Development and Validation of Spectrophotometric Assay Methods for 'Ribavirin' by Using Three Effective Reagents

[1]N. Murali Krishna, [2]C. Rambabu, [3]M. V. Satyanarayana

[1]Department of Chemistry, V. R. Siddhartha Engineering College, Kanuru, Vijayawada, India.

[2]Department of Chemistry, Acharya Nagarjuna University, Guntur District, India.

[3]Department of Chemistry, P. V. P. Siddhartha Institute of Technology, Vijayawada, India.

[1]nmkchem29@gmail.com, [2]rbchintala@gmail.com, [3]mvsku@yahoo.com

Abstract- In the present study, three simple spectrophotometric methods are developed and validated in order to determine Ribavirin, a synthetic nucleoside analogue. Apart from the simplicity, the methods are of low cost and effective. Three reagents namely Alizarin Red dye, p-chloroanilic acid and 1,10-phenanthroline are used in the development of the methods. The drug was determined in both pure form as well as in formulations. The drug is a base and hence forms an ion-association complex with Alizarin Red which is an acidic dye. The heteroatom nitrogen of ribavirin donates electrons to the π-acceptor p-chloroanilinic acid and forms a charge transfer complex. Further, the drug forms an orange coloured chromogen with 1,10-phenanthroline and ferric chloride. The absorption maxima of the new complexes formed are found to be 510, 528 and 440 nm for the complexes with 1,10-phenanthroline, p-chloroanilic acid and Alizarin Red respectively. The labeled amounts of the drug in the formulation as well as the results obtained in case of the newly proposed methods are in agreement. The results obtained in the present study infer that the proposed methods provide simple, low cost and quick process for the quantitative determination of ribavirin using visible spectrophotometric analysis.

Keywords —Ribavirin, Visible spectrophotometry, Parachloroanilic acid, Alizarin Red, 1,10-phenanthroline, validation

I. INTRODUCTION

Ribavirin [1-5] with chemical name 1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide is a synthetic nucleoside analogue. The chemical structure of ribavirin is shown in figure 1. It is an antiviral drug that is indicated for different infections like severe RSV infection, hepatitis C infection and other viral infections. Ribavirin is a prodrug. When this drug is metabolized, it resembles purine RNA nucleotides. In this form, it interferes with RNA metabolism required for viral replication.

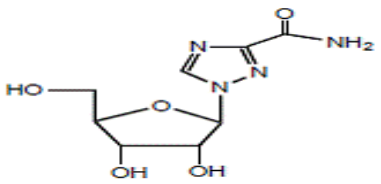

Figure 1: Chemical structure of Ribavirin

Many analytical methods [6-18] have been reported for the determination of ribavirin in various biological fluids and a very few in pharmaceutical dosage forms. This motivated the authors to develop inexpensive visible spectrophotometric method that may be considered for routine determination of ribavirin in pure and tablet dosage forms. To the knowledge of the authors, only two visible spectrometric methods [6,7] have been so far reported in the literature for the determination of ribavirin in pharmaceutical dosage forms and this fact prompted the them to develop accurate and inexpensive UV-Visible spectrophotometric methods for the determination of ribavirin in pure and tablet dosage forms. The present study describes three new visible spectrophotometric methods, which are based on reactivity of the functional groups of ribavirin with three organic reagents to form colored species which have good stability, showing the feasibility for spectrophotometric assessment of ribavirin in pure and pharmaceutical dosages.

II. MATERIALS AND METHODS

Ribavirin is white in colour and crystalline in nature. Its solubility is found to be good in water, while it is slightly soluble in alcohol. It is sold with brand names, Copegus, Rebetol, Ribasphere, Vilona and Virazole. Each capsule is found to contain a white powder in a white, opaque, gelatin capsule. The weight of ribavirin is 200 mg in a capsule, along with inactive ingredients like microcrystalline cellulose, lactose monohydrate, croscarmellose sodium, and magnesium stearate.

