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In vitro analysis of rifampicin and its effect on quality control tests of rifampicin containing dosage forms

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The chemical stability of rifampicin both in solid state and various media has widely been investigated. While rifampicin is appreciably stable in solid-state, its decomposition rate is very high in acidic as well as in alkaline medium and a variety of decomposition products were identified. The literature reports on highly variable rifampicin decomposition in acidic medium. Hence, the objective of this investigation was to study possible reasons responsible for this variability. For this purpose, filter validation and correlation between rifampicin and its degradation products were developed to account for the loss of rifampicin in acidic media. For analysis of rifampicin with or without the presence of isoniazid, a simple and accurate method was developed using high performance chromatography recommended in FDC monographs of the United States Pharmacopoeia. Using the equations developed in this investigation, the amount of rifampicin degraded in the acidic media was calculated from the area under curve of the degradation products. Further, it was proved that in a dissolution study, the colorimetric method of analysis recommended in the United States Pharmacopoeia provides accurate results regarding rifampicin release. Filter type, time of injection as well as interpretation of data are important factors that affect analysis results of rifampicin in *in vitro* studies and quality control.

1. Introduction

In the literature, there are numerous reports on the instability of rifampicin (RIF) alone or in combination with isoniazid (INH) in acidic medium. However, the degradation rates are highly variable indicating 6–50% *in situ* degradation in solution of standard drugs or in the formulations (Jindal et al. 1994; Shishoo et al. 1999; Singh et al. 2000a, 2001). One of the study report 97% and 98% degradation of RIF in combination with INH from suspension formulations at 4 °C and 40 °C, respectively after 28 days (Seifart et al. 1991). This is in direct contrast with the earlier report by Seydel where the degradation rate of RIF was found to be temperature dependent and Arrhenius plots were generated (Seydel 1970). Further, wide variations in decomposition of RIF (7.5–33%) and INH (1.4–5.3%) were observed in the marketed formulations when studies were carried out in 0.1 N HCl at 37 °C for 50 min (Singh et al. 2000a). In a commercial RIF tablet formulation procured from the market and analysed for RIF was found to be degraded upto 7% in solid state (Singh and Mohan 2003). In context of these reports, the question arises why, provided RIF alone or in the presence of INH is decomposing by a specific mechanism, the above-mentioned reports showed such a variability. This prompted us to doubt if RIF or its degradation products were analyzed accurately and if the results were interpreted correctly?

The stability of RIF in aqueous solution has been widely investigated and the conditions and transformation products are reported (Seydel 1970; Gallo and Radaelli 1976). RIF in acidic solution degrades to 3-formyl rifamycin (3F-RIF), whereas in the presence of INH, degradation of RIF is increased two-threefold and yielding a hydrazone (HYD) between 3F-RIF and INH (Seydel 1970; Shishoo et al. 1999; Singh et al. 2000b). These reactions were found to be pH, temperature and time dependent (Gallo and Radaelli 1976; Seydel 1970). USP specifies 0.1 N HCl as the dissolution medium for RIF alone and RIF plus INH in fixed dose combination (FDC), whereas simulated gastric fluid without pepsin is recommended for three drug FDC (USP 26, 2003). A number of studies has shown that acidic dissolution media are not appropriate to study the dissolution of a RIF containing formulation because of the unstable nature of the drug in these media (Garbo et al. 1989; Jindal et al. 1994; Shishoo et al. 1999). However, in all these studies, release was calculated based on the RIF peak only without considering the decrease in RIF peak due to degradation. Although the presence of other degradation products was observed in a stability indicating HPLC or HPTLC method, there was no attempt to determine the amount of RIF that went into solution and degraded from the formulations. This approach resulted in underestimation of release from the formulations and hence degradation was suggested as the possible cause for reduced bioavailability. In addition, because of the known instability of RIF in acidic medium, dissolution medium comprised of 0.4%w/v sodium lauryl sulfate was suggested as a dissolution medium where RIF was found to stable and also has sufficient solubility (Gharbo et al.

