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Validated Stability-Indicating HPLC and HPTLC Methods for the Determination of Ritonavir in Bulk Powder and in Capsules

MOHAMMAD H. ABDELHAY, AZZA A. GAZY, RASHA A. SHAALAN* AND HEBA K. ASHOUR

Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, University of Alexandria, Alexandria, Egypt

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ABSTRACT

Two sensitive and reproducible methods were described for the quantitative determination of the antireteroviral drug ritonavir in the presence of its degradation products. The first method was based on high performance liquid chromatographic (HPLC) separation of the drug from its stress degradation products with the use of a reversed phase Agilent Eclipse XDB-C18 column (5 µm, 4.6 × 150 mm) and a mobile phase consisting of acetonitrile : 0.05 M phosphoric acid (55 : 45, v/v) at a flow rate of 1.0 mL/min. The retention time of the drug was found to be 4.82 ± 0.002 min. Quantification was achieved with diode array detection (DAD) at 210 nm based on peak area and a linear calibration curve in the concentration range of 1-500 µg/mL. The proposed method made use of diode array detection as a tool for peak purity and identification. The second method involved a high performance thin layer chromatographic (HPTLC) separation followed by densitometric measurement of the spots at 240 nm. The separation was carried out on Fluka TLC aluminium sheets of silica gel with fluorescent indicator (254) nm and the mobile phase was acetonitrile - water (1 : 2, v/v), adjusted to pH 5.0 using 1 M orthophosphoric acid solution . The proposed procedure gave compact spots for ritonavir (retention factor, R_f = 0.41 ± 0.014). The linear regression equation was generated by least-squares treatment of the calibration data in the range of 0.8-12.5 µg/spot. The reliability and analytical performance of the proposed methods, including linearity, range, precision, accuracy, detection and quantitation limits, were statistically validated. The proposed methods were applied to Norvir capsules and no chromatographic interference was observed. When ritonavir was subjected to stress conditions; according to ICH guidelines, the proposed methods could effectively separate the drug from its degradation products, and were thus considered as good stability-indicating procedures.

Key words: HPLC, HPTLC, ritonavir, soft gelatin capsules, stability-indicating

INTRODUCTION

Ritonavir (RIT, Figure 1) chemically known as [5S,8S, 10S,11S]-10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid 5-thiazolylmethyl ester[1], is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. RIT was originally developed as an inhibitor of HIV protease. It is one of the most complex inhibitors and is now rarely used for its own antiviral activity, but remains widely used as a booster of other protease inhibitors[2,3]. More specifically, RIT is used to inhibit a particular liver enzyme that normally metabolizes protease inhibitors, cytochrome P450-3A4. RIT is frequently prescribed with antiretroviral drugs, not for its antiviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors.

The analytical method for RIT is published in BP 2010[4] and USP 2011[5] , in which the bulk powder is assayed using HPLC. Literature on RIT determination focused on

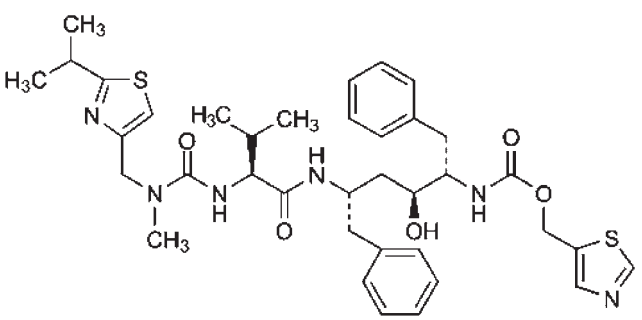

Figure 1. Structure of Ritonavir (RIT).

* Author for correspondence. E-mail:rasha_shaalan@yahoo.com

chromatographic methods, with HPLC as the predominant method, and dealt with the determination of RIT in biological fluids. Most of them are applied for the quantitation of RIT in plasma[6-13], serum[14,15], salivary fluid and CSF[16]. All these HPLC methods depended on either mass spectrometric detection[6-10] or UV detection[10-13]. Two novel methods were reported using sub 2-μm particle columns[6,8], while most of the methods made use of C18 columns[9,11-13].

Capillary electrophoresis was also one of the tools applied for the separation of RIT from other protease inhibitors[17] or the quantitation of RIT in serum[18].

Several HPLC methods had been reported for the determination of RIT and most of them involved the quantification of RIT in biological fluids[6-16]. However, none of them suggested an application to its capsules. To our knowledge, no article related to the stability-indicating chromatographic determination of RIT in pharmaceutical dosage form has been reported. Few methods studied the physical stability of RIT[19-22] and only one method has discussed its chemical stability under different conditions[23]. The ICH Q1A guideline entitled "Stability testing of new drug substances and products" requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance[24]. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, such as pH, light, oxidation and dry heat followed by separation of the drug from its degradation products. The method is expected to allow the analysis of individual degradation products. An ideal stability-indicating method is one that quantifies the standard drug alone and also resolves its degradation products. Consequently, the implementation of an analytical methodology to determine RIT in pharmaceutical dosage form in the presence of its degradation products is a pending challenge. Therefore, it was thought necessary to study the stability of RIT towards acidic, alkaline and oxidative degradation processes. The aim of this work was to develop stability-indicating chromatographic methods for the determination of RIT in the presence of its degradation products for the assessment of the purity of the bulk drug and the stability of its bulk dosage form using HPLC and HPTLC-densitometry.

The first method was based on high performance liquid chromatographic separation of the drug from its degradation products using a reversed phase, Agilent Eclipse XDB-C18 column at ambient temperature. The second method was a very viable stability-indicating one based on high performance thin layer chromatographic (HPTLC) separation followed by densitometric measurement of the spots at 240 nm. The advantage of HPTLC is that several samples could be run simultaneously using a small quantity of the mobile phase system, thus reducing analysis time and cost per analysis.