A UV-VIS Spectrophotometer from Elico (Model: SL-160) with 1.0 cm quartz cuvettes was utilized for spectral measurements. Electronic balance from Shimadzu (Model: AUW-220D) with 0.001 g Readability, 200 g Capacity, 0.001 g Repeatability and 0.002 g was used to weigh the required amount of the drug and the reagents. pH measurements were carried out using a digital pH meter received from Systronics model 362.

Analytical grade chemicals were used in carrying out the experimental part and double distilled water was used for the purpose of preparing the solutions. Three methods were proposed in the present study labeled as M1, M2 and M3 using the reagents 1,10-phenanthroline, parachloroanilic acid and Alizarin red dye respectively. The method M1 involves use of 0.01 M solution of 1,10-phenanthroline, 0.003 M solution of ferric chloride and 0.2 M solution of o-phosphoric acid. In the method M2, p-chloroanilic acid was used. In order to prepare this solution, 100 mg of p-chloroanilic acid was dissolved in 100 mL of 1,4-dioxane, and the solution was kept in dark when not in use. The method M3 involves an aqueous solution of Alizarin Red dye prepared by dissolving 0.1 g of the solid in 100 ml of distilled water.

100 mg of ribavirin was accurately weighed and dissolved in 10 mL of double distilled water and then made to 100 mL mark by adding double distilled water. Thus, a clear solution of ribavirin was obtained with a concentration of 1 mg/mL. To get the working standard solution of concentration 100 µg/mL, calculated volume of the above stock solution was used and diluted step-wise. Rebetol capsules containing 200 mg of ribavirin in each capsule were used for the analysis of drug in formulations. The capsules were opened and collected powder equivalent to 100 mg of ribavirin and transferred to 100 mL calibrated flask. The contents were shaken thoroughly for 15-20 minutes after adding about 30 mL of double distilled water. Then the drug was extracted into the liquid phase. After the extraction of the drug, the solution was made up to the mark using distilled water and mixed well. Then the solution was filtered through Whatman filter paper 41. Then the filtrate was made upto the mark using double distilled water in a volumetric flask of capacity 100 mL. From this filtrate, a suitable volume was diluted with water and the resulting solution was used in the determination of the drug ribavirin according to the recommended procedures.

III. RESULTS AND DISCUSSION

A. Optimization studies

Different parameters were considered for the study of the drug molecule. In order to obtain the optimum conditions for each and every parameter for the development of colour, each parameter was varied at a time while keeping the remaining parameters fixed and constant. Then the effect of varied parameter on the absorbance of the coloured species was studied. The experiments carried out to obtain the optimum conditions in case of all the parameters are detailed below. Based on the results, the conditions were included in the procedures.

M1: For establishing the optimum conditions required for quick formation of coloured complex which is significant quantitatively and exhibiting very high stability and sensitivity, the authors have conducted controlled experimental studies by altering one variable and keeping the other parameters, such as volume of Fe(III), o-phenanthroline and o-phosphoric acid, temperature, heating time, sequence of reagent addition and nature of solvents for final dilution. The details of all the parameters are presented in Table 1.

Table 1 Optimum conditions established in method M1

Parameter for the study of effect	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	490-530	510	---
Volume of Fe(III)	0.5 to 2.0 mL	1.0 mL	Erratic results beyond this range
Volume of o-Phenan-throline	1.5 to 3.0 mL	1.0 mL	Erratic results beyond this range
Temperature	60-80 ^{o}C	80 ^{o}C	Boiling waterbath was required for uniform temperature and maximum colour development
Heating time	5-25 min.	10 min.	No coloured complex below 25 min.
Volume of o-phosphoric acid	1.0-3.0 mL	2.0 mL	In order to for complex with excess of ferric iron, minimum volume required is 1.0 mL
Nature of solvent for final	Distilled water	Distilled water	Other solvents are found not to

dilution			increase the colour intensity of the final product.
Stability of colored species after final dilution	---	24 hours	Beyond 24 h, absorbance of coloured product was decreased

