Original Article

TJPS
The Thai Journal of Pharmaceutical Sciences
39 (2), April-June 2015: 35-63

Development and validation of a stability-indicating ultra-performance liquid chromatographic method for the determination of rifampicin in bulk drug and capsules

N. Swamy[1], K. Basavaiah[1*], P. Vamsikrishna[1] and G. Krishnamurthy[2]

[1]*Department of Chemistry, Manasagangotri, University of Mysore, Mysuru-570 006, Karnataka, India*
[2]*Department of Chemistry, PES College of Science, Arts and Commerce, Mandya-571 401, Karnataka, India*

Abstract

A rapid method based on ultra-performance liquid chromatography (UPLC) was developed and validated for the specific determination of anti-tuberculosis drug rifampicin (RIF) in bulk form and capsules. The UPLC determination was carried out on a reversed-phase Waters Acquity UPLC BEH C_{18} (100 × 2.1 mm; 1.7 µm particle size) column within a short run time of 5.0 min using a mobile phase consisting of a mixture of formic acid buffer of pH 4.5 and acetonitrile (40:60 v/v) at a flow rate of 0.4 mL/min with UV-detection at 235 nm. Calibration graph was linear from 5.0 to 80 µg/mL RIF with regression coefficient (r) value of 0.9997. The limit of detection (S/N = 3) and limit of quantification (S/N = 10) were 0.03 and 0.09 µg/mL, respectively. The method was validated according to current *ICH* guidelines including the assay of pharmacopoeial standard capsules. Intra-day and inter-day precisions were 1.05 - 1.48 % and 1.02 - 1.52 % respectively; and the accuracy expressed as % relative error was ≤ 2.02 %. The developed UPLC method is comparable in terms of accuracy and precision with that of a published method. To demonstrate the stability-indicating ability of the method, RIF active substance was subjected to acid-, base-, H_2O_2-, heat- and light-induced degradation; and the study revealed that the drug was highly susceptible to acid- and peroxide-induced degradation, and moderately susceptible to base-induced degradation, but remained stable to other stress conditions. The method provided rapid, sensitive and reproducible quantification of RIF to facilitate drug monitoring during treatment.

Keywords: Rifampicin, UPLC, Stability-indicating, Assay, Capsules

Introduction

Being potentially hepatotoxic rifampicin (RIF) [Figure 1], 3-[(4-methyl-1-piperazinyl) imino] methyl rifamycin SV, is an established first-line anti-tuberculosis agent derived from rifampicin SV and its use in other serious infections, such as HIV, is increasing [1-3]. It is metabolized in liver mainly by deacetylation and is excreted with its metabolites in bile. Hence, care must be taken when RIF is used in patients with liver disease and in such circumstances, monitoring the serum concentrations of RIF is of value in optimizing the dose [4, 5].

Different techniques have been in general use for the determination of RIF in pharmaceutical products. Methods for the quantification of RIF in pharmaceutical

Correspondence to: K. Basavaiah, UGC-BSR Faculty Fellow
Department of Chemistry, University of Mysore,
Manasagangotri, Mysuru-570006, Karnataka, India
Tel: +91 82 1241 9659; FAX: +91 82 1251 6133
Email: kanakapurabasavaiah@gmail.com

Received: 08 March 2015
Revised: 20 May 2015
Accepted:22 June 2015

Academic Editor: Pornchai Rojsitthisak

dosage forms in association with other antituberculosis drugs such as isoniazid and pyrazinamide are based on techniques such as UV-spectrometry and its variants [6-12], linear sweep and cyclic voltammetry [13], differential pulse polarography [14, 15], horseradish peroxidase-based amperometry [16], continuous flow-chemiluminescence spectrometry [17, 18], HPTLC [19, 20], and HPLC [21, 22].

RIF in bulk drug and dosage form when present alone, has been assayed by visible spectrophotometry [23-33], UV-spectrophotometry [34], nuclear magnetic resonance spectrometry [35], differential pulse polarography [36, 37] and HPLC [38].

HPLC is perhaps the most widely used technique for assaying drugs in dosage forms. However, analysis with HPLC with UV-detection could be significantly improved by the use of ultra-performance liquid chromatography (UPLC). UPLC is a relatively new technique using analytical bridged ethane hybrid (BEH) C_{18} columns packed with 1.7 µm particles, which offers the advantages of increased speed, improved sensitivity, selectivity and specificity compared to HPLC [39]. The literature reveals that, despite these advantages, UPLC has not been applied to the determination of RIF in pharmaceuticals.

International Conference on Harmonization (*ICH*) guidelines [40] recommend performing stress-testing of the drug substance that can help in identifying the likely degradation products, in establishing the degradation pathways, and validating the stability-indicating power of the analytical method used [41]. Stability-indicating methods can be used for evaluating the drug in the presence of its degradation products, excipients and additives [42]. However, to the best of our knowledge, none of the reported methods for RIF is stability-indicating. Therefore, in the present study, the development and validation of a stability-indicating UPLC/UV method for the determination of RIF in bulk drug and capsules has been described. The developed method was validated in accordance with the *ICH* guidelines [43].

