Stability-Indicating Thin-Layer Chromatographic Method for Quantitative Determination of Ribavirin

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Abstract

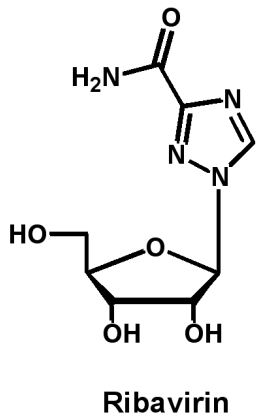
A simple and accurate stability-indicating thin-layer chromatographic (TLC) method is developed and validated for the quantitative determination of ribavirin (RBV) in its bulk and capsule forms. The method employs TLC aluminum plates precoated with silica gel 60F-254 as a stationary phase. The solvent system used for development consists of chloroform–methanol–acetic acid (60:15:15, v/v/v). The separated spots are visualized as bluish green spots after being sprayed with anisaldehyde reagent. RBV is subjected to different accelerated stress conditions. The drug is found to undergo degradation under all stress conditions, and the degradation products are well resolved from the pure drug with significantly different R_f values. The optical densities of the separated spots are found to be linear with the amount of RBV in the range of 5–40 µg/spot with a good correlation coefficient (r = 0.9980). The limit of detection and limit of quantitation values are 1.40 and 4.67 µg/spot, respectively. Statistical analysis proves that the method is repeatable and accurate for the determination of RBV in the presence of its degradation products. The method meets the International Conference on Harmonisation/Food and Drug Administration regulatory requirements. The proposed TLC method is successfully applied for the determination of RBV, pure and in capsules, with good accuracy and precision; the label claim percentages are 98.8% ± 1.5%. The results obtained by the proposed TLC method are comparable with those obtained by the official method.

Introduction

The quality of a pharmaceutical product, in terms of purity and stability of the active substance and/or finished product, is vital for the effective and safe delivery of its therapeutic value to the patients. This is because the presence of impurities and/or potential degradation products may cause changing of chemical, pharmacological, and/or toxicological properties of the active drug (1–3). In general, pharmaceuticals are sensitive to environmental factors, such as temperature, humidity, and light. These factors usually vary during manufacturing, transportation, storage, and distribution of the finished product. For these reasons, stability testing of the active substance and the finished product is necessary for providing information about potential degradation products, possible degradation pathways of the drug, compatibility of the drug with the excipients in the finished product, and the long-term effects of the environmental factors on the active drug and its finished products. The results of stability testing are important in developing proper manufacturing processes, selecting proper packaging, storage conditions, the product's shelf life, and determining the expiration date (4–7).

Ribavirin (RBV), 1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide (Figure 1), is an antiviral agent that is currently available and intensively used in our community. It is a synthetic guanosine analogue that can inhibit the replication of a wide range of DNA and RNA viruses, including influenza A and B, parainfluenza, respiratory syncytial virus, paramyxo-viruses, HCV, and HIV-1 (8). RBV, in combination with interferon α-2b, has been approved for the treatment of chronic hepatitis C infection in patients with compensated liver disease (9–12). It also decreases the mortality of Lassa fever and other viral hemorrhagic fevers (8, 13).

Analytical methods that have been reported for the determination of RBV include high-performance liquid chromatography (HPLC) (14–17), gas chromatography (18), capillary electrophoresis (19), radioimmunoassay (20), fluorimetry (21,22),



Ribavirin

Figure 1. Chemical structure of ribavirin (RBV).

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and spectrophotometry (23,24). None of the reported methods was described as a stability-indicating method for the determination of RBV in the presence of its degradation products, which may result from the decomposition of RBV under inappropriate conditions. Therefore, the aim of the present study was directed to the development of a stability-indicating chromatographic method for stability evaluation and quantitative determination of RBV in the presence of its degradation products. Thin-layer chromatography (TLC) is a routine analytical technique for the separation and identification of drugs due to its simplicity (it requires a less sophisticated apparatus), low cost, and need for minimal sample cleanup. The analysis can be performed without a remote area, and in some cases, it can be used away from laboratory. It also uses a limited volume of solvents. For these reasons, the development of a stability-indicating TLC method was considered in the present study.