M2: In establishing optimum conditions for the rapid formation of the stable charge transfer complexes, the present studied drug was allowed to react with different volumes of the reagents (0.5-3.0 ml of 0.1 % p-chloroanilic acid). Maximum absorbance values were obtained for sample only when 1.0 ml of the reagent was used. At higher volumes of the reagent the greater absorbance for blank and lower absorbance for color complexes were observed. Therefore, 1.0 ml of the reagent was used throughout the investigation. Formation of charge transfer complex was instantaneous and the absorbance values of ribavirin-reagent were stable for at least 4 hours. The details are shown in Table 2.

M3: The optimum conditions were kept constant basing on the investigations on effects of different parameters, nature of acid, quantities of acid as well as dye, selection of organic solvent, relative quantities of organic phase and aqueous phase, time of shaking, temperature, intensity of the coloured species in the organic phase and its stability in the organic phase. The authors conducted controlled impediments by obtaining absorbance values (λ_{max}) at 440 nm of a series of solutions changing one parameter and keeping the other parameters constant. The results are incorporated in Table.3.

Table 2 Optimum conditions established in method M2

Parameter for the study of effect	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	510-540	528	---
Volume of p-chloroanilic acid	0.5 to 3.0 mL	1.0 mL	Low absorbance values beyond this range
Solvent for final dilution	Acetonitrile	Acetonitrile	Better absorbance values observed with this solvent
Stability of coloured species after final dilution	4 hours	---	After 4 h, the intensity of coloured product begins to decrease

Table 3 Optimum conditions established in method M3

Parameter for the study of effect	Optimum range	Conditions in procedure	Remarks
λ_{max} (nm)	430-460	440	---
Buffer	HCl	0.1 M HCl	Low absorbance values when pH is varied or [HCl] is other than 0.1 M
Volume of Alizarin Red	1.0 to 2.5 mL	2.0 mL	2.0 ml was required for covering wide range of limits of Beer's law
Choice of organic solvent for extracting coloured complex	$CHCl_3$	$CHCl_3$	Selective extraction of drug-dye coloured complex from the aqueous phase is effective with this solvent
Stability of coloured species in organic solvent	---	14 hours	The complex is stable up to 14 hours

B. Recommended Procedures

Based on the above systematic study of different variables detailed above, the procedures for the assay of ribavirin in the bulk samples are proposed as follows.

M1: A series 10.0 mL calibrated tubes were taken and aliquots of standard ribavirin with a concentration of 0.5-2.5mL, 100µg/mL are transferred into them. To all these solutions, 1.0 mL of ferric chloride solution and 1.0 mL of 1,10-phenanthroline solution were added. Later, distilled water was added to all the tubes so that the total volume of the mixtures is 3.0 mL in each tube. These solutions were heated in a water bath maintained at 90 ^{o}C for about 10 minutes. After heating, the solutions were cooled to ambient temperature and then 2.0 mL of H_3PO_4 solution was added in each tube. As a result of this process, an orange coloured complex was produced. Following the same procedure, a reagent blank was also prepared. Then the absorbance values of all the complexes were measured after 5 minutes at a wavelength of 510 nm. From the Beer-Lambert's plot (figure 2), the quantity of ribavirin was calculated.

