization (WHO), Coronavirus Disease (COVID-19) Dashboard: Situation Report, 24. September. 2021.
2. Zhu N, Zhang D, Wang W, et al. China Novel Coronavirus Investigating and Research Team A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 2020; 382:72733.
3. Li G, Clercq ED. Therapeutic options for the 2019 novel coronavirus (2019-nCoV). Nat Rev Drug Discov 2020; 19:14950.
4. Liu W, Zhou P, Chen K, et al. Efficacy and safety of antiviral treatment for COVID-19 from evidence in studies of SARSCoV-2 and other acute viral infections: a systematic review and meta-analysis. CMAJ 2020; 192: E73444.
5. Fernandez H, Banks G, Smith R, et al. RBVN: a clinical overview. Eur J Epidemiol 1986; 2:14.
6. Morgenstern B, Michaelis M, Baer PC, et al. RBVN and interferon-beta synergistically inhibit SARS-associated coronavirus replication in animal and human cell lines. Biochem Biophys Res Commun 2005; 326:9058.
7. Momattin H, Mohammed K, Zumla A, et al. Therapeutic options for Middle East respiratory syndrome coronavirus (MERS-CoV)-possible lessons from a systematic review of SARS-CoV therapy. Int J Infect Dis 2013; 17: e7928.
8. Stockman LJ, Bellamy R, Garner P, et al. SARS: Systematic Review of Treatment Effects. PloS Med 2006; 3: e343.
9. Tong S, Su Y, Yu Y, et al. RBVN therapy for severe COVID-19: a retrospective cohort study. Int J Antimicrob Agents. 2020;56(3):106114.
10. The United States Pharmacopeia, 34th edition. The National Formulary, 29th Edition, Asian ed. The Official Compendia of Standards, The United States Pharmacopeial Convention, Rockville, MD, (2011); pp. 1186–1187, 1759, 2153–2154, 2544–2546, 3099, 3260, 4141–4142, 3433–3434.
11. The British Pharmacopoeia, Her Majesty's Stationery Office, London, (2010); pp. 46 –48, 57 –59, 365–366, 671–673, 1089–1091, 1241– 1243, 1370–1371, 1837–1838, 2080–2081.
12. Milovanovic, G.A., Cakar, M.M., Vucic, N.B., Jokanovic, M.; Selective indicator reaction for kinetic determination of traces of manganese(II), RBVN and tiazofurin; Mikrochimica Acta, (2000); 135: 173–178.

13. El-Din, M.K.S., El-Brashy, A.M., Sheribah, Z.A., El-Gamal, R.M.; Spectrophotometric determination of acyclovir and RBVN in their dosage forms; Journal of AOAC International, (2006); 89: 631–641.
14. Darwish, I.A., Khedr, A.S., Askal, H.F., Mohamed, R.M.; Application of inorganic oxidants to the spectrophotometric determination of RBVN in bulk and capsules; Journal of AOAC International, (2006); 89: 341–351.
15. El-Brashy, A.M., Sheribah, Z.A., Sharaf El-Din, M.K., El-gamal, R.M.; Kinetic determination of RBVN in drug formulations; International Journal of Biomedical Science, (2007); 3: 65 –71.
16. Han, J.Y; Analysis of RBVN and its intermediate substances by high-performance capillary electrophoresis; Yaown Fenxi Zazhi, (2001); 21: 160–162.
17. Wang, J.X., Feng, S.M.; High-performance liquid-chromatographic determination of the RBVN content in its preparations; Yaown Fenxi Zazhi, (1995); 15: 48 –49.
18. Shah, Y., Joshi, S., Jindal, K.C., Khanna, S.; Stability indicating HPLC method for tRBVN and its pharmaceutical dosage forms; Drug Development and Industrial Pharmacy, (1994); 20: 85 –91.
19. Liang, G.L.; HPLC determination of ganciclovir and RBVN in eye drops; Yaown Fenxi Zazhi, (2006); 26: 1308–1310.
20. Wu, H., Liu, F.; Determination of RBVN and related substances in ribavirin injection by RP-HPLC; Anhui Yiyao, (2009); 13: 31–33.
21. Wei, W., Wang, G.; Determination of RBVN in its injection by polarimetry; Yiyao Gongye, (1988); 19: 178–179.
22. Tao, H., Amatjan, M.X.; Development of a near IR method for rapid determination of RBVN tablets; Yaown Fenxi Zazhi, (2008); 28: 1705–1708.
23. Lu, Y., Zhang, Z.Y., He, D.Y., Hu, Y.F.; Flow-injection chemiluminescence method for determination of RBVN based on the enhancement of luminol-persulfate reaction; Analytical Letters, (2003); 36: 1587–1595
24. Darwish, I.A., Askal, H.F., Khedr, A.S., Mahmoud, R.M.; Stabilityindicating thin-layer chromatographic method for the quantitative determination of RBVN; Journal of Chromatographic Science, (2008); 46: 4–9.
25. Yeh, L.T., Nguyen, M., Lin, C.C.; A sensitive and specific method for the determination of total RBVN in human red blood cells by liquid chromatography-tandem mass spectrometry; Journal of Chromatographic Science, (2003); 41: 255–260.
26. Svensson, J.O., Bruchfeld, A., Schvarcz, R., Stahle, L.; Determination of RBVN in serum using highly selective solid-phase extraction and high-performance liquid chromatography; Therapeutic Drug Monitoring, (2000); 22: 215–218.
27. Granich, G.G., Krogstad, D.J., Connor, J.D., Desrochers, K.L., Sherwood, C.; High-performance liquid chromatography (HPLC) assay for RBVN and comparison of the HPLC assay with radioimmunoassay; Antimicrobial Agents and Chemotherapy, (1989); 33: 311–315.
28. Paroni, R., Sirtori, C.R., Borghi, C., Kienle, M.G.; High-performance liquid-chromatographic determination of RBVN in serum and urine and of its urinary metabolite 1,2,4-triazole-3-carboxamide; Journal of Chromatography and Biomedical Applications, 1(420), (1987); 64: 189–196.
29. Austin, R.K., Trefts, P.E., Hintz, M., Connor, J.D., Kagnoff, M.F.; Sensitive radio-immunoassay for the broad-spectrum antiviral agent RBVN; Antimicrobial Agents and Chemotherapy, (1983); 24: 696–701.
30. Liu, Y.F., Xu, C., Yan, R.Z., Lim, C., Yeh, L.T., Lin, C.C.; Sensitive and specific LC-MS/MS method for the simultaneous measurements of viramidine and RBVN in human plasma; Journal of Chromatography B, (2006); 832: 17 –23.
31. ICH. (2005). Q2 (R1), harmonized tripartite guideline, validation of analytical procedures: text and methodology, In Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.

32. Center for Drug Evaluation and Research (CDER). (1994). Reviewer Guidance: Validation of Chromatographic Methods.