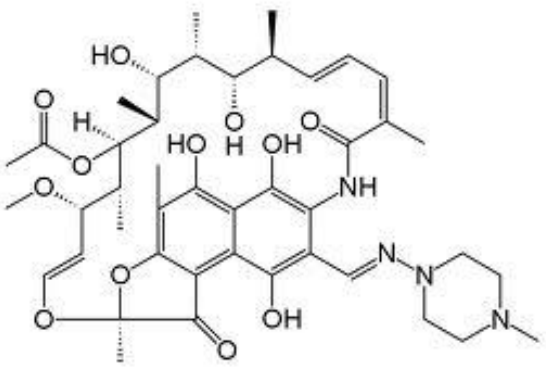

Figure 1 Chemical structure of rifampicin

Materials and methods

Reagents and materials: Analytical reagent grade triethylamine (Qualigens Fine Chemicals, Mumbai, India), HPLC grade acetonitrile, chloroform, formic acid and analytical reagent grade sodium hydroxide, hydrochloric acid, hydrogen peroxide were purchased from Merck Pvt. Ltd. Mumbai, India. Doubly distilled water filtered through a 0.45 µm filter (Millipore India Pvt. Ltd., Bangalore) was used throughout the investigation. Sodium hydroxide solution (NaOH, 5M) was prepared by dissolving required quantity in water. Hydrochloric acid (HCl, 5M) was prepared by appropriate dilution of concentrated acid (Sp. gr. 1.18) with water. A 3% solution of H_2O_2 was prepared by diluting required volume of the commercially available 30% reagent with water.

Pharmaceutical grade RIF (99.9% purity) was procured from Lupin Limited, Tarapur, Maharashtra, India, and was used as received. R-Cin 300 and R-Cin 450 capsules (Lupin Limited, Chikaltana, Aurangabad, India) were purchased from local commercial sources.

Apparatus: UPLC analysis was performed using a Waters Acquity UPLC separation module equipped with binary solvent delivery pump, an auto sampler and tunable UV (TUV) detector (Waters Corporation, Milford, USA). The output signal was monitored and processed using Empower-2 software. The Chromatographic column used was Waters Acquity UPLC BEH C_{18} (100 × 2.1 mm; 1.7 µm). Isocratic elution process was adopted throughout the analysis. The pH of the solutions was measured by pH meter (Control Dynamics, Model APX 175E/C, Digital pH meter). For the sample solution preparation, an ultrasonic instrument was used for sonification.

Mobile phase preparation: Two mL of formic acid was dissolved in 1000 mL of water, pH adjusted to 4.5 with triethylamine. A 40 mL portion of this resulting buffer was mixed with 60 mL of acetonitrile (40:60 v/v), shaken well and filtered using 0.22 µm nylon membrane filter, the resulting solution was used as solvent system for chromatography.

Instrumental parameters: Chromatographic separation was achieved on Waters Acquity UPLC BEH C_{18} (100 × 2.1 mm; 1.7 µm) and the isocratic flow rate of mobile phase was maintained at 0.4 mL/min. The column temperature was kept to 30 °C. The injection volume was 2 µL. Eluted sample was monitored at 235 nm and the run time was 5 min and the retention time of the analyte was about 1.943 min.

Preparation of standard RIF solution: Milli-Q water and acetonitrile in the ratio 50:50 v/v was used as a diluent. A stock standard solution containing 100 µg/mL RIF was prepared by dissolving accurately weighed 10 mg of drug in 100 mL diluent in a calibrated flask and filtered through a 0.22 µm nylon membrane filter.

Procedures:

Procedure for calibration graph: Working standard solutions equivalent to 5.0 - 80 µg/mL of RIF were

prepared by transferring 2.5 - 40 mL of stock standard solution (100 µg/mL) into separate 50 mL volumetric flasks and diluting to volume with the diluent. Two µL volume was injected automatically into the chromatograph in triplicate and chromatograms were recorded. Calibration graph was constructed by plotting the mean peak area against RIF concentration. The concentration of the unknown was computed from the regression equation derived using mean peak area-concentration data.

Procedure for capsules: Contents of twenty capsules were pooled and pulverized. A quantity of powder equivalent to 25 mg of RIF was accurately weighed out into a 100 mL volumetric flask. About 50 mL diluent were added, sonicated for 20 min, and then diluted to the final volume with the same diluent to yield a concentration of 250 µg/mL. A small portion of this solution (~10 mL) was withdrawn and filtered through a 0.2 µm nylon membrane filter to ensure the absence of particulate matter. This filtered extract was appropriately diluted to get the final concentration of 50 µg/mL RIF, and injected in five replicates as described earlier.