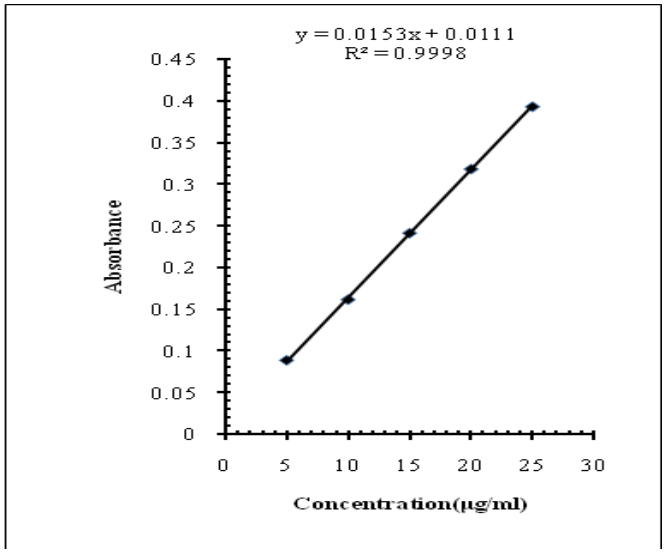

Figure 2: Beer's law plot of ribavirin with 1,10-phenanthroline-Fe(III)/Phosphoric acid

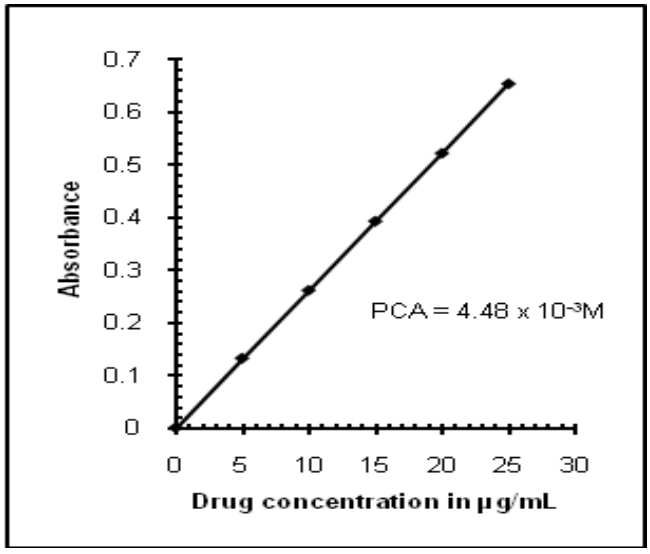

Figure 3: Beer's law plot of ribavirin with p-chloroanilic acid

M2: Different aliquots of standard ribavirin solutions (0.5-2.5 mL; 100 µg/mL) were accurately transferred into a series of 10.0 mL volumetric flasks and the total volume was adjusted to 3.0 mL by adding adequate quantity of acetonitrile. To each flask was then added 2.0 mL of 0.1 % p-chloranilic acid, and the contents were mixed well and kept aside for 10 minutes. The mixture was diluted to the volume with acetonitrile and the absorbance was measured at 518 nm against a reagent blank prepared simultaneously. The concentration of the ribavirin was read from the computed from the respective regression equation derived using the Beer's law data (figure 3).

M3: 250 mL separating funnels were taken and aliquots of standard solution of ribavirin of concentration 100 µg/mL were added to them in the volume range from 0.5 to 2.5 mL. To these solutions, 2.0 mL of 0.1 % of Alizarin red solution was added. Then, 1.0 mL of 0.1 N hydrochloric acid solution was added. Later, using distilled water, the total volume of aqueous solution in all the funnels was made to 13.0 mL. After making the total volume to 13.0 mL in all the funnels, 10.0 mL of chloroform was added to each separating funnel and the mixtures were thoroughly shaken for about two minutes. After thorough mixing, the two phases were separated and the chloroform phase was subjected to absorbance measurement at the wavelength of 440 nm along with corresponding reagent blank. Using the calibration curve (figure 4), the quantity of ribavirin present in the sample solution was calculated.

C. Spectral characteristics

In order to ascertain the maximum absorption (λ_{max}) of the colored species produced in the methods described above, fixed quantities of ribavirin were considered and colours were produced individually by following the procedures proposed in this paper. The absorption spectra were recorded on a spectrophotometer in the wavelength range of 340-900 nm along with similar reagent blank or distilled water. The results obtained by following this procedure are presented graphically in figures 5 to 7 respectively.