MATERIALS AND METHODS

I. *Instrumentation*

The HPLC-DAD system consisted of Agilent (Santa Clara, CA, USA) 1200 series (quaternary pump, vacuum degasser and diode array and multiple wavelength detector G1315 and G 1365 C/D) connected to a computer installed with Agilent ChemStation software. A rheodyne manual injector with a 20-μL loop was used. The column used was reversed phase Agilent Eclipse XDB-C18 (4.6 × 150 mm, 5 μm).

A CAMAG Linomat TLC-Applicator and a CAMAG Linomat syringe (100 μL) were used for the application of solutions onto silica gel/TLC cards (DC-Alufolien-Kieselgel with fluorescent indicator 254 nm, layer thickness of 0.2 mm, FlukaChemica®) of size 200 × 200 mm. The plates were developed in CAMAG twin trough chromatographic tanks (150 × 200 × 300), and scanned densitometrically using CAMAG TLC-Scanner 3 (Version 4.06), supported with UV-lamp short wavelength 254 nm and interfaced to an IBM computer installed with CAMAG-TLC-Software (CATS).

II. *Materials*

The authentic sample of Ritonavir (RIT) was kindly provided by Pharco Pharmaceuticals, Alexandria, Egypt. Analytical grade orthophosphoric acid, sodium hydroxide, hydrochloric acid and 30% hydrogen peroxide were used. HPLC grade acetonitrile (LAB-SCAN analytical sciences, Ireland) was used. Point forty-five micrometer cellulose nitrate membrane filters (Sartorius Stedium Biotech GmbH 37070 Goettingen, Germany) were used. Norvir® capsules labeled to contain 100 mg of ritonavir, (Abbott Diagnostics, Austria) were assayed in this study.

III. *General Procedures and Calibration Graphs*

(I) *HPLC Method*

The mobile phase was prepared by mixing acetonitrile: 0.05 M Phosphoric acid (55 : 45, v/v). The mobile phase was filtered and degassed by passing through a 0.45-μm pore size membrane filter prior to use. The flow rate was 1.0 mL/min and the injection volume was 20 μL, the eluent was monitored using diode array detection (DAD) from 190 nm to 400 nm, and chromatograms were recorded at 210 nm. All determinations were performed at room temperature.

RIT stock standard solution (12.5 mg/mL) was prepared in acetonitrile. RIT working standard solution (0.5 mg/mL) was prepared by dilution of 1 mL of RIT stock standard solution with acetonitrile into a 25-mL volumetric flask. The working solutions were prepared by dilution of the standard solutions with the mobile phase to obtain a concentration range of 1-500 μg/mL. Triplicate 20-μL injections were made for each concentration and chromatographed under the described LC conditions. The peak areas were plotted against the corresponding concentrations to obtain the calibration curve.

(II) *HPTLC Densitometry*

The final solutions were prepared by dilution of accurate volumes of the RIT stock standard solution (12.5 mg/mL) with acetonitrile to obtain a concentration range of 0.8-12.5 µg/spot.

1. *Sample Loading*

The solutions were applied to the marked start edge of the TLC plate at a height of 15 mm from the lower edge of the plate using the specified TLC CAMAG linomat syringe. The sample volume for all solutions was 5 µL and spotting was performed in the form of bands of 6-mm width. Each solution was applied in duplicate and allowed to air-dry for 15 min.

Table 1. Analytical parameters for the determination of RIT using the proposed chromatographic methods

Parameter	HPLC	HPTLC
Linearity Range	1-500 (µg/mL)	0.8-12.5 (µg/spot)
Intercept(a)	46.78	1143.11
% y-intercept[1] (Peak area at 100% target concentration)	0.50 (9327.5)	12.35 (9255.4)
Slope (b)	46.90	1622.46
RSD% of slope	0.21	1.12
Correlation Coefficient(r)	0.99998	0.99968
S_a[2]	20.91	123.24
S_b[3]	0.01	18.16
$S_{y/x}$[4]	56.22	215.10
LOD	0.14 (µg/mL)	0.25 (µg/spot)
LOQ	0.47 (µg/mL)	0.76 (µg/spot)

[1] $\%\text{y-intercept} = \frac{\text{y-intercept}}{\text{peak area at 100\% target concentration}} \times 100$

[2] S_a: Standard deviation of intercept

[3] S_b: Standard deviation of slope

[4] $S_{y/x}$: Standard error

2. *Chromatogram Development*

The mobile phase used was acetonitrile-water in the volume ratio 1 : 2, adjusted to pH 5.0 using 1 M orthophosphoric acid solution. Twenty milliliters of the mobile phase were poured into the TLC chamber that was lined with a thick filter paper on a single side to assist in the chamber saturation. The chamber was covered with a lid and pre-saturated with the mobile phase for 15 min at room temperature (25 ± 2°C). The sample-loaded TLC plates were transferred to the chamber and then developed ascendingly for not less than 18-cm of migration distance of the mobile phase from the start line (approximately 40 min). The developed TLC plates were air dried for about 15 min.

3. *Chromatogram Scanning and Data Processing*

The dried plates were densitometrically scanned at 240 nm. The TLC chromatogram was captured by the scanner and loaded into the CAMAG TLC software. In the software, the series of spots were selected as tracks. Each track was evaluated based on the position of the spot and its peak area. Quantity calibration was then performed by pre-assignment of the concentration of the investigated drug. The calibration graph correlated the concentration to the corresponding peak area.

The accuracy and precision study was performed for both HPLC and HPTLC by preparing three replicates for three selected concentrations of RIT (Table 2). Aliquots of the stock solution of RIT were diluted with the mobile phase to reach the selected concentrations. Solutions were either injected into the column, or applied to the TLC plate and then assayed using the appropriate procedure, and the recovered concentrations were calculated from the previously constructed calibration graphs.