Selectivity by placebo and synthetic mixture analyses: A placebo blank of the composition [34]: urea (10 mg), sodium oxalate (15 mg), camphor (10 mg), glucose (10 mg), lactose (20 mg), sucrose (15 mg) and ascorbic acid (10 mg) was made by uniform mixing, 20 mg was transferred in to a 100 mL volumetric flask, and its solution prepared as described under "*procedure for capsules*" using the diluent, and analyzed as described earlier. To assess the role of the inactive ingredients on the assay of RIF, a synthetic mixture was separately prepared by mixing 10 mg of RIF with 10 mg placebo mentioned above, and homogenized. The drug was extracted and solution was prepared as described under the '*procedure for capsules'*. The solution after appropriate dilution was analyzed following the recommended procedure.

Sample preparation for forced degradation: A 2 mL aliquot of 100 µg/mL RIF solution was transferred into three different 10 mL calibrated flasks and added 2 mL of 5M HCl, 5M NaOH or 3 % H_2O_2 and the flasks were heated for 3 hours on a water bath maintained at 80 °C. Then the solutions were cooled and neutralized by adding base or acid, the volume in each flask was brought to the mark with diluent, and the appropriate volume (2 µL) was injected for analysis. Solid state thermal degradation was carried out by exposing pure drug to dry heat at 105 °C for 48 hours. For photolytic degradation studies, pure drug in solid state was exposed to 1.2 million lux hours in a photo stability chamber. The sample after exposure to heat and light was used to prepare 100 µg/mL solutions in diluent and the chromatographic procedure was followed.

Results

Method development and Optimization: The main objective of this study was to develop an UPLC method using isocratic conditions for the determination of RIF in drug substance and drug product. The wavelength, 235 nm was selected for the detection of the eluted species, because the drug has sufficient absorption at this wavelength, and low concentrations of RIF may be detected. Furthermore, the calibration curves obtained at 235 nm exhibited good linearity. The mobile phase very often used is the mixture of phosphate buffer and acetonitrile in varying proportions. The runtime was too long with the higher pH (above 5.0) and higher proportion of buffer in the mobile phase. To solve this problem, several mobile phases were tested varying their composition and pH to obtain a symmetric peak within a reasonable time. The proposed mobile phase consisting of formic acid buffer of pH 4.5 (adjusted with triethylamine) and acetonitrile (40:60 v/v) yielded a higher sensitivity with a shorter runtime. The Acquity UPLC BEH C_{18} (100 × 2.1 mm; 1.7 µm particle size) column was produced good peak shape and symmetry. The injection volume was varied between 1 and 5 µL and finally 2 µL was chosen, because larger volumes resulted in wider peaks without much enhancement of the signal-to-noise ratio. After several trials, flow rate of the mobile phase was kept at 0.4 mL/min, and the column temperature was maintained at 30 ^oC.

Validation of the method: The proposed method was validated for specificity, accuracy and precision, limits of detection (LOD) and quantification (LOQ), linear range and robustness and ruggedness as per the *ICH* guidelines [43].

System suitability: The system suitability test was used to ensure that the UPLC system and procedure are adequate for the analysis. The parameters of this test were column efficiency (number of theoretical plates) asymmetry of chromatographic peak and reproducibility as RSD of peak area of seven injections of standard solutions. While performing the system suitability test, %RSD of the peak areas was < 2 %, the number of theoretical plates was 2231 ± 10.6 and the USP tailing factor was ≤ 2.0. These results are summarized in Table 1.

Specificity: The ability of the method to separate and accurately measure the peak of interest indicates the specificity of the method. This was checked by injecting in five replicates of RIF standard, RIF capsule extract, placebo blank and synthetic mixture solutions. There was no additional peak in the chromatograms for capsule

Table 1 System suitability test results

Parameter	Specification	Observed value
Retention time, min (mean ± RSD; n = 7)	≥ 1.65 and ≤ 2.15 ± 0.1 min	1.92 ± 0.18
USP tailing	≤ 2.0	1.27
No. of theoretical plates	≥ 2000	2231 ± 10.6
Area (%RSD, n = 7)	≤ 5 %	0.37 %

Table 2 Results of accuracy and precision study

RIF taken (µg/mL)	Within-day (n = 7)			Between-day (n = 5)		
	Percent recovered	%RE	%RSD	Percent recovered	%RE	%RSD
20	99.21	0.80	1.05	98.70	1.30	1.45
40	98.39	1.60	1.48	97.99	2.02	1.52
60	99.21	0.79	1.13	101.2	1.20	1.02

RE-Relative error; RSD- Relative standard deviation

extract and synthetic mixture solutions at 1.9 min; and the chromatogram of the placebo blank did not show any peak at all. The analysis of the synthetic mixture solution yielded a percent recovery of 97.45 ± 0.64 (n = 5). It is implied from these studies that there is interference from the coformulated substances in the capsules, ensuring the specificity of the method.