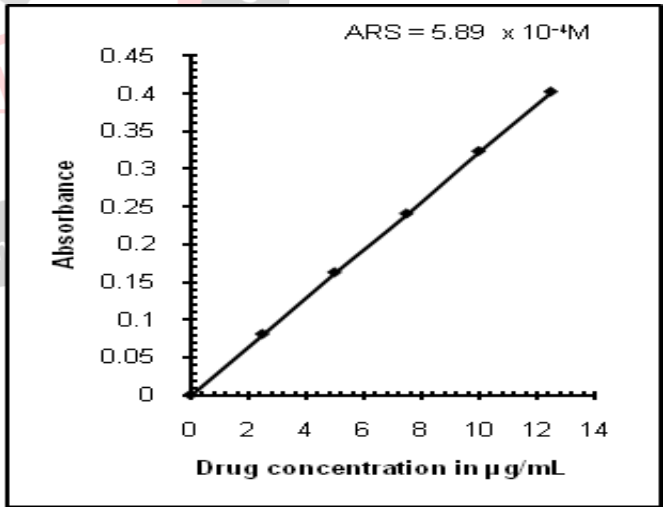

Figure 4: Beer's law plot of ribavirin with Alizarin Red-Chloroform

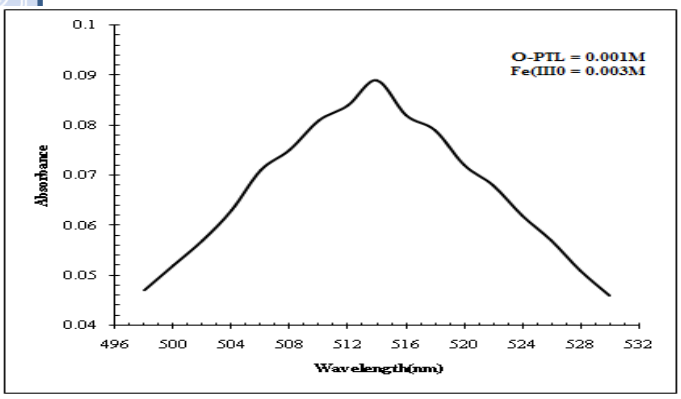

Figure 5: Absorption spectrum of ribavirin with 1,10-phenanthroline-Fe(III)/Phosphoric acid