IV. *Applications*

(I) *Forced Degradation and Stability Indicating Study of RIT*

Table 2. Accuracy and precision for the analysis of RIT in bulk form using the proposed chromatographic methods

A) HPLC-DAD Method

Results / Conc[n.]	Intra-day(n = 9)			Inter-day(n = 9)		
Nominal value (µg/mL)	Found ± SD (µg/mL)	RSD (%)	Er(%)	Found ± SD (µg/mL)	RSD (%)	Er(%)
20	20.42 ± 0.059	0.29	2.01	20.36 ± 0.663	0.65	1.80
60	60.74 ± 0.555	0.91	1.24	60.97 ± 1.022	1.68	1.61
100	102.58 ± 0.424	0.41	2.58	102.67 ± 0.445	0.43	2.67

B) HPTLC Method

Results / Conc[n.]	Intra-day(n = 9)			Inter-day(n = 9)		
Nominal value (µg/spot)	Found ± SD (µg/spot)	RSD (%)	Er(%)	Found ± SD (µg/spot)	RSD (%)	Er(%)
1.0	1.01 ± 0.012	1.21	1.10	1.03 ± 0.026	2.54	2.95
2.5	2.48 ± 0.024	0.98	-0.70	2.53 ± 0.024	0.94	1.09
10.0	10.08 ± 0.123	1.22	0.81	10.18 ± 0.114	1.12	1.76

Forced degradation studies under different conditions were carried out on the RIT standard according to the following conditions:

1. *Acidic and Basic Conditions (for HPLC and HPTLC)*

RIT solutions were treated with 1 mL of 1 M methanolic HCl or 1 M methanolic NaOH. A set of solutions was kept at room temperature for 24 h and another set was placed in a water bath at 85°C for 2 h. After the specified time intervals, all solutions were neutralized by adjusting the pH to 7.0 and then diluted with the mobile phase to reach a final concentration of 50 µg/mL of RIT.

2. *Oxidation with H_2O_2 (for HPLC Only)*

RIT solutions were treated with 1 mL of hydrogen peroxide 30%. One solution was kept at room temperature for 24 h and another was placed in a water bath at 85°C for 2 h. Both solutions were kept protected from light. After the specified time intervals, the solutions were diluted with the mobile phase to reach a final concentration of 50 µg/mL of RIT. After the previous treatments, the solutions were filtered with a 0.45-µm filtration disc prior to injection to the column.

(II) *Assay of Norvir Capsules*

Norvir® capsules are soft gelatin capsules that contain 100 mg of RIT. One capsule was punctured and the liquid content was carefully squeezed into a beaker. The soft gelatin shell was washed with acetonitrile into the beaker, sonicated for 30 min and the solution was delivered quantitatively into a 100-mL volumetric flask. The beaker was washed with acetonitrile and the wash was transferred into the flask. The volume was finally diluted to 100 mL with acetonitrile. For HPLC, further dilutions were made to obtain solutions of final concentration within the linearity range 1-500 µg/mL, and the general procedure was then followed. For HPTLC, 5 µL of Norvir® sample solution was applied to the plate concurrently with RIT standard solution. The TLC plates were then treated in a manner similar to the previously described procedure.

RESULTS AND DISCUSSION

I. *HPLC Method*

A stability-indicating HPLC-DAD method was developed to provide rapid and reliable quality control analysis of RIT in soft gelatin capsules. The method involved the use of RP-C18 column with isocratic elution using a mobile phase consisting of acetonitrile : 0.05 M phosphoric acid (55 : 45, v/v). Using a mobile phase flow rate of 1.0 mL/min, RIT displayed typical peak characteristics at 210 nm with acceptable retention time (t_R = 4.82 min) (Figure 2). The proposed method had the advantage of using a simple mobile phase without the need for pH adjustment.

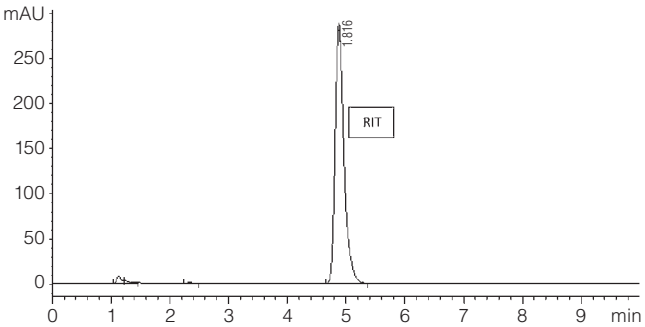

Figure 2. Typical HPLC chromatogram of a 20-µL injection of 60 µg/mL RIT at 210 nm.

(I) *Optimization of Chromatographic Conditions*

Several mobile phases were tried using various proportions of different aqueous phases and organic modifiers. The best chromatogram was obtained using the above mentioned mobile phase (Figure 2). Decreasing the acetonitrile content in the mobile phase led to longer retention times and excessive peak tailing. Increasing the acetonitrile content yielded a RIT peak that was very close to the solvent peak. Methanol was tried as an organic modifier and different phosphoric acid strengths were tried (0.025 and 0.075 M). Phosphoric acid was substituted by acetic acid in some trials. In all these trials, the chromatograms showed broad asymmetric peaks and/or increased retention times and, consequently, fewer theoretical plates for the eluted peaks. Quantitation was achieved using DAD based on peak area measurements. RIT exhibits UV absorption mainly below 260 nm. The previously described chromatographic conditions showed symmetrical peaks at 4.82 ± 0.002 min, with retention factor (K') of 1.86 and column performance (apparent efficiency expressed by the number of theoretical plates, N) of 5930. The resolution of standard RIT in the presence of degradation products was satisfactory. Ultimately, a mobile phase consisting of acetonitrile : 0.05 M phosphoric acid (55 : 45, v/v) was selected for validation purposes and stability studies.