Experimental

Apparatus

The UVP scanner and software (Gel Works 1D Advanced Version 3.01) were obtained from Ultra Violet Products, Ltd. (Cambridge, U.K.). A 12-mL test tube atomizer from Desaga GmbH (Wiesloch, Germany) was connected to a positive–pressure outlet valve of a membrane pump (Cole-Parmer, Chicago, IL). A 25-µL TLV spotting syringe was obtained from LKB (Bromma, Sweden). A standard-type TLC tank and minivials lined with a silylated tetrafluoroethylene cap (1 mL) were products of Alltech (Mallinckrodt Chemical Works, New York, NY). A hot air oven (WTB binder 7200 Tuttingen, Schwa Bach, Germany), a UV lamp (Vilber lournate, Marne-lavallee Cedex, France), and a laboratory-made heating block were used.

Materials

TLC aluminum plates (20 × 20 cm, 0.25 mm layer thickness), precoated with silica gel 60F-254, were obtained from Merck (Darmstadt, Germany). *p*-Dimethylaminobenzaldehyde (anisaldehyde, Sigma-Aldrich) and nylon sample spraying filtration discs (0.45 µm) were obtained from Riedel-de Haën AG (Seelze, Germany). *p*-Methoxyacetophenone (Riedel-de Haën AG), *p*-methylbenzaldehyde (Merck), 4-aminobenzophenone (Sigma Chemicals Co., Norwalk, CT), *p*-nitroacetophenone (BDH, Poole, U.K.), *p*-methoxycinnamaldehyde (Merck), and vanillin (BDH) were used. RBV reference standard was obtained from T3A (Assiut, Egypt) and used as received. RBV capsules (T3A) are labeled to contain 200 mg ribavirin per capsule. All solvents were HPLC-grade, and other chemicals used throughout this study were of analytical grade.

Preparation of standard solution

An accurately weighed amount (1 g) of RBV was transferred into a 10-mL calibrated flask and dissolved in approximately 5 mL of distilled water. The resulting solution was completed to the mark with the same solvent to provide a stock standard solution containing 100 mg/mL. A measured volume (0.6 mL) of the stock solution was diluted to 10 mL to give 6 mg/mL as a working solution. Different volumes of this stock solution were transferred into a 10-mL calibrated flask and diluted to 10 mL with distilled water to obtain the working standard solutions of concentrations suitable for analysis. For analysis, 5 µL were applied to the TLC plate.

Preparation of capsule samples

The contents of 20 capsules were mixed, and an accurately-weighed amount of the contents equivalent to 100 mg of RBV was transferred into a 10-mL volumetric flask. Four milliliters of water were added, and the contents of the flask were shaken for approximately 2 min. The solution was then diluted to the mark with the same solvent. The contents of the flask were filtered through a 0.45 µm filtration disc, and 6 mL of the filtrate were diluted to 100 mL to give a concentration of 6 mg/mL. For analysis, 5 µL were applied to the TLC plate.

Preparation of detection reagent

The anisaldehyde reagent (5%, v/v) was prepared by transferring 0.5 mL of anisaldehyde to 9 mL of ethyl alcohol. Then, 0.1 mL of glacial acetic acid was added, followed by 0.5 mL of concentrated sulfuric acid, which was added dropwise. The solution was then cooled to be ready for spraying.

Methods and procedures

Sample loading

The samples were applied to the marked start edge of the TLC plate at a height of 1.5 cm from the lower edge of the plate using the specified TLC-Hamilton glass syringe. The sample volumes for all experiments were 5 µL. The plate was then allowed to be air-dried for 10 min before it was transferred to the TLC tank for the development.