Linearity: Linearity of the method was studied by analyzing standard solutions at four different concentration levels ranging from 5 to 80 µg/mL. The calibration graph was constructed by plotting the mean peak area against the corresponding concentration injected. The regression equation (y = mx + b) obtained by the method of least squares was y = 37712.8x + 2534.5 (r = 0.9997), where y is the mean peak area measured and x is concentration of RIF µg/mL. The standard deviations of slope and intercept were 14536.72 and 524.03, respectively. Further, low values of standard deviations of slope and intercept indicated that the points tend to be very close to the expected values. The high value correlation coefficient indicated a good linearity.

Limits of detection (LOD) and quantification (LOQ): The LOD and LOQ values were determined based on a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions of analyte with known concentrations, and were found to be 0.03 and 0.09 µg/mL, respectively. The precision study was also carried out at the LOD and LOQ levels, by injecting seven replicates of RIF solution. The calculated %RSD of the peak areas at LOD and LOQ concentration levels were found to be < 10 % and < 5 %, respectively.

Accuracy and precision: The accuracy of the method was determined by analyzing standard RIF solution at three concentration levels in seven replicates on the same day (intra-day) and on five consecutive days under the optimized conditions. The accuracy expressed as % relative error (%RE) was ≤ 2.02 % implying good accuracy of the proposed method (Table 2).

The within-day precision of the method was determined for peak area by repeat analysis (seven identical injections) of the standard solution containing the drug at three different concentration levels. The day-to-day precision was established by performing the analysis over a five-day period on solution prepared freshly each day. The low RSD values indicate the good precision of the method (Table 2).

Robustness: Robustness of the method was determined by performing the analysis under slightly altered chromatographic conditions. The impact on retention time, peak asymmetry and % assay was evaluated by changing flow rate of the mobile phase (0.4 and 0.4 ± 0.05 mL/min), mobile phase composition (45:55, 40:60 and 35:65 buffer: acetonitrile v/v), column temperature (30 and 30 ± 2 °C) and detection wavelength (235 and 235 ± 2 nm). In each case, the %RSD values were calculated for the obtained peak area. The number theoretical plates and tailing factors were compared with those obtained under the optimized conditions. These results are given in Table 3.

Ruggedness: The ruggedness of the method was demonstrated by carrying out the analysis by different operators using different columns of similar type. No marked changes in the vital system suitability parameters were observed as shown by the %RSD values of < 1 % in Table 3.

Solution stability and mobile phase stability: Stability of RIF solution was investigated by injecting the standard solution into the chromatographic system at the time intervals of 0, 12 and 24 h, and the RSD values were calculated (Table 3). The mobile phase stability was studied by preparing the drug solution afresh with the mobile phase stored at room temperature at various intervals of time. The solution prepared at 0, 12 and 24 hours was injected and RSD of the peak areas were calculated (Table 3). The results showed that in both cases the peak areas, retention times, tailing factors and number of theoretical plates remained almost unchanged (%RSD < 1 %) and no significant degradation was observed within the indicated period. This showed that both drug solution and mobile phase were stable for at least 24 hours, which was sufficient to complete whole analytical process.

Application to RIF capsules: The developed method was applied to the determination of RIF in commercially available capsules. Results of determination of RIF content in capsules of two strengths were in good agreement with the labeled amount. The same batch capsules were analyzed by the reported method [8]. Capsule extract equivalent to 100 µg/mL RIF was prepared in methanol and 5 mL of this extract was diluted to 10 mL with phosphate buffer of pH 7.4, and absorbance measured at 475 nm *vs* buffer. The results obtained were

Table 3 Results of method robustness, ruggedness, solution stability and mobile phase stability

Condition altered	Modification	Mean peak area ± SD*	%RSD	Mean Rt ± SD*	%RSD	Mean Theoretical plates ± SD*	%RSD	Mean tailing factor ± SD*	%RSD
Results of method robustness									
Column temperature	28, 30, 32 ºC	2,007,625 ± 4,489	0.22	1.929 ± 0.005	0.20	2,226 ± 21.8	0.52	1.21 ± 0.01	0.83
Mobile phase composition	(Buffer:ACN*) 45:55 40:60 35:65	1,998,861 ± 8,791	0.44	1.918 ± 0.007	0.28	2,196 ± 19.1	0.46	1.19 ± 0.01	0.84
Flow rate	0.35, 0.40, 0.45 mL/min	2,000,790 ± 9,421	0.47	1.899 ± 0.004	0.12	2,241 ± 17.5	0.41	1.18 ± 0.005	0.42
Wavelength	233, 235, 237 nm	2,001,784 ± 5,059	0.25	1.911 ± 0.005	0.20	2,234 ± 16.3	0.38	1.20 ± 0.004	0.34
Results of method ruggedness									
Analysts (n = 3)	-	1,998,861 ± 8,063	0.40	1.927 ± 0.02	0.89	2,216 ± 19.6	0.46	1.21 ± 0.004	0.33
Columns (n = 3)	-	2,003,330 ± 6,354	0.32	1.936 ± 0.02	0.79	2,235 ± 20.7	0.49	1.18 ± 0.005	0.42
Results of solution stability									
Time interval	0 hr	1,998,981 ± 8054	0.34	1.931 ± 0.02	0.45	2,215 ± 20.6	0.45	1.17 ± 0.003	0.39
	12 hr	1,998,863 ± 7054	0.35	1.941 ± 0.01	0.39	2,227 ± 20.1	0.48	1.19 ± 0.004	0.37
	24 hr	2,001,254 ± 7291	0.32	1.929 ± 0.03	0.37	2,222 ± 20.3	0.44	1.15 ± 0.002	0.35
Results of mobile phase stability									
Time interval	0 hr	1,998,872 ± 7051	0.36	1.944 ± 0.02	0.34	2,217 ± 20.1	0.48	1.19 ± 0.004	0.34
	12 hr	2,000,027 ± 7529	0.38	1.945 ± 0.04	0.39	2,245 ± 19.2	0.46	1.18 ± 0.005	0.46
	24 hr	1,998,813 ± 7152	0.33	1.934 ± 0.01	0.41	2,226 ± 18.5	0.42	1.20 ± 0.003	0.37