(II) *Stability Indicating Aspects*

According to ICH guidelines, forced degradation experiments were carried out on RIT in order to produce the possible relevant degradants and test their chromatographic behavior using the optimized HPLC method.

Hydrolytic degradation studies using both strong acidic and basic media as well as oxidative degradation studies on RIT were conducted either at room temperature or with the aid of heating. In strong acidic medium and with the aid of heating, degradation of RIT was observed from the reduction of its peak area at 4.82 ± 0.002 min which decreased by 88% of the expected intact area and the appearance of a major degradation peak at about 5.47 min. In comparison keeping the drug in contact with HCl for a long period of time (24 h) at room temperature resulted in limited degradation. In both

cases, minor degradation products co-eluted with the solvent peak at about 1.192 and 2.3 min. Figure 3A and 3B show the chromatograms of RIT after treatment with 1 M HCl.

Similarly, sodium hydroxide attacked RIT and a major degradation product peak eluted at about 5.47 min. Other minor degradation products peaks eluted in the time range between 1.2 and 1.8 min. Degradation increased when the drug solution was kept in contact with NaOH for 24 h at room temperature observed from the peak areas of RIT at 4.82 ± 0.002 min and that of the major degradation peak at 5.47 min (Figure 4A and 4B).

It was noteworthy to mention that the major degradation peak eluted at 5.47 min for both acidic and basic degradation in which an absorption spectrum very similar to that of the RIT intact drug was observed. This suggested that the drug chromophore was not altered. In a previous report discussing the stability of RIT using LC-MS-MS[23], it was reported that the drug was degraded due to the presence of carbamate and urea linkage, which were susceptible to hydrolysis.

The situation was different in the case of oxidative degradation. RIT was relatively resistant to degradation with hydrogen peroxide at room temperature as indicated from its peak area compared to that of the standard of the same concentration, while upon heating at 80 ± 5°C for 2 h, RIT underwent degradation up to 82%. Figures 5A and 5B illustrate the chromatograms of RIT after treatment with 30% hydrogen peroxide.

In all these forced degradation experiments, RIT was successfully separated from all the degradation products as confirmed by the resolution values calculated for each

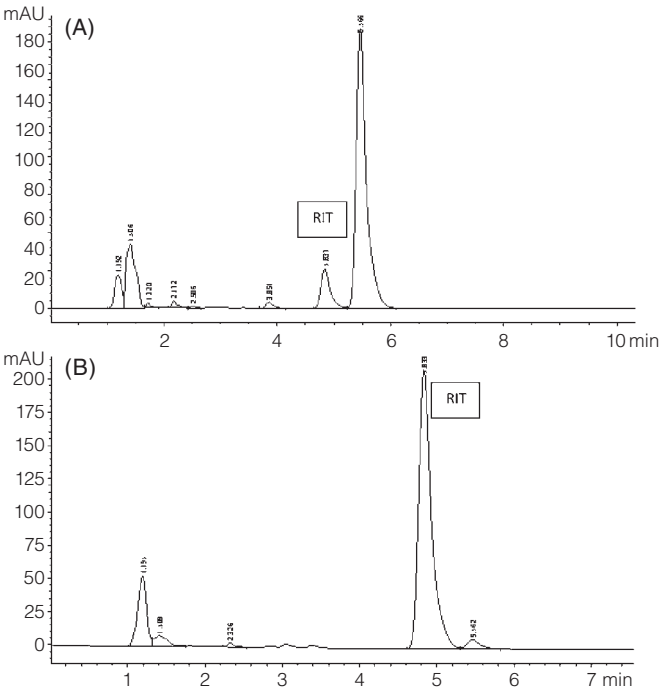

Figure 3. HPLC chromatograms of 50 µg/mL RIT after exposure to acid degradation with the aid of heating (A) and at room temperature (B).

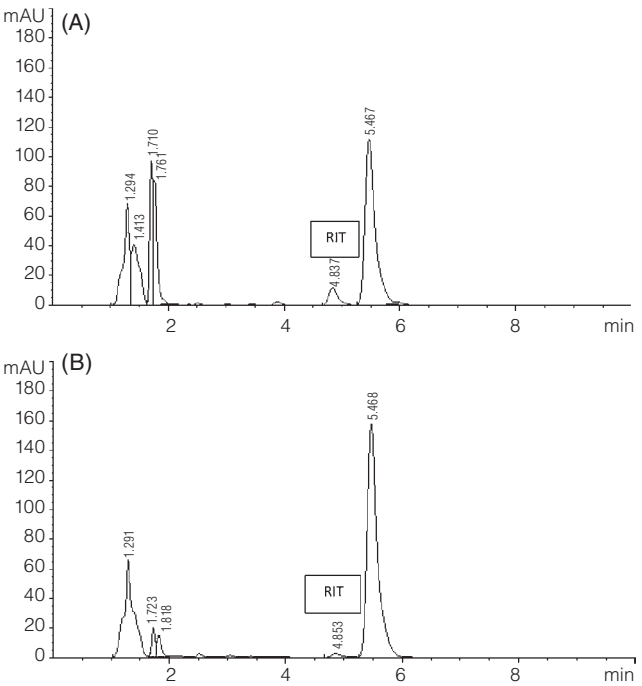

Figure 4. HPLC chromatograms of 50 µg/mL RIT after exposure to alkaline degradation with the aid of heating (A) and at room temperature (B).