Chromatogram development

The mobile system used was chloroform–methanol–glacial acetic acid (60:15:15, v/v/v). Fifty milliliters of the mobile system were poured into the TLC tank that was lined with a thick filter paper on a few sides to help with the chamber saturation. The tank was then covered with a lid and pre-saturated with the mobile system vapor for at least 30 min at room temperature (5 ± 25°C) before use. The sample-loaded TLC plate was transferred to the TLC tank; the TLC plate was then developed for no less than a 15-cm migration distance of the solvent from the start line (approximately 30 min). The developed TLC plate was air-dried for about 10 min in the laboratory hood.

Visualization of spots

Spraying of the detection reagent was achieved by using a double-nozzle sprayer connected to the positive pressure outlet valve of a membrane pump. The distance between sprayer and the plate was approximately 30 cm. The plate was sprayed with approximately 8 mL anisaldehyde reagent, air-dried for 10 min in the laboratory hood, and then transferred to the hot-air oven at 80°C for 10 min. Bluish-green spots with a light orange background were developed.

Forced degradation

Boiling

One milliliter of RBV solution (200 mg/mL) was transferred to minivials. The vials were capped and inserted into the heating block and heated at 100°C for 30 min. The vials were cooled

before opening, and a volume of 5 µL (1 mg sample) was applied onto the TLC plate. Five microliters of standard RBV solution (3 mg/mL) were applied.

Acid and alkali hydrolysis

An aliquot of 1 mL of RBV solution (200 mg/mL) was transferred to minivials. The solution was mixed with 1 mL of 1 N hydrochloric acid or 1 N sodium hydroxide. The prepared solutions were heated at 60°C for 30 min with intermittent shaking. The samples were cooled to room temperature (25 ± 5°C) and neutralized with an amount of acid or base equivalent to that of the previously added amount. From the resulting neutral solution, 5 µL (1 mg) was applied to the TLC plate. Five microliters of standard RBV solution (3 mg/mL) were applied.

Oxidation

One milliliter of RBV solution (200 mg/mL) was transferred to minivials. The vial content was then mixed with 1 mL of 10% hydrogen peroxide solution and heated at 60°C for 30 min with intermittent shaking. A volume of 5 µL (1 mg) was applied to the TLC plate. Five microliters of standard RBV solution (3 mg/mL) were applied.

Irradiation with UV light

A sample powder of RBV (200 mg) was exposed to UV light for seven days. The vial contents were dissolved in 2 mL water. The solution was filtered with a syringe filtration disk and diluted to 20 mL with a suitable solvent to obtain a claimed concentration of 5 mg/mL. Five microliters of standard RBV solution (3 mg/mL) were applied.

Heating of capsule contents (incompatibility testing)

The contents of one capsule of RBV were transferred into minivials (two vials for each product). One of them was mixed with 10 µL of water, and the other was left to dry. The vials were capped tightly and allowed to stand in a heating incubator at 60°C for 14 days. The vial contents were dissolved in water. The solutions were filtered with a 0.45-µm filtration disc and diluted to obtain a claimed concentration of 3 mg/mL. A volume of 5 µL was applied to the TLC plate.

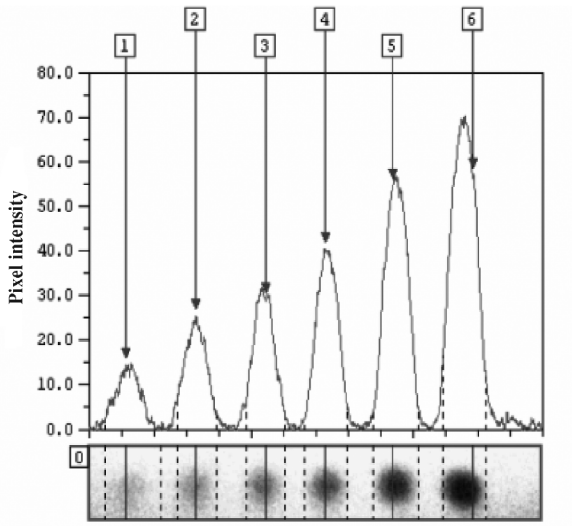

Figure 2. One-point calibration curve of on-plate derivatized RBV. The concentrations of RBV were 5, 10, 15, 20, 30, and 40 µg/spot for spots 1–6, respectively.