*ACN-acetonitrile
*Mean value of three determinations at RIF concentration of 40 µg/mL

Table 4 Results of analysis of capsules by the proposed method and statistical comparison of the results with the published method

Brand name of capsules	Nominal amount, mg	%RIF found[#] ± SD		*t*- value	*F*- value
		Published Method	Proposed method		
R-Cin 300[*]	300	98.9 ± 0.63	99.46 ± 0.54	1.5	1.36
R-Cin 450[*]	450	100.7 ± 0.74	99.63 ± 0.82	2.17	1.23

[*]Marketed by Lupin Limited, Chikaltana Aurangabad, India
[#]Mean value of five determinations
Tabulated t-value at 95% confidence level is 2.78
Tabulated F-value at 95% confidence level is 6.39

compared statistically by the Student's *t*-test and the variance-ratio *F*-test. The calculated *t*- and *F*-values did not exceed the tabulated values of 2.77 and 6.39 at the 95% confidence level and for four degrees of freedom (Table 4), indicating close similarity between the proposed method and the reference method with respect to accuracy and precision. The results obtained are presented in Table 4.

Recovery study: In order to further demonstrate the suitability and accuracy of the method, recovery studies were performed *via* standard-addition technique. Pre analyzed capsule content was spiked with pure RIF at three different levels and the total was found by proposed method. The experiment at each level was repeated three times. The percent recoveries of the pure drug added which are compiled in Table 5 reveal that the commonly added additives urea, sodium oxalate, camphor, glucose, lactose, sucrose and ascorbic acid did not interfere in the assay method. This was amply demonstrated by a single peak due to RIF in the chromatogram of the capsule solution.

Stability indicating property: RIF was subjected to acid-, base- and hydrogen peroxide-induced degradation in solution state, and light- and heat-induced degradation in solid state. The inference was drawn by measuring the peak area of RIF solution after subjecting to forced degradation. From the area, percentage recovery of RIF was calculated in each case. The study revealed that RIF was completely degraded in acid- and peroxide-induced stress conditions, moderately degraded under base-induced stress condition; and remained stable under other stress conditions (Figure 3).

Conclusion

In conclusion, a stability-indicating UPLC method has been developed and validated for the assay of RIF in bulk drug and capsules for the purpose of product quality assessment. The method is rapid, selective, accurate and precise for RIF quantitation. A single chromatographic run took less than 5 min. The method does not require extensive sample treatment and involves a UPLC system employing an inexpensive mobile phase. The UV-detection response was linear for the concentrations studied. There was no interference from matrix sources. The proposed assay method is suitable for regular determination of RIF and for checking the stability of its formulations. Due to its speed, reliability and sensitivity, the method can be conveniently applied to the determination of the drug in body fluids after appropriate sample treatment.

Table 5 Results of recovery study by standard addition technique

Capsules studied	RIF in capsules, (µg/mL)	Pure RIF added, (µg/mL)	Total found, (µg/mL)	%Pure RIF recovered ± SD
R-Cin 300	29.84	15.0	43.29	96.56 ± 0.58
	29.84	30.0	60.80	101.6 ± 0.89
	29.84	45.0	77.16	103.8 ± 0.72
R-Cin 450	29.89	15.0	43.25	96.36 ± 0.82
	29.89	30.0	60.79	101.5 ± 0.76
	29.89	45.0	78.93	105.4 ± 0.69