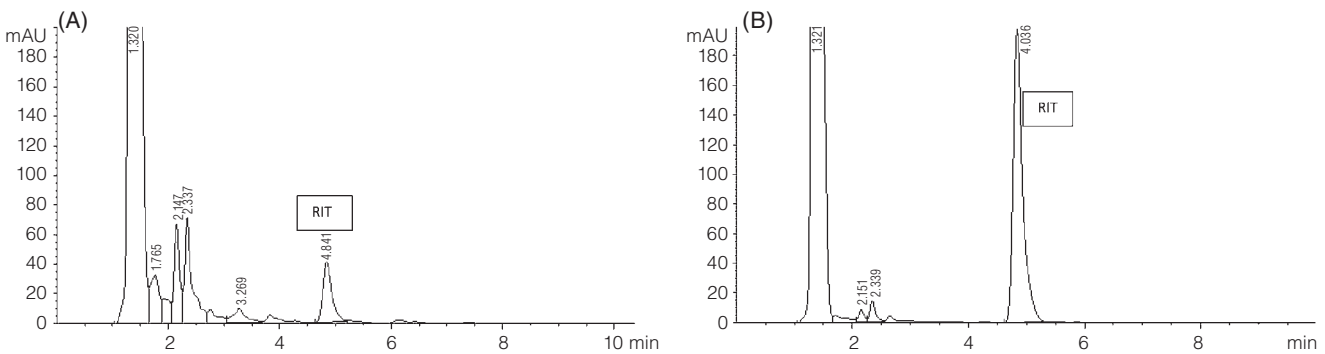

Figure 5. HPLC chromatograms of 50 µg/mL RIT after oxidative degradation with the aid of heating (A) and at room temperature (B).

chromatogram (> 2). In addition, the identity and purity of RIT were confirmed with the use of DAD, and no signs of co-elution from any of the degradation products was detected.

II. *HPTLC-Densitometric Method*

The present work is concerned with the application of the HPTLC densitometric technique in the determination of RIT in its pure form and in capsules. The initial method development was conducted on the pure drug using a working standard solution. A number of preliminary trials were performed to establish the final chromatographic conditions. Different mobile phase systems were tested, including acetonitrile/methanol (1 : 1, v/v), acetonitrile/water (1 : 2, v/v) and acetonitrile/phosphate buffer in three different ratios. Acetonitrile/water (1 : 2, v/v) adjusted to pH 5 was found to give optimum separation between pure RIT and its degradation products. The effect of pH on the separation ability of the mobile phase was investigated by changing the pH from 3 to 6 at one pH unit interval. The best resolution and sharp peaks were obtained at pH 5.0. In addition, the proportion of acetonitrile in the mobile phase was changed from 10 to 70%, and the best results were obtained using acetonitrile/water (1 : 2, v/v). The proposed procedure gave compact spots for RIT with an R_f of 0.41 ± 0.01 (Figure 6).

(I) *Stability Indicating Aspects*

An ideal stability-indicating assay method is one that quantifies the standard drug alone and resolves its degradation products. As described in the experimental section different stress conditions were applied, including acidic and alkaline degradation.

The densitogram of the acid-degraded RIT solution under heating conditions showed two degraded peaks at R_f = 0.68 and R_f = 0.75 (Figure 7). The concentration of the drug was found to change from the initial concentration as confirmed by a reduction in the peak area by 75% of the expected response, hence indicating that RIT undergoes degradation under acidic conditions.

On the other hand, degradation in acidic medium at room temperature for 24 h showed reduced degradation. Only one

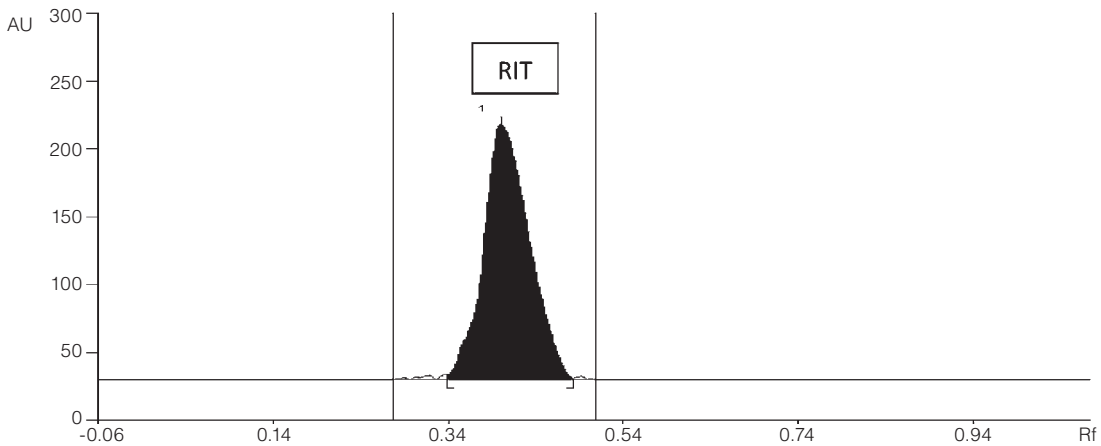

Figure 6. Densitogram of standard RIT (7.5 µg/spot-5-µL band), peak 1 (R_f = 0.41 ± 0.01).