Data processing and treatment

The TLC chromatogram, as an image, was captured by the scanner and loaded into the Gel Works software. In the software, the following operations were performed: the series of spots to be manipulated were selected as a lane-by-lane creation function. Once the lane was created, a chromatogram was generated. The generated chromatogram was a function of spot position with the corresponding optical density represented as pixel intensity.

The background of the TLC chromatogram, if any, was subtracted. After background subtraction, the signals of the chromatogram were assigned with numbers according to the sequence of the corresponding spots in the previously selected band.

Quantity calibration was then performed by pre-assignment of the concentration of the active material of each spot. Once the authentic known concentrations were assigned, the calibration graph was automatically generated. The generated graph correlated the concentration to the corresponding signal intensity (represented as raw volume).

Once these operations were performed, a report was given by the software. Figure 2 illustrates an example of a one-point calibration curve; however, in the analysis, a three-point calibration curve was used.

Results and Discussion

Method development

The initial method development was conducted on the pure drug using a working standard solution. Actual chromatographic conditions were established after a number of preliminary experiments involving the selection of a proper mobile system and detection reagent. Different mobile systems were tested, and the selection of the mobile phase system depended on its ability to give good separation between pure RBV and its possible impurities and/or degradation products. The proper system consisted of chloroform–methanol–acetic acid (60:15:15, v/v/v). This system gave compact spots for RBV [Retention factor (R_f) value of 0.45 ± 0.02, five replicates]. Several spraying reagents were tested for the detection of RBV on TLC plates. These reagents were *p*-methoxyacetophenone, 4-aminobenzophenone, *p*-nitroacetophenone, *p*-dimethylamino benzaldehyde (anisaldehyde), *p*-methoxycinnamaldehyde, *p*-methylbenzaldehyde, and vanillin. The reagents, except anisaldehyde, gave faint spots, dark backgrounds, and/or the reaction required a long heating time for spot development. Anisaldehyde gave bluish-green spots with a faint pinkish background. The background could be subtracted by the Gel Works software. Therefore, anisaldehyde was selected for the detection of RBV in all subsequent procedures.

Method validation

Linearity and limits of detection and quantitation

Using the previously mentioned optimal mobile system, chromatogram development, and detection reagent, the three-point calibration graph correlating the optical density as pixels

(expressed as raw volume) to the corresponding RBV concentration was constructed. Regression analysis for the results was carried out using the least-square method. The results revealed a good linear calibration fit in the range of 5–40 µg/spot. The calibration equation, generated automatically once the method of calculation was defined, was:

$$OD = 1.26 + 2.088\ C\ (r = 0.9980)$$

where OD is the optical density (pixel intensity), C is the concentration of RBV, and r is the regression coefficient. The calibration curve was valid only for the calculation of concentration of RBV separated on the same plate.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by fitting the interday standard deviations (SDs) calculated for each calibration curve. The intercept was then equal to SD0 (the estimated SD at a concentration of zero). LOD was then defined as 3SD0, and LOQ was defined as 10SD0. The LOD and LOQ values were 1.40 and 4.67 µg/spot, respectively.

System suitability, precision, and accuracy

System suitability testing was performed on TLC plates using a standard RBV solution of 40 µg/spot. The precision of the proposed method was determined by replicate analysis of nine separate samples of the same solution; the results are shown in Figure 3. The relative standard deviation (RSD) of the calculated R_f values was close to zero. As well, the RSD of the raw volumes (pixel intensities) did not exceed 1.64%. This good level of precision was suitable for quality control analysis of RBV. Accuracy was checked by analysis of RBV sample solutions at three concentration levels (5, 10, and 20 µg/spot) by the calibration curve generated on the same TLC plate. The error did not exceed 0.44% (Table I).