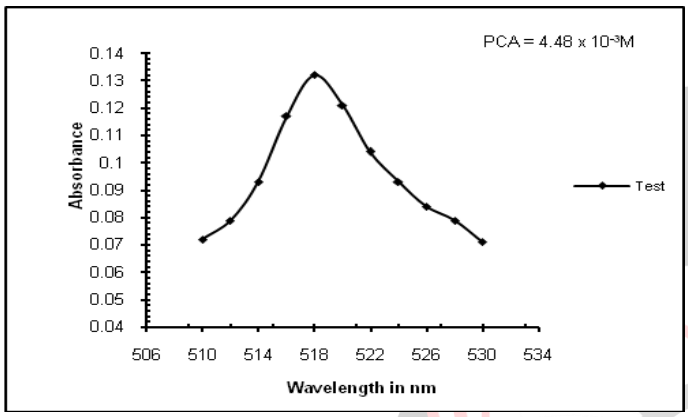

Figure 6: Absorption spectrum of ribavirin with p-chloroanilic acid

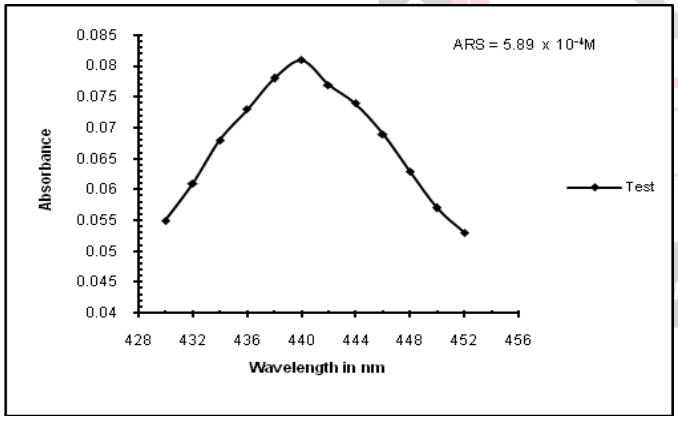

Figure 7: Absorption spectrum of ribavirin with Alizarin Red-Chloroform

D. Method validation

Optical characteristics: The plots of Beer's law for all the systems considered for the study were recorded including the respective reagent blank solutions are shown in figures 8 to 10. Table 4 shows the Sandell's sensitivity, optimum photometric range, absorptivity and Beer's law limits in case of all the three proposed methods. Further, the slope, intercept and correlation coefficient values were also obtained by using least square regression analysis.

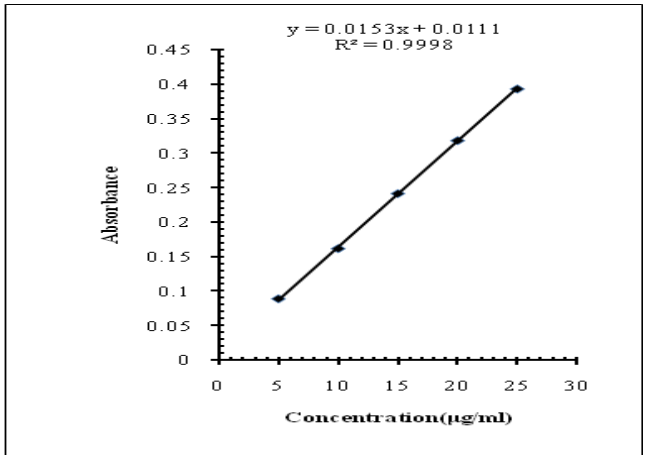

Figure 8: Beer's law plot of ribavirin with 1,10-phenanthroline-Fe(III)/Phosphoric acid

Precision: In case of each of the proposed methods, absorbance values were obtained by for six replicates with fixed amount of ribavirin and based on the results the precision was ascertained. The relative standard deviation as well as range of error in terms of percentage (at 0.05 and 0.01 confidence limits) were obtained for the three methods proposed.