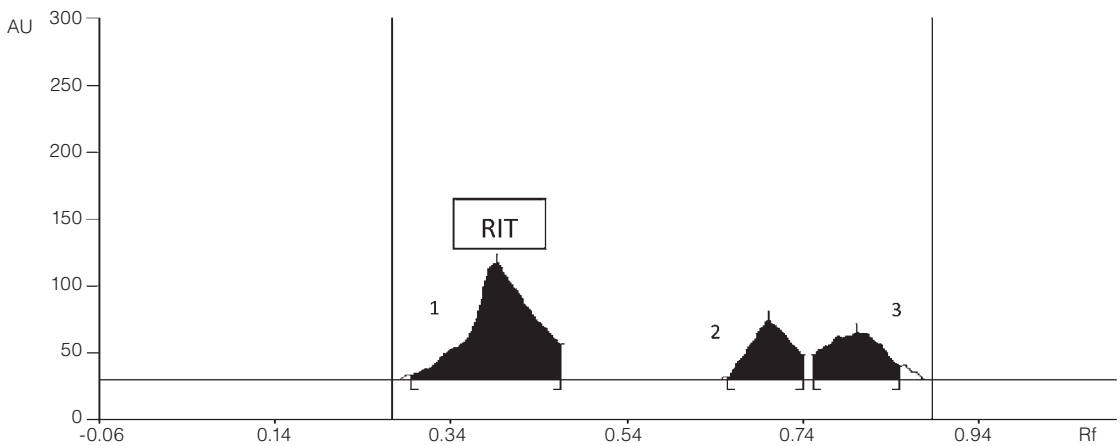

Figure 7. Densitogram of acid (1 M HCl, 80° C for 2 h)-treated RIT (2.5 µg/spot); peak 1 (standard, R_f = 0.41), peak 2 (degraded, R_f = 0.68), peak 3 (degraded, R_f = 0.75).

additional peak appeared at R_f = 0.60 (Figure 8), and the peak area of the intact RIT drug suffered from only 10% reduction.

The samples degraded in alkaline medium, with the aid of heating at 80°C for 2 h, showed additional peaks at R_f values of 0.55 and 0.73 (Figure 9). The spots of the degraded products were well-resolved from the drug spot. Similarly, the densitogram of the alkaline-degraded RIT solution at room temperature for 24 h (Figure 10) showed an additional spot at R_f = 0.73. The reduction in the peak area was almost up to 80%. The above experimental findings indicate that RIT is susceptible to acid-base hydrolysis. The higher R_f values of acid and base-degraded components indicated that they were more polar than the analyte itself.

III. *Statistical Analysis of Results*

(I) *Concentration Ranges and Calibration Graphs*

Under the optimal experimental chromatographic conditions, linear relationship exists between the integrated peak area and the corresponding concentration of RIT. The performance data and statistical parameters including linear regression equations, concentration ranges, correlation coefficients (r) and other statistical parameters such as the standard deviations of the intercept (S_a), the slope (S_b) and standard deviations of residuals ($S_{y/x}$) are listed in Table 1 for both the HPLC and HPTLC methods. Regression analysis for the calibration curves showed good linear relationships over the concentration range of 1-500 μg/mL as judged by the correlation coefficient value (r = 0.99998) for HPLC and over the concentration range of 0.8-12.5 μg/spot as judged by the correlation coefficients value (r = 0.99968) for HPTLC, the RSD% of slope did not exceed 0.21 and 1.12% for HPLC and HPTLC, respectively, and the y-intercept values were less than 2% of the response for the target value of the analyte[25].

Unlike HPLC methods, for which linearity of detector response over a wide range of concentration of analyte can be obtained, the calibration curve of UV-detector response versus a wide range of concentration for HPTLC often does not follow linear regression. With HPTLC, the analyte

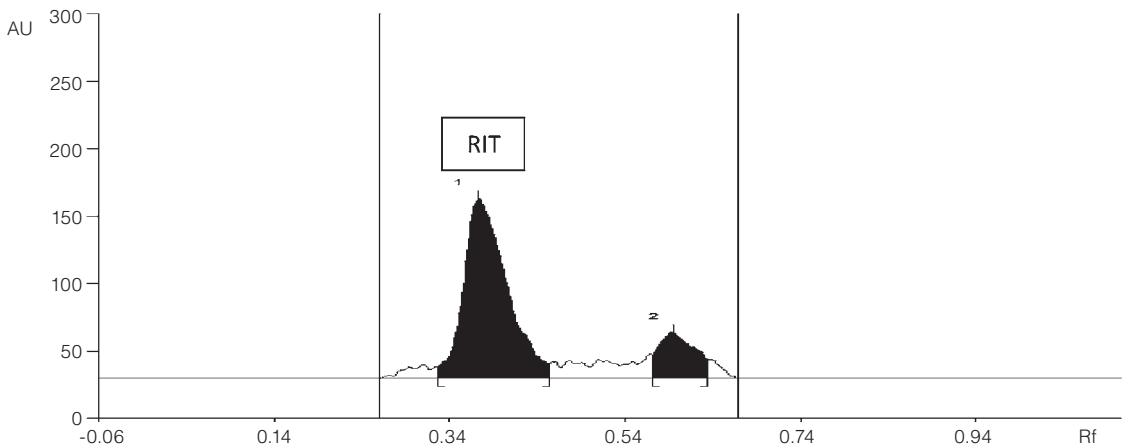

Figure 8. Densitogram of acid (1 M HCl, ambient temperature for 24 h)-treated RIT (2.5 μg/spot); peak 1 (standard, R_f = 0.41), peak 2 (degraded, R_f = 0.60).