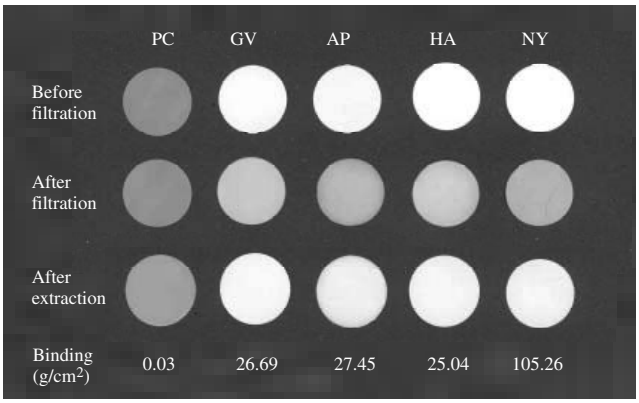

Fig. 1: Filter validation for RIF.
PC: Polycarbonate, GV: low protein binding polyvinylidene, AP: Glass fibre, HA: Mixed cellulose esters and NY: Nylon filter
Note: Polycarbonate filters being thin and translucent, have acquired the colour of background

1989; Jindal et al. 1994). However, this method would not simulate *in vivo* conditions and the quality of the formulations studied will be questionable as RIF being a zwitterion shows pH dependent solubility. In addition, RIF is known to have surfactant properties in acidic medium (Gallo and Radaelli 1976). All these properties of RIF influence its solubility behaviour. Hence the ideal situation would be to correct for the loss due to degradation and determine the release of RIF after correcting it for degradation in gastric fluids. For this purpose, USP recommends calculations based on the reference vessel where all the active ingredients of RIF formulations are dissolved at time zero and kept in the dissolution bath for the time specified which is regarded as standard solution (USP 26, 2003). Release of RIF is then calculated with reference to absorbance values of the standard solution at 475 nm. Further, RIF and INH both are highly permeable molecules as indicated by excellent oral bioavailability and high P_{eff} values and hence the amount of RIF degraded or its interaction with INH *in vivo* will be very low as both the molecules are being absorbed immediately as soon as going into solution. Hence, to study the dissolution of RIF containing formulations, the release should be calculated on the basis of the amount of RIF that went into solution and is available for the absorption after correcting the loss because of the degradation in the acidic medium. Furthermore, RIF being a hydrophobic drug binds to surfaces. Hence, it is important to study the binding properties of RIF to the filters so that accurate results could be obtained.
In the light of the above discussion, the objective of this investigation was to study the influence of the factors affecting RIF analysis and deriving a method that can account for the degradation products and thus can be used for accurate *in vitro* evaluation of RIF contained in dosage forms.

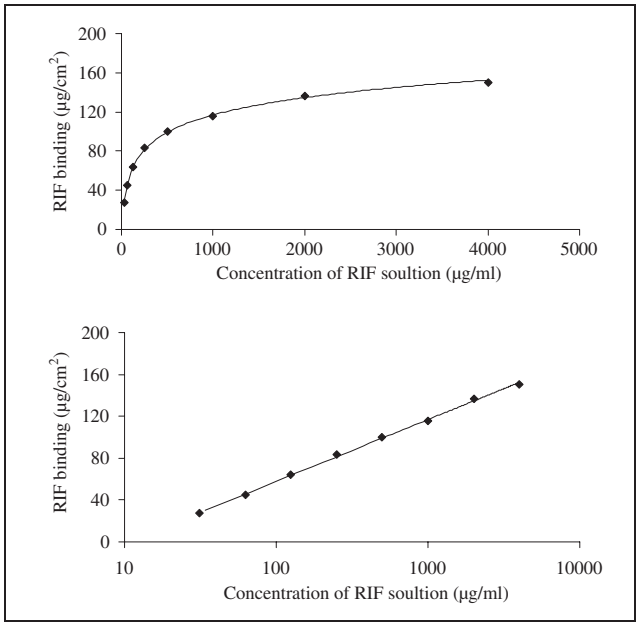

Fig. 2: RIF binding on the nylon filter from solutions of different concentrations.

2. Investigations, results and discussion

2.1. Filter validation

RIF, due to its hydrophobic nature, shows the tendency of binding to various surfaces and hence to minimize the loss of drug and to obtain accurate results, filter validation is necessary. For filter validation, five commonly used filters for hydrophobic substances were used. Figure 1 shows the amount of RIF that was bound to these filters. Out of five filters tested, polyvinylidene, glass fibre and mixed cellulose ester filters have shown binding equal to 25 µg/cm^2. The nylon filter has shown the highest binding of 105 µg/cm^2 while binding to polycarbonate filter was minimal (0.03 µg/cm^2).
When RIF binding as a function of concentration of RIF solution was studied using nylon filter, a log-linear relationship was observed (Fig. 2). The equation for RIF binding on nylon filter was $y = 25.96\text{Ln}(x)-61.24$ with a correlation of 0.998. Thus, RIF binding on filter is a function of the concentration and hence will have influence in the analysis of RIF.