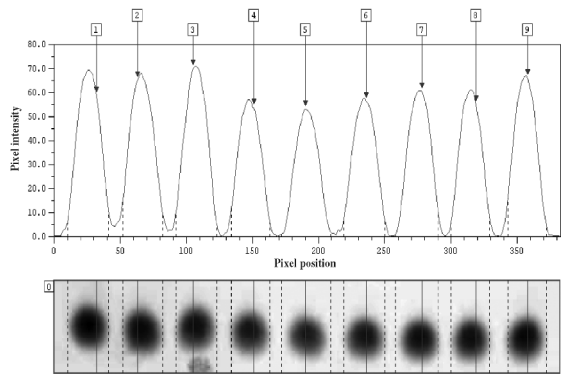

Figure 3. Replicate analysis of 40 µg/spot of RBV by TLC for testing of system suitability and method precision.

Table I. Accuracy of the Proposed TLC Method for Analysis of Ribavirin

Nominated conc. (µg/spot)	Measured conc. (µg/spot)	RSD (%)	Deviation* (%)
5	4.99 ± 0.40	0.98	0.09
10	9.99 ± 0.52	1.12	0.24
20	19.98 ± 0.98	1.79	0.44

* Deviation (%) = (nominated conc. – mean measured conc.) × 100/nominated conc. Values are mean of three determinations ± SD.

Selectivity

In order to check the selectivity of the proposed TLC method, the ability of the mobile system to resolve the major compound from possible degradation product was performed. A sample containing the degradation product was prepared by melting an authentic sample of the drug (200 mg) on a hot plate. Then the melted material was dissolved in 1 mL water. Five microliters (1 mg) of the sample solution were then applied to the plate and analyzed concurrently with two different concentrations of the standard solutions that have not been subjected to the stressed conditions. The results revealed that the proposed method was able to completely discriminate the major compound from the possible degradation products, confirming the selectivity of the method.

Robustness and ruggedness

In order to measure the extent of the method's robustness, the most critical parameters were interchanged while keeping the other parameters unchanged, and in parallel, the chromatographic profile was observed and recorded. The chromatographic parameters were interchanged within the range of 1–10% of the optimal recommended conditions. The studied parameters were: the composition of the mobile phase, the amount of spraying reagent, and the heating temperature and time. The results indicated that the small change in the composition of the mobile phase did not significantly affect the resolution of RBV from the degradation products.