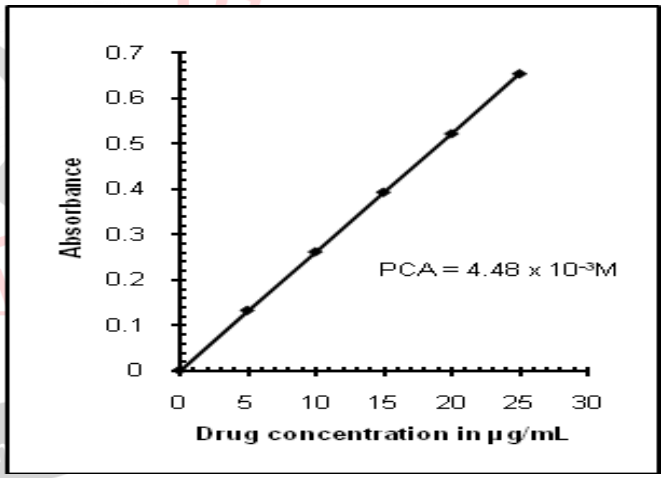

Figure 9: Beer's law plot of ribavirin with p-chloroanilic acid

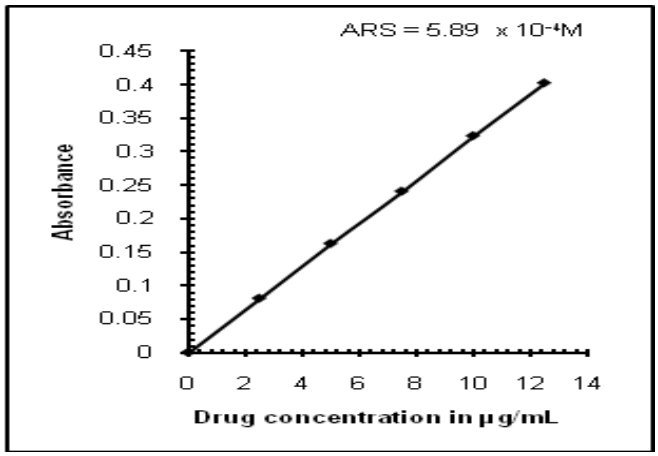

Figure 10: Beer's law plot of ribavirin with Alizarin Red-Chloroform

Accuracy: Table 5 shows the results in terms of percentage error of the methods proposed. These results were obtained by determining the accuracy of each method. For this purpose, the bulk samples of ribavirin at different concentrations within the Beer's law limits were considered and analyzed.

Analysis of formulations: Apart from the analysis of pure drug, the commercial samples containing drug molecules were considered. The formulations considered are Rebetol-200 mg tablets. They were successfully analyzed for the drug using the developed methods. The comparison of suggested methods and the reference method [7] for formulations was done statistically using F-test and t-test. The results of the recovery experiments are presented in Table 5.

Nature of coloured complexes: Based on the results of the studies on development of new methods discussed above, an attempt was made to predict the reaction mechanism involved in the proposed methods by exploiting appropriate functional moieties in ribavirin with various reagents. The probable reaction schemes for each proposed methods are described below. In case of the method M1, oxidation occurs followed by formation of complex of ribavirin with 1,10-phenanthroline, $FeCl_3$ and H_3PO_4. It leads to formation of an orange coloured compound having absorption maximum of 510 nm. Method M2 involves reaction between N of ribavirin which acts as electron donor and p-chloroanilic acid which is a π-acceptor leading to formation of a charge transfer complex. When method M3 is considered, ribavirin forms an ion-association complex with Alizarin Red, an acidic dye. The complex can be extracted into chloroform from the aqueous medium.

Table 4: Regression as well as optical characteristics, accuracy and precision of the proposed methods for ribavirin

S. No.	Parameter	Method, M1	Method, M2	Method, M3
1	λ_{max}(nm)	510	518	440
2	Beer's law limits (µg/mL)	5.0 - 25.0	5.0 - 25.0	2.0 - 10.0
3	Molar absorptivity (1 $mol^{-1}.cm^{-1}$)	2.808×10^4	7.310×10^4	2.130×10^4
4	Sandell's sensitivity (µg/cm^2/0.001 absorbance unit)	0.0139	0.03708	0.03546
5	Optimum photometric range (µg/mL)	5.5 - 22.5	6.5 - 20.0	3.0 - 7.5
6	Regression equation (Y = a + bc); slope (b)	0.0153	0.1298	0.0200
7	Intercept (a)	0.111	0.0022	0.00365
8	Correlation coefficient (r)	0.9998	0.9999	0.9998
9	Relative standard deviation (%)*	1.678	0.9545	2.0382
10	% Range of error (confidence limits)	---	---	----
11	0.05 level	1.688	1.0018	2.1393
12	0.01 level	1.650	1.5711	3.3550

Table 5: Assay and recovery of ribavirin in dosage forms

Method	Pharmaceutical Formulation	Labeled Amount (mg)	Proposed Method			Found by reference method[15]±S.D	%Recovery by proposed method*
			Amount found** (mg) ±S.D	t-value	F-value		
M1	Rebetol	200	199.95±0.10	0.131	2.56	199.96±0.16	99.99±0.98
M2			199.90±0.12	0.921	2.254		96.96±0.95
M3			199.94±0.13	0.236	1.523		99.97±0.81

*Average ± standard deviation of six determinations the t and F- values refer to comparison of the proposed method. Theoretical values at 95. % confidence limits t = 2.365 and F = 4.88.