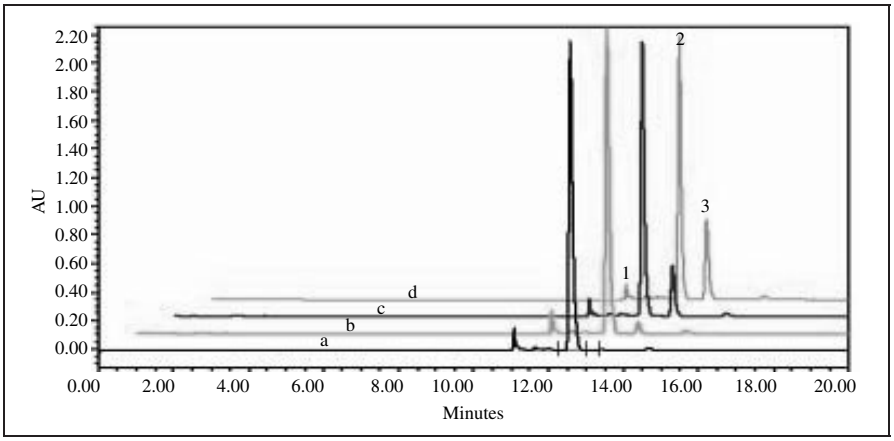

Fig. 3:
Chromatograms representing degradation of RIF in 0.01 N HCl and 25 °C (a: 0 min, b: 63 min, c: 402 min, d: 663 min). 1: peak due to change in mobile phase composition during gradient run, 2: RIF peak and 3: 3F-RIF peak.

Table 1: AUC values of RIF, 3F-RIF and summation of these two obtained after repeated injection of RIF solution in 0.01 N HCl maintained at 25 °C

Time (min)	AUC values ($\times 10^{-6}$)		
	RIF	3F-RIF	Sum-RIF
0	15.18	0.05	15.24
21	14.96	0.21	15.17
42	14.78	0.36	15.14
63	14.63	0.52	15.14
233	13.19	1.63	14.81
276	12.97	1.87	14.84
360	12.33	2.34	14.67
381	12.23	2.44	14.67
402	12.10	2.55	14.66
642	10.73	3.65	14.38
663	10.62	3.76	14.38
% Change*	30.40	24.66	5.74

Sum-RIF represents summation of AUC values of RIF and 3F-RIF.
% Change is the decrease in AUC values of RIF and sum-RIF in relation to zero minute in the given time. In case of 3F-RIF, it represents % increase in the given time.
All the AUC values are represented as AUC ($\times 10^{-6}$) for the simplicity in the presentation

2.2. Correlation between RIF and 3F-RIF

When RIF solution in 0.01N HCl was injected from the same vial, it was seen that, as a function of time, RIF peak decreased while another peak at 13.4 min increased which was very small at time zero (Fig. 3). Based on degradation pathway of RIF in acidic medium, the second peak was attributed to 3F-RIF (Gallo and Radaelli 1976). Table 1 represents the AUC values of RIF and 3F-RIF peaks along with the summation of these two AUCs. As is evident from Table 1, at 25 °C RIF in 0.01 N HCl was degraded to 30.4% however, AUC of 3F-RIF represents only 24.7% of this degradation. Further, the sum of RIF and 3F-RIF shows 5.7% decrease in 11 h. This indicates that in the mobile phase used in this method and at 238 nm, molar absorptivity of 3F-RIF is less than RIF and hence the simple sum of two peaks does not represent the total RIF content initially present. To determine the correlation between RIF and 3F-RIF, the decrease in AUC of RIF was calculated as a function of time with respect to time zero AUC of RIF and given in Table 2. In the time

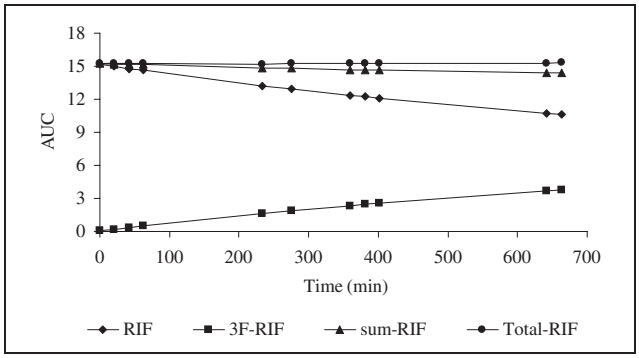

Fig. 4: RIF degradation in 0.01N HCl at 25 °C
Y-axis is represented as (AUC $\times 10^{-6}$)