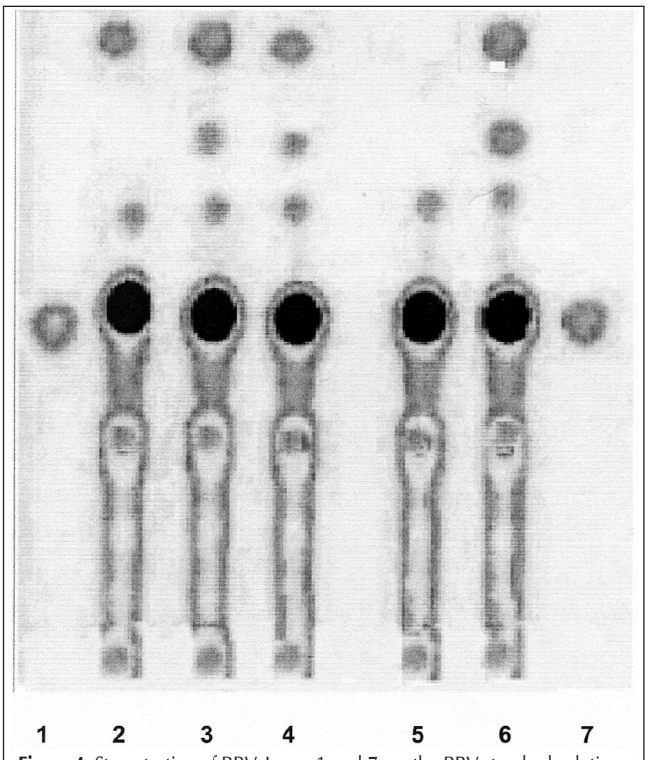

Figure 4. Stress testing of RBV. Lanes 1 and 7 are the RBV standard solution. Lanes from 2 to 6 are samples that have been subjected to alkali hydrolysis, oxidation, acid hydrolysis, UV irradiation, and boiling, respectively.

Ruggedness

The ruggedness of the method was evaluated by applying the analysis of RBV solutions using two different TLC plates of different layer thicknesses (0.25 and 0.20 mm) from two different manufacturers, Merck and Fluka Chemie GmbH (Buchs, Germany). Both plates yielded the same resolution efficiency and R_f values; however, the thinner-layer plate required a longer time for the solvent to reach the marked front. A compact spot without tailing was observed in both cases. Attention should be paid to the amount of samples delivered from the spotting syringe because this step expresses the material concentration. A calibrated spotting device could be useful to avoid sample volume variation. However, the loaded volume area variation did not make any difference in the results.

Sample solution stability

According to the experimental procedure previously described, the preparation and loading of 20 samples on the plate would take approximately 30 min, which is enough time to allow for much material degradation, if it is degradable. Therefore, it was necessary to investigate the solution stability during the analysis time. The results revealed that no degradation occurred during run time, and the solution was stable for at least 2 h.

Stability-indicating study

The International Conference of Harmonization (ICH) guideline entitled "Stability Testing of Drug Substances and Products" requires stress testing to be carried out to elucidate the inherent stability characteristics of the active substance and provide a rapid identification of differences that might result from changes in the manufacturing processes or source sample (4). Susceptibility to oxidation, hydrolytic stability, and photolytic stability are the required tests. An ideal stability-indicating method is one that quantitates the standard drug alone and also resolves its degradation products. A relatively high concentration (1 mg/5 µL spot volume) of RBV was prepared in water. As described in the Experimental section, different stress conditions were applied: boiling, acid, base hydrolysis, oxidation, and irradiation with UV light. From this investigation, it was clear that in the cases of oxidation, boiling, and acid hydrolysis, the degradation profile was the same, and the same four degradation products were obtained (Figure 5). The R_f values of these products were 0.3, 0.6, 0.7, and 0.85, respectively. In the case of alkaline hydrolysis, only three degradation products (at R_f values of 0.3, 0.6, and 0.85) were produced; the degradation product of R_f 0.7 was not produced. Irradiation with UV light yielded degradation products at R_f values 0.3 and 0.6; however, the other degradation products (at R_f values of 0.7 and 0.85) were not produced. In all cases, the method was able to completely separate the degradation products from the major drug. This confirmed the selectivity and stability-indicating properties of the proposed method.

In order to estimate the concentration of the produced degradation products relative to the intact drug, one level of concentration was used for matching. Figure 5 illustrates the detection and estimation of the one-lane degradation profile obtained when boiling, UV irradiation, and alkaline hydrolysis were applied. Table II shows the calculated amounts as percentages of the degradation products in each particular stress condition. As shown, the greatest sensitivity of RBV was observed with boiling; however, the least sensitivity was observed with irradiation with UV light.

The compatibility of RBV with the exipients used was also studied in the presence and absence of moisture. The stress testing results revealed that RBV was compatible with the combined exipients, because no more degradation products were observed when the stress testing experiments carried out on RBV-containing capsules.