*Mean value of three determinations

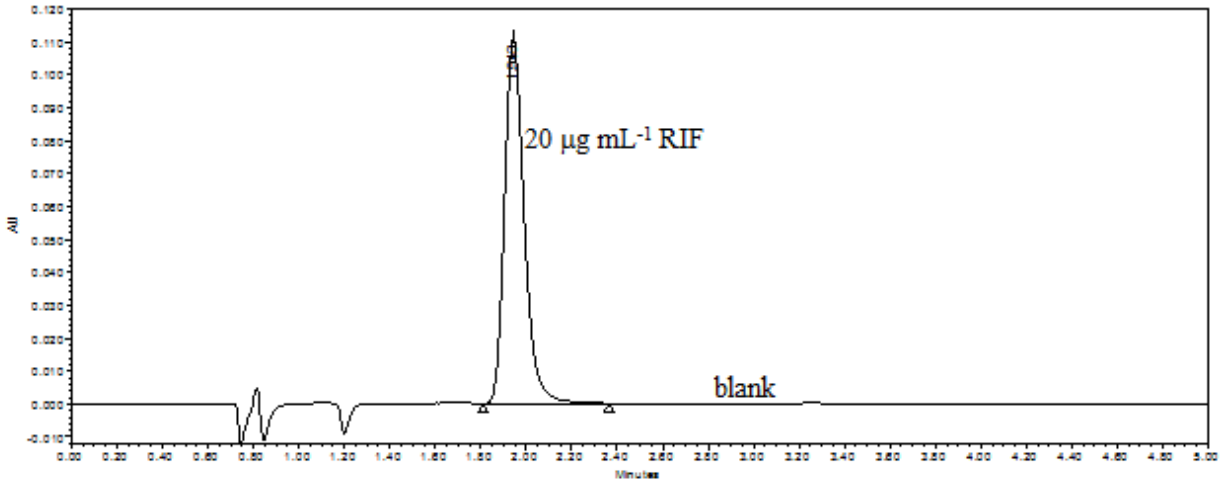

Figure 2 Overlaid chromatograms of: (a) blank, (b) 20 µg/mL RIF

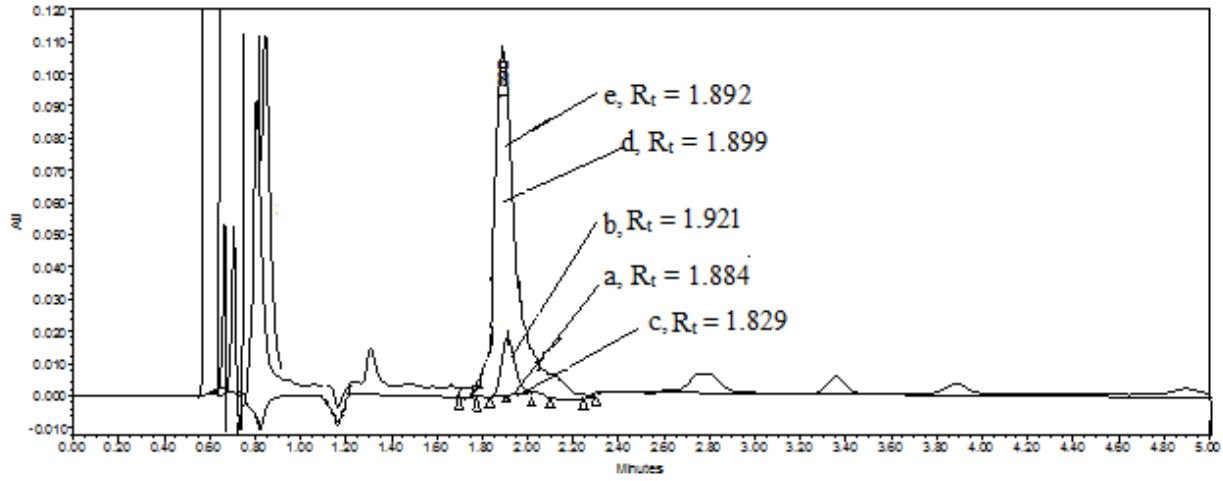

Figure 3 Overlaid chromatograms of RIF after forced degradation (20 µg/mL) (a) acid degradation, (b) base degradation, (c) oxidative degradation, (d) thermal degradation, and (e) photolytic degradation

Acknowledgement

The authors express their gratitude to the Quality Control Manager, Lupin Ltd., Tarapur, Maharashtra, India, for gift sample of rifampicin. Prof. K. Basavaiah thanks UGC, New Delhi, India, for the award of UGC-BSR faculty fellowship. Two of the authors (NS and PV) thank the authorities of the University of Mysore, Mysore, for providing research facilities and N. Swamy is grateful to the University of Mysore for awarding a UGC NON-NET Ph.D fellowship.