zero chromatogram, 3F-RIF was also seen in significant amounts (due to time required in preparation of solution and injection) and hence increase in 3F-RIF peak with respect to first injection was corrected by subtracting the time zero value from AUC of 3F-RIF at each time point. There exists a constant relationship between the decrease in AUC of RIF and corrected AUC of 3F-RIF and at any time the ratio of two AUC values was found to be constant with a mean of 1.2387. This ratio can also be correlated to molar absorptivity ratio of RIF and 3F-RIF at 238 nm in the mobile phase used for analysis. Thus, for unknown samples, RIF that was degraded to 3F-RIF can be calculated by applying this correction factor and total RIF that was present initially can be determined by eq. (1):

$$\text{Total-RIF} = \text{RIF} + (1.2387 \times \text{3F-RIF}) \qquad \text{eq. (1)}$$

Total RIF from the solution that was degraded up to 30.4% over a period of 11 h was calculated by eq. 1 and is given in the last column of Table 2. At any time given, total RIF thus calculated was constant with a mean of 15.25 AUC units with a very low CV of 0.16%. Decrease in AUC of RIF, increase in 3F-RIF and its summation are also presented in Fig. 4 along with the total RIF calculated using eq. (1). As is evident from Fig. 4, total RIF was unchanged irrespective of degradation of RIF over a period of time.
Apart from degradation, another important factor in the analysis of RIF from solution or quality control samples is the time required to prepare the sample and time of injection.

Table 2: Deriving the correlation between RIF and 3F-RIF using AUC values as a function of time

Time (min)	AUC values ($\times 10^{-6}$)					
	RIF	Dec in RIF	3F-RIF	Corr 3F-RIF[1]	Dec RIF/Corr 3F-RIF[2]	Total RIF[3]
0	15.18	0.00	0.05	0.00	–	15.25
21	14.96	0.19	0.21	0.16	1.23	15.25
42	14.78	0.40	0.36	0.31	1.30	15.23
63	14.63	0.56	0.52	0.46	1.21	15.26
233	13.19	2.00	1.63	1.57	1.27	15.20
276	12.97	2.21	1.87	1.82	1.21	15.29
360	12.33	2.85	2.34	2.29	1.25	15.23
381	12.23	2.95	2.44	2.39	1.24	15.26
402	12.10	3.08	2.55	2.50	1.23	15.26
642	10.73	4.45	3.65	3.59	1.24	15.25
663	10.62	4.56	3.76	3.71	1.23	15.28

[1] As 3F-RIF peak was found in zero time injection, to derive the relationship between RIF and 3F-RIF, AUC of 3F-RIF was corrected for zero time AUC by subtracting zero time AUC from all subsequent AUC values of 3F-RIF. These corrected 3F-RIF values represent increase in AUC of 3F-RIF as a result of RIF decomposition starting from zero time injection.
[2] Correlation between RIF and 3F-RIF was derived by dividing decrease in AUC values of RIF by increase in 3F-RIF AUC starting from zero time injection. This ratio was found to be constant over a period of experimental time i.e. 663 min with a mean of 1.2387.
[3] Total RIF at any given point that was originally present was calculated using equation 1. Total RIF was found to be constant with a mean of 15.25 AUC units with a very low CV of 0.16% (Fig 4). By this method, amount of RIF that was degraded to 3F-RIF can be calculated.
Abbreviations: Dec: decrease, Corr: corrected, CV: coefficient of variation
All the AUC values are represented as (AUC $\times 10^{-6}$)