Application to the analysis of capsules

It is evident from the previously obtained results that the proposed method gave satisfactory results with the analysis of RBV in bulk. Thus, RBV capsules were subjected to the analysis for their contents of RBV by the proposed method as well as the official method (14). The label claim percentage was 98.75 ± 1.5%. These results were compared to those obtained from the official method by statistical analysis with respect to the accuracy (t-test) and precision (F-test). No significant differences were found between the calculated (t- and F-values were 2.02 and 4.05, respectively) and theoretical values of both the proposed and the

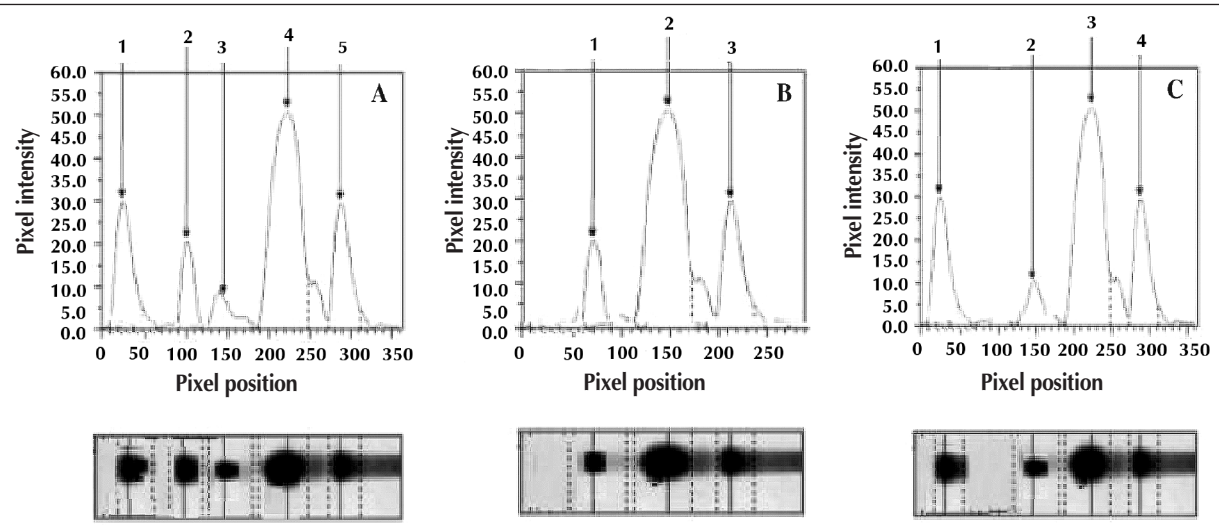

Figure 5. Chromatograms and data processed by the software for RBV subjected to boiling (A), UV irradiation (B), and alkaline hydrolysis (C).

Table II. Calculated Amounts of Degradation Products Produced by Stress Conditions Applied to Ribavirin

Stress condition	Degradation (%) for individual spots of R_f values: 0.30	0.60	0.70	0.85	Total degradation (%)
Boiling	2.3	0.4	2.3	3.2	8.2
Acid hydrolysis	1.2	0.3	1.1	1.6	4.2
Alkali hydrolysis	1.0	0.2	—*	1.0	2.2
Oxidation	1.8	1.0	1.9	2.5	7.2
UV light	0.7	0.2	—	—*	0.9

* No degradation product was produced.

reported methods at 95% confidence level; the theoretical values of t- and F- (at $n = 3$) were 3.18 and 9.01, respectively. This indicated similar accuracy and precision in the analysis of RBV in capsule form.

Conclusion

The present study represents the first report that deals with the development of a stability-indicating method for the determination of ribavirin. This study is a typical example of the development of a stability-indicating assay, established following the recommendations of ICH/Food and Drug Administration guidelines. The proposed method involved the simplest chromatographic technique, thin-layer chromatography. From an economical point of view, the method involved inexpensive equipment, a scanner and software. As well, all the analytical reagents are inexpensive and available in any analytical laboratory. Statistical analysis proved that the method is suitable for the determination of RBV in bulk and capsule forms without any interference from the degradation products, and it is recommended for routine use in quality control industry laboratories.

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Manuscript received July 4, 2007;
revision received January 29, 2007.