References

[1] A. G. Gilmann, L. S. Goodmann. In the Pharmaceutical Basis of Therapeutics, 4th Edn. The Mc Millan Co. 1333, 1970.
[2] J. Melnick. Adelbergs, *Med. Microbio.* 21: 171- 1998.
[3] P. F. Barnes and S. A. Barrows. Tuberculosis in 1990s, *Ann. Intern. Med.* 119: 400-410, 1993.
[4] S. Oldfield, J. D. Borg, H. J. Stiles and B. M. Buckley. Measurement of rifampicin and 25-desacetylrifampicin in biological fluids using high-performance liquid chromatography with direct sample injection, *J. Chrom.* 377: 423-429, 1986.
[5] A. B. M. Jamaluddin, G. Sarwar, M. A. Rahim and M. K. Rahman. High-performance liquid chromatographic assay of rifampicin in human serum, *J. Chrom.* 525: 495-497, 1990.
[6] G. C. He´ctor and O. C. Alejandro. Simultaneous determination of rifampicin, isoniazid and pyrazinamide in tablet preparations by multivariate spectrophotometric calibration, *J. Pharm. Biomed. Anal.* 20: 681–686, 1999.
[7] M. A. Espinosa, V. M. I. Acedo, D. P. A. Muñoz, F. Salinas and C. F. Cañada. Comparative study of partial least squares and a modification of hybrid linear analysis calibration in the simultaneous spectrophotometric determination of rifampicin, pyrazinamide and isoniazid, *Anal. Chim. Acta.* 427: 129–136, 2001.
[8] S. A. Benetton, E. R. M. Kedor-Hackmann, M. I. R. M. Santoro and V. M. Borges. Visible spectrophotometric and first-derivative UV spectrophotometric determination of rifampicin and isoniazid in pharmaceutical preparations, *Talanta.* 47: 639–643, 1998.
[9] A. R. Rote and A. K. Sharma. Simultaneous spectrophotometric determination of rifampicin, isoniazid and pyrazinamide by first-