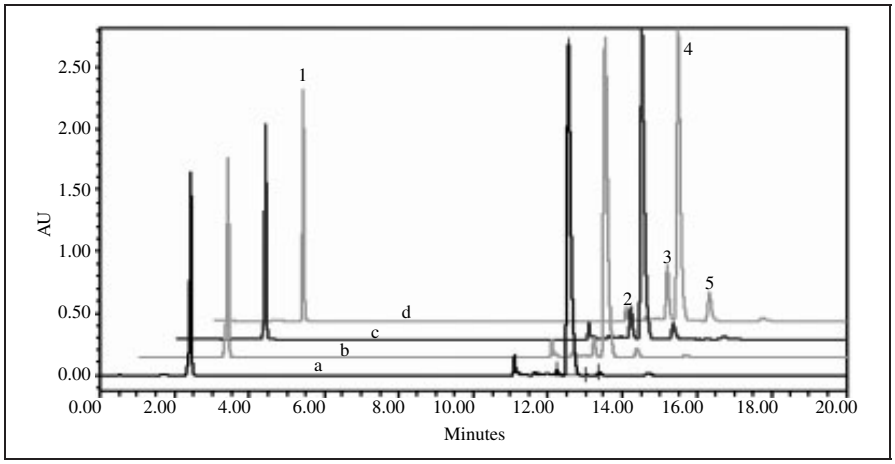

Fig. 5:
Chromatograms representing degradation of rifampicin in presence of isoniazid at 0.01 N HCl and 25 °C (a: 0 min, b: 63 min, c: 170 min, d: 339 min). 1: INH peak, 2: peak due to change in mobile phase composition during gradient run, 3: HYD peak, 4: RIF peak and 5: 3F-RIF peak

This can be appreciated from Table 2 that at time zero RIF area was 15.18 but after correction for 3F-RIF present in time zero injection, the initial amount of RIF went into solution corresponded to 15.25 AUC units. In this analysis, although extreme care was taken to minimize the time required to prepare the solution, 3F-RIF was observed in the first injection. Hence, delay in the injection of samples in the chromatograph is an important parameter in RIF analysis that affects the accuracy and interpretation of assay results.

Table 3: AUC values of RIF, 3F-RIF, HYD and summation of these three obtained after repeated injection of RIF plus INH solution in 0.01 N HCl maintained at 25 °C

Time (min)	AUC values (x 10^{-6})			
	RIF	3F-RIF	HYD	Sum-RIF
0	19.76	0.07	0.05	19.89
21	19.51	0.19	0.21	19.92
42	19.17	0.30	0.38	19.85
63	18.91	0.40	0.55	19.86
84	18.62	0.49	0.72	19.84
170	17.57	0.86	1.31	19.73
212	17.08	1.03	1.58	19.69
339	15.78	1.53	2.29	19.60
360	15.60	1.62	2.44	19.65
381	15.40	1.69	2.55	19.64
% Change	22.09	8.57	12.91	1.26

In RIF plus isoniazid solution, Sum-RIF represents summation of AUC values of RIF, 3F-RIF and HYD. RIF in presence of isoniazid degraded to 22.09% in 381 min with the increase in peak intensities of 3F-RIF (8.57%) and HYD (12.91%). Sum-RIF shoes 1.26% decrease in AUC values.
All the AUC values are represented as (AUC × 10^{-6})

2.3. Correlation between RIF, 3F-RIF and HYD

It is well known that INH in acidic medium increases the degradation rate of RIF two-to threefold. Decomposition of RIF in acidic conditions in the absence of INH stops at the formation of 3-F RIF, while the reaction in presence of INH proceeds to a hydrazone (HYD) between 3F-RIF and INH. In HPLC, the new peak that emerged before the RIF peak was regarded as HYD (Fig. 5). To determine the correlation between RIF and HYD, a similar experiment was conducted up to 6.5 h. In presence of INH, RIF in 0.01 N HCl was degraded to 22.09% in 6.5 h accompanied by an increase of 8.57% in 3F-RIF and 12.91% in the HYD peak area, whereas the sum of these three peaks corresponded to only 1.26% decrease (Table 3). Similar to the earlier correlation, the decrease in RIF area was calculated with respect to time zero injection and given in Table 4. 3F-RIF and HYD areas were corrected for time zero presence by subtracting its AUC from all the time points. 3F-RIF's contribution to the decrease in RIF area was determined from the correlation of RIF and 3F-RIF and the decrease due to HYD was calculated (Table 4).

Table 4: Deriving the correlation between RIF and HYD for the degradation of RIF in presence of INH in acidic medium