** Average of six determinations.

IV. CONCLUSION

The proposed visible spectrophotometric methods developed in the present study for the assay of ribavirin were observed to be relatively simple, selective, sensitive and also economical. The developed methods show high degree of sensitivity, selectivity, reproducibility and high recovery, when compared with previously reported methods. The statistical parameters and recovery studies data clearly indicate the reproducibility and accuracy of the proposed methods. The sample recoveries in formulations were in good agreement with their respective label claims. Therefore, it is concluded that these developed methods could be considered as alternative methods for the determination of ribavirin in pure and formulations by the quality control laboratories of pharmaceutical industries.

ACKNOWLEDGMENT

One of the authors, Dr. N. Murali Krishna is grateful to the Principal and Management of V. R. Siddhartha Engineering College, Vijayawada, for their support and encouragement in research activities.

REFERENCES

[1] S. Crotty, C. Cameron, R. Andino, *J. Mol. Med.*, vol. 80, no. 2, pp. 86-95, 2002.

[2] G. S. Ascio, L. H. Leble, B. H. Vaha, K. A. Chan, *J. Antimicrob. Chemother.*, vol. 66, no. 6, pp. 1215-1222, 2011.

[3] D. G. Bausch, C. M. Hadi, S. H. Khan, J. J. Lertora, *Clin. Infect. Dis.*, vol. 51, no. 12, pp. 1435-1441, 2010.

[4] S. C. Sweetman, Martindale – The complete drug reference, 1, 36th Edition, Pharmaceutical Press, London, UK, 2009.

[5] The United States Pharmacopoeia, 34th Edition, The National Formulary, 29th Edition, Asian Ed., The Official Compendia of Standards, The United States Pharmacopeia Convention, Rockville, MD, pp. 186-187, 1759, 2153-2154, 2544-2546, 3099, 3260, 4141-4142, 3433-3434, 2011.

[6] M. K. S. El-Din, A. M. El-Brashy, Z. A. Sheribah, R. M. Gamal, *Journal of AOAC International*, vol. 89, pp. 631-641, 2006.

[7] I. A. Darwish, A. S. Khedr, H. F. Askal, R. M. Mohammed, *Journal of AOAC International*, vol. 89, pp. 341-351, 2006.

[8] Z. S. Wilson, Z. B. Hai, A. Thomas, J. Xiangyu, N. Weng, *J. Pharm. Anal.*, vol. 29, no. 2, pp. 83-94, 2002.

[9] A. M. Lou, P. L. Lori, *J. Med. Chem. Sci.*, vol. 15 pp. 1150, 1972.

[10] L. Z. Zhi, Y. Y. Xi, Y. P. Huai, F. C. Tie, X. L. Qiu, *BMC*, vol. 22, no. 12, pp. 1409-1415, 2008.

[11] T. Y. Li, N. Y. Mai, C. L. Chin, *J. Chromatogr. Sci.*, vol. 41, no. 5, pp. 255-260, 2003.

[12] A. Manel, M. Darius, C. Matthias, M. Thomas, *AAC*, vol. 57, no. 7, pp. 3147-3158, 2013.

[13] D. Antonio, I. Allende, S. Marco, G. Daniel, *J. Chromatogr. B*, vol. 835, no. 2, pp. 127-130, 2006.

[14] A. Avolio, A. Nicolo, M. Simiele, S. Turini, D. Agnesod, L. Boglione, *J. Pharm. Biomed. Anal*., vol. 66, no. 10, pp. 376-380, 2012.

[15] J. X. Wang, S. M. Feng, *Yaown Fenxi Zazhi*, vo. 15, pp. 48-49, 1995.

[16] A. Suman, K. Naveen, M. Ramana, *International Journal of Research in Pharmaceutical and Biomedical Sciences*, vol. 2, no. 2, pp. 704-709, 2011.

[17] Rim Said Haggag, Said Fathalla Belal, Ismail Ibrahim Hewala, Ola Ahmed El Rouby, *Journal of Chromatographic Science*, vol. 52, pp. 493-500, 2014.

[18] G. Raveendrababu, A. Lakshmana Rao, J. Venkateswara Rao, *Int. J. Pharm. Pharm. Sci.*, vol. 7, no. 2, pp. 60-63, 2015.