derivative UV spectrophotometry in combined pharmaceutical dosage forms, *Indian J. Pharm. Sci.* 59: 119-123, 1997.
[10] M. Y. Rasha and M. M. Hadir. A new hybrid double divisor ratio spectra method for the analysis of ternary mixtures, *Spectrochim. Acta. Part A.* 70: 1152–1166, 2008.
[11] L. F. Marcellos, A. F. Faria, M. V. N. Souza, M. R. Almeida, G. P. Sabin, R. J. Poppi and M. A. L. Oliveira. Simultaneous analysis of first-line anti-tuberculosis drugs in tablets by UV spectrophotometry compared to capillary zone electrophoresis, *Cent. Eur. J. Chem.* 10: 1808-1816, 2012.
[12] R. B. Kakde, A. V. Kasture and S. G. Wadodkar. Spectrophotometric determination of rifampicin and isoniazid in pharmaceutical preparations, *Indian J. Pharm. Sci.* 64: 24-27, 2002.
[13] T. Wahdan. Voltammetric method for the simultaneous determination of rifampicin and isoniazid in pharmaceutical formulations, *Chem. Anal. (Warsaw).* 50: 457-464, 2005.
[14] A. Z. Karim and S. A. Payam. Simultaneous polarographic determination of isoniazid and rifampicin by differential pulse polarography method and support vector regression, *Electrochim. Acta.* 55: 6570–6576, 2010.
[15] M. A. L. Alonso, O. R. Dom´ınguez and M. J. M. Arcos. Resolution of ternary mixtures of rifampicin, isoniazid and pyrazinamide by differential pulse polarography and partial least squares method, *Anal. Chim. Acta.* 449: 167–177, 2001.
[16] M. A. A. Alonso, J. M. Kauffmann and M. J. M. Arcos. HRP-based biosensor for monitoring rifampicin, *Biosen. Bioel.* 18: 1165-1171, 2003.
[17] L. Baoxin, H. Yuezhen, L. Jiagen and Z. Zhujun. Simultaneous determination of rifampicin and isoniazid by continuous-flow chemiluminescence with artificial neural network calibration, *Anal. Bioanal. Chem.* 383: 817–824, 2005.
[18] S. A. Halvatzis, P. M. M. Timotheou and T. P. Hadjiioannou. Continuous-flow chemiluminometric determination of dihydralazine, rifampicin and rifamycin SV by oxidation with N-bromosuccinimide, *Anal. Chim. Acta.* 272: 251-263, 1993.
[19] D. H. Shewiyoa, E. Kaaleb, P. G. Rishab, B. Dejaegherc, V. J. Smeyers and H. Y. Vander. Optimization of a reversed-phase-high-performance thin-layer chromatography method for the separation of isoniazid, ethambutol, rifampicin and pyrazinamide in fixed-dose combination antituberculosis tablets, *J. Chromatogr. A.* 1260: 232–238, 2012.
[20] J. Ali, N. Ali, Y. Sultana, S. Baboota and S. Faiyaz. Development and validation of a stability-indicating HPTLC method for analysis of antitubercular drugs, *Acta. Chromatogr.* 18: 168-179, 2007
[21] S. Gunasekaran and E. Sailatha. Estimation of pyrazinamide, isoniazid and rifampicin in pharmaceutical formulations by high performance liquid chromatography method, *Asian J. Chem.* 21: 3561-3566, 2009.
[22] E. Calleri, E. De Lorenzi S. Furlanetto G. Massolini and G. Caccialanza. Validation of a RP-LC method for the simultaneous determination of isoniazid, pyrazinamide and rifampicin in a pharmaceutical formulation, *J. Pharm. Biomed. Anal*. 29: 1089–1096, 2002.
[23] S. Sadeghi and E. Karimi. Spectrophotometric determination of rifampicin through chelate formation and charge transfer complexation in pharmaceutical preparation and biological fluids, *Chem. Pharm. Bull.* 54: 1107-1112, 2006.
[24] C. S. P. Sastry, T. E. Divakar and U. V. Prasad. Spectrophotometric determination of rifampicin with uranyl or thorium(IV), *J. Inst. Chem. (India)* 58: 17-18, 1986.
[25] C. S. P. Sastry, T. E. Divakar and U. V. Prasad. Spectrophotometric determination of rifampicin with some metal ions, *Indian Drugs*. 22: 604-606, 1985.
[26] T. P. Gandhi, A. A. Patel, P. R. Patel and V. C. Patel. Colorimetric estimation of rifampicins in formulations and biological fluids by metallic ions, *Indian Drugs* 16: 10-12, 1978.
[27] B. S. Reddy and C. S. P. Sastry. Ion-pair extraction method for ethambutol, ethionamide and rifampicin determination, *J. Inst. Chem. (India)* 55: 69-70, 1983.
[28] M. G. Shereen, M. B. Salah and M. E. Abdel-Hamid. Comparative spectrophotometrc analysis of rifampicin by chelate formation and charge-transfer complexation, *Anal. Lett.* 25: 725-743, 1992.
[29] I. C. Shukla, P. K. Dwivedi, S. Kumar, B. K. Singh and A. Dubey. Application of ammonium metavanadate for the determination of some tubercular and adrenocortical steroid drugs, *J. Ind. Chem. Soc.* 84: 100-102, 2007.
[30] C. S. P. Sastry, T. E. Divakar and U. V. Prasad. Spectrophotometric determination of rifampicin with chloranil, *Indian J. Pharm. Sci.* 47: 45-46, 1985.
[31] T. E. Divakar, U. V. Prasad and C. S. P. Sastry. Spectrophotometric estimation of tetracyclines and rifampicin using *p*-N,N-dimethylphenylenediamine and chloramine T, *Indian Drugs* 22: 328-329, 1985.
[32] G. R. Rao, S. S. N. Murty and E. V. Rao. Spectrophotometric determination of rifampicin in pharmaceutical dosage forms, *Indian Drugs* 22: 484-488, 1985.
[33] T. E. Divakar, S. Sunitha, G. K. Deepthi, T. Benzamin and N. P. Babu. Assay of rifampicin in bulk and its dosage forms by visible spectrophotometry using chloranilic acid, *Int. J. Chem. Environ. Pharm. Res.* 3: 64-67, 2012.
[34] N. B. Barsoum, M. S. Kamel and M. M. A. Diab. Spectrophotometric determination of isoniazid and rifampicin from pharmaceutical preparations and biological fluids, *Res. J. Agri. Bio. Sci.* 4: 471-484, 2008.
[35] A. A. Salem, H. A. Mossab and B. N. Barsoum. Quantitative determinations of levofloxacin and rifampicin in pharmaceutical and urine samples using nuclear magnetic resonance spectroscopy, *Spectrochim. Acta Part A.* 62: 466–472, 2005.
[36] H. Younghee and S. Sunmi. Electrochemical behaviour and differential pulse polarographic determination of rifampicin in the pharmaceutical preparations, *Arch. Pharm. Res.* 24: 100-104, 2001.
[37] M. A. A. Lomillo, O. D. Renedo and M. J. A. Martinez. Optimization procedure, applying the experimental-design methodology, for the determination of rifampicin after metal complexation by differential pulse adsorptive stripping voltammetry, *Helvet. Chim. Acta.* 85: 2430-2439, 2002.
[38] Y. Shah, S. Khanna, K. C. Jindal and V. S. Dighe. Determination of rifampicin and isoniazid in pharmaceutical formulations by HPLC, *Drug. Dev. Ind. Pharm.* 18: 1589-1596, 1992.
[39] L. Novakova, L. Matysova, and P. Solich. Advantages of applications of UPLC in pharmaceutical analysis, *Talanta*. 68: 908-918, 2006.
[40] *ICH* [Stability Testing of New Drug Substances and Products (Q1AR2), International Conference on Harmonization, Food and Drug Administration, USA, November 1996 and February 2013.
[41] *ICH* [Validation of Analytical Procedures: Methodology (Q2AR1), International Conference on Harmonization, Food and Drug Administration, USA, November 1991 and November 2005.
[42] M. Bakshi and S. Singh. Development of validated stability-indicating assay methods-critical review, *J. Pharm. Biomed. Anal.* 28: 1011-1040, 2006.
[43] *ICH* Harmonized Tripartite Guidelines, Validation, Analytical Procedures, Text and Methodology, Q2 (R1), Geneva, Switzerland, 2005.