Time (min)	AUC values (x 10^{-6})									
	RIF	Dec in RIF	3F-RIF	Corr 3F-RIF[1]	HYD	Corr HYD[1]	Dec in RIF due to 3F-RIF[2]	Dec in RIF due to HYD[3]	Dec RIF/ corr HYD[4]	Total RIF[5]
0	19.76	0.00	0.07	–	0.05	–	–	–		19.91
21	19.51	0.25	0.19	0.12	0.21	0.16	0.15	0.10		19.96
42	19.17	0.60	0.30	0.22	0.38	0.33	0.28	0.32	0.97	19.90
63	18.91	0.85	0.40	0.32	0.55	0.50	0.40	0.45	0.91	19.93
84	18.62	1.14	0.49	0.42	0.72	0.66	0.52	0.62	0.94	19.92
170	17.57	2.20	0.86	0.78	1.31	1.25	0.97	1.23	0.98	19.87
212	17.08	2.68	1.03	0.95	1.58	1.53	1.18	1.50	0.98	19.87
339	15.78	3.99	1.53	1.46	2.29	2.23	1.81	2.18	0.98	19.86
360	15.60	4.16	1.62	1.54	2.44	2.38	1.91	2.25	0.95	19.93
381	15.40	4.37	1.69	1.62	2.55	2.50	2.01	2.36	0.95	19.93

[1] 3F-RIF and HYD AUCs were corrected for zero time AUC by subtracting respective zero time AUC from all subsequent AUC values of 3F-RIF and HYD.
[2] Contribution of 3F-RIF in the decrease in AUC of RIF was determined by equation 1.
[3] Decrease due to HYD was determined by subtracting decrease due to 3F-RIF from initial decrease in RIF
[4] Correlation between RIF and HYD was derived by dividing decrease in AUC of RIF due to HYD by corrected HYD AUC for zero time injection. This ratio was found to be constant over a period of experimental time with a mean of 0.9544.
[5] Total RIF was then calculated by equation 2 and found to be constant with 0.03% CV (Fig. 6).
Abbreviations: Dec: decrease, Corr: corrected, CV: coefficient of variation
All the AUC values are represented as (AUC × 10^{-6})

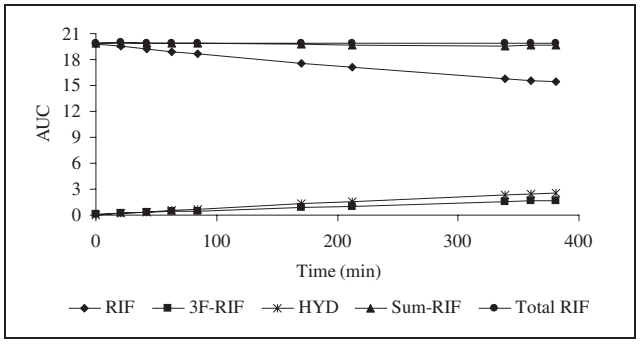

Fig. 6: RIF degradation in presence of INH in 0.01 N HCl at 25 °C
Y-axis is represented as (AUC × 10^{-6})

There exists a correlation between decrease in RIF AUC due to HYD and AUC of HYD having a mean ratio of 0.9544. Thus for the determination of total-RIF corrected for its degradation products in presence of INH, eq. (1) can be modified as

$$\text{Total-RIF} = \text{RIF} + (1.2387 \times \text{3F-RIF}) + (0.9544 \times \text{HYD}) \quad \text{eq. (2)}$$

When total RIF was calculated using eq. (2), it was found to be constant with % CV of 0.03 (Table 4 and Fig. 6). In presence of INH, summation of RIF, 3F-RIF and HYD almost corresponded to the total RIF as molar absorptivity of HYD is more than RIF and hence accounted for the decreased absorptivity of 3F-RIF.

2.4. Determination of total RIF in presence of INH at 0.1 N HCl

The rate of RIF degradation is enhanced at lower pH (Gallo and Radaelli 1976) and hence RIF alone or in combination with INH shows increased degradation at 0.1 N HCl. Further 0.1 N HCl or simulated gastric fluid is the dissolution medium for RIF containing formulations. As RIF in 0.1 N HCl dissolves very fast because of its high solubility, RIF powder corresponding to 0.2 mg/ml and INH corresponding to 0.1 mg/ml were dissolved in 0.1 N HCl immediately before injection. Repeated injections were given over a period of 3 h from the same vial, which was maintained at 25 °C in an autosampler. Similar decomposition pattern was observed in 0.1 N HCl, however, rate of degradation was increased (36% in 3 h). When the derived correction factors were applied for RIF plus INH solution in 0.1N HCl, the total RIF content obtained was constant irrespective of the high degradation rate in this medium (Table 5). As RIF is degraded faster in 0.1 N HCl, time of injection becomes more important. This can be appreciated from Table 5 where RIF was decomposed to 5% in 21 min.

Generally, for deriving such type of correlations complex instrumentation such as photodiode array (PDA) or software as partial least square (PLS) regression (Espinosa-Mansilla et al. 2001) are used. In this method we have suggested a simple method