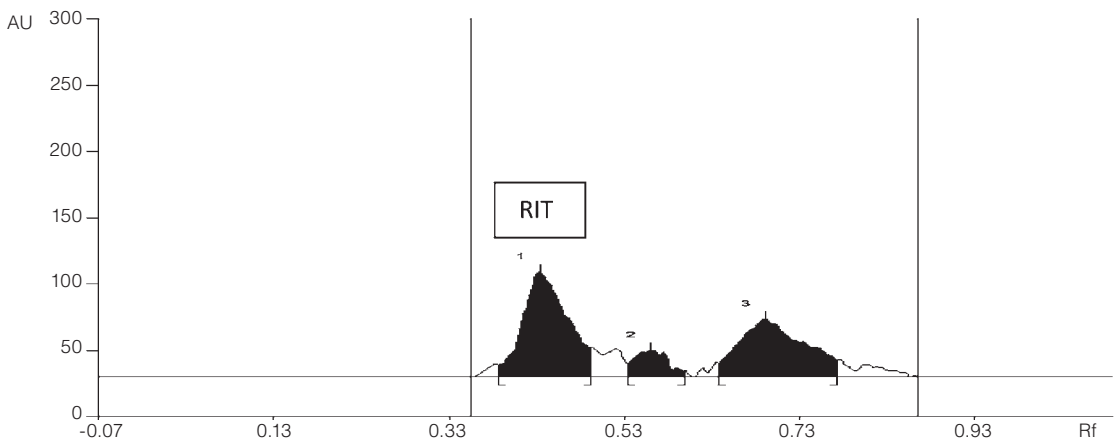

Figure 9. Densitogram of base (1 M NaOH, 80°C for 2 h)-treated RIT (2.5 μg/spot); peak 1 (standard, R_f = 0.41), peak 2 (degraded, R_f = 0.55), peak 3 (degraded, R_f = 0.73).

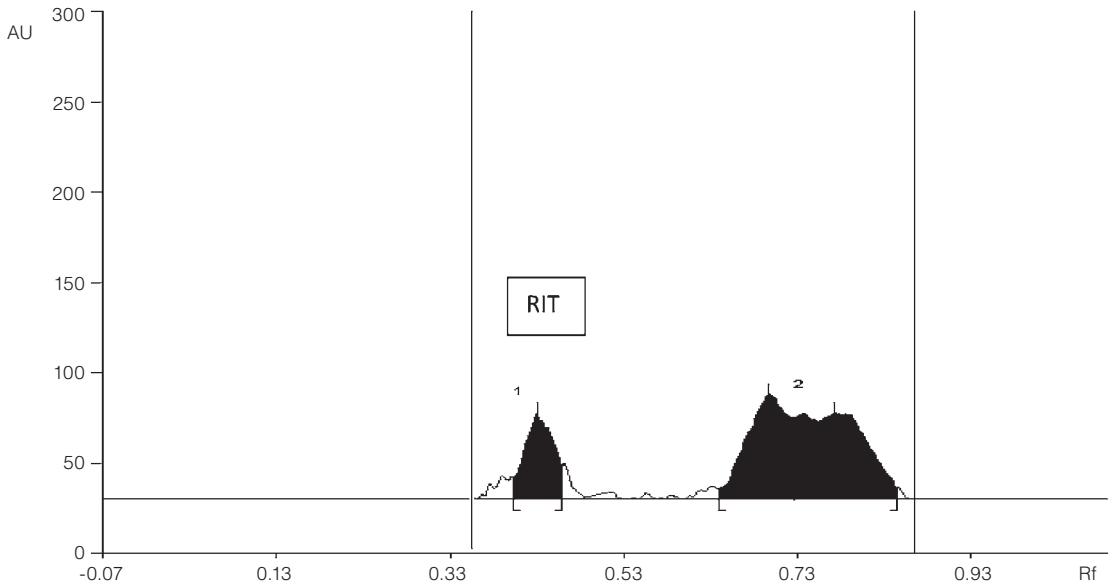

Figure 10. Densitogram of base (1 M NaOH, ambient temperature for 24 h)-treated RIT (2.5 µg/spot); peak 1 (standard, R_f = 0.41), peak 2 (degraded, R_f = 0.73).

interacts with the surface of the stationary phase where scattering and absorption tend to take place with high concentration of analyte[26].

(II) *Detection and Quantitation Limits*

The limit of detection (LOD) was defined as the concentration which has a signal-to-noise ratio of 3 : 1. For the limit of quantitation (LOQ), the ratio considered was 10 : 1. The LOD and LOQ values of RIT are given in Table 1. The LOQ value was verified by its nearness to the lower concentration of the working range (1 µg/mL) for HPLC. Both LOD and LOQ values confirmed the sensitivity of the proposed HPLC procedure.

For HPTLC, the limit of detection (LOD) and the limit of quantitation (LOQ) were mathematically calculated according to the formulae[27]:

$$LOD = \frac{3.3 \times \sigma}{S}$$

$$LOQ = \frac{10 \times \sigma}{S}$$

where σ is the standard error and S is the slope of the calibration plot. These values are presented in Table 1 and indicated high sensitivity of the proposed HPTLC method.

(III) *Precision and Accuracy*

The precision and accuracy were examined at three concentration levels for the analyte (20, 60 and 100 µg/mL for HPLC; 1.0, 2.5 and 10.0 µg/spot for HPTLC) and five replicate determinations for each concentration. The assay results performed on the same day and on three consecutive days showed good precision and accuracy as indicated by the RSD% and Er% values (Tables 2A and 2B).

(IV) *Robustness*

The robustness of an analytical procedure is a measure of its capability to remain unaffected by small but deliberate variations in method parameters. This was performed in the case of the HPLC method by making small changes in the mobile phase composition (± 2%), flow rate (± 0.1 mL/min) and working wavelengths (± 2 nm). These variations did not have any significant effect on the measured responses or the chromatographic separation. The RSD% of the measured peak areas for the studied variations were found to be 0.23, 0.48 and 0.66 when changing the mobile phase composition, flow rate and working wavelength were changed, respectively. The studied changes in the case of HPTLC were the acetonitrile content in the mobile system (± 10%) and the time for saturation of the development chamber (± 5 min). The RSD % of the measured peak areas for the studied variations was found to be 0.77 and 0.90, respectively.

(V) *Specificity and Selectivity*

Specificity of the HPLC and HPTLC methods were assessed by comparing the chromatograms obtained from standard solutions with those obtained from sample solutions of capsules. As the retention time t_R and R_f values of the standard drug and the drug in the capsule test solution were the same, this indicated that the methods were specific. On the other hand, the use of photodiode array detector in the case of HPLC allowed the confirmation of the specificity of the method by comparison with the reference drug spectrum.

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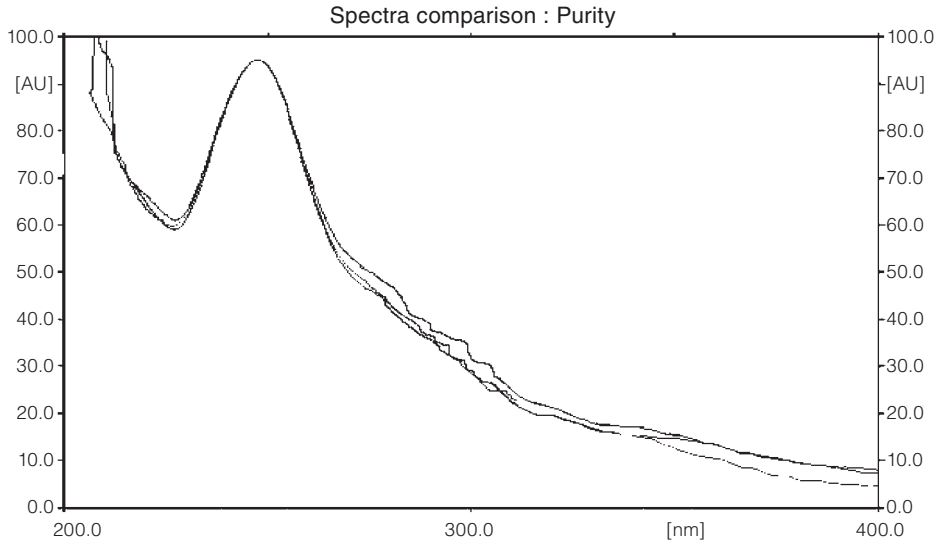

Figure 11. UV spectra of the HPTLC eluted peak due to RIT at the peak start, apex and peak end.

In addition the peak purity of RIT was assessed in the case of HPTLC by comparing its respective spectra at the peak start, apex and peak end positions of the spot (Figure 11). The specificity of the method was performed by analyzing the drug standard and sample solutions of capsules. The spot of RIT was compared with its R_f values in both cases.

Selectivity was demonstrated by the separation of RIT from forced degradation products and formulation additives in both methods. The use of the photodiode array detector in HPLC allowed the confirmation of the selectivity of the method by comparison with the reference drug spectrum. The method proved to be selective in the separation of the investigated drug. Selectivity was also demonstrated by the separation of RIT from forced degradation products and formulation additives.

To check the selectivity of the proposed HPTLC method, the ability of the mobile phase to resolve the major compound from possible degradation products was tested. A sample containing the forced degradation solution was applied to the plate and analyzed concurrently with two different concentrations of the intact standard solutions. The results revealed that the proposed method could completely discriminate the major intact drug from the forced degradation products, hence proving the selectivity of the method.

(VI) *Stability of Standard and Sample Solutions*

The stability of the working solutions of the analyte in the HPLC mobile phase was examined, and no chromatographic changes were observed within 5 h at room temperature. In the case of HPTLC, there was no indication of compound instability in the sample solution, when the freshly-prepared solutions and those stored under refrigeration (4°C) for 12 h, 24 h and 7 days were applied on the same HPTLC plate. The densitograms were evaluated after development and no additional spots were detected. The stock solutions were stable for at least one week when kept under refrigeration.

Table 3. Application of the proposed chromatographic methods for the determination of RIT in Norvir® soft gelatin capsules

Results \ Method	HPLC method	Comparison referee method[d]	HPTLC method
%Recovery ± SD[a]	98.03 ± 0.71	98.34 ± 0.61	97.34 ± 0.77
RSD%[b]	0.72	0.62	0.79
Er%[c]	-1.97	-1.66	-2.66
Variance (S^2)	0.50	0.37	0.59
t-calculated	0.73		2.27
F-calculated	1.32		1.56

[a] Mean % recovery ± SD for five determinations.
[b] % relative standard deviation.
[c] % relative error.
[d] Comparison Referee HPLC method[(11)].
Theoretical values for t and F at $P = 0.05$ are 2.31 and 6.39, respectively.

IV. *Assay of Capsules*

The developed stability-indicating HPLC and HPTLC procedures were applied for the assay of RIT in soft gelatin capsules. Table 3 shows the results obtained for the proposed methods and a comparison referee HPLC method[(11)]. The results obtained by the proposed methods were statistically compared with those of the comparison referee method using the Student's *t*-test and the variance ratio *F*-test. The calculated *t*- and *F*-values did not exceed the theoretical values which indicated a good agreement between the proposed and comparison referee methods. No interfering peaks were observed in the chromatograms or densitograms of RIT capsules.

CONCLUSIONS

A simple and rapid HPLC-DAD assay method has been developed for the quality- control and quantitation of RIT capsules. The method can be used to determine concentrations as low as 1 µg/mL. It can also be used as a quality control tool to monitor the exact content of RIT capsules. Moreover, the method was extended to study the degradation behavior of RIT under stress hydrolytic and oxidative conditions. Few reports were published concerning the degradation behavior of RIT and its forced degradation products, of which, one recent report used LC-MS-MS[23]. The current study is comparable to the reported work[23]. In addition, the active pharmaceutical ingredient eluted earlier at 4.82 min in the proposed study compared to 9.0 min.

The proposed HPTLC method is simple, accurate and stability-indicating as it separates the drug from its degradation products. The method was used for the determination of RIT in soft gelatin capsules, without any interference from excipients and in the presence of its acidic and alkaline degradation products. The chromatographic method was validated as per ICH guidelines. The HPTLC method uses a minimal volume of solvents and is suitable for use in quality control laboratories where time and cost are factors of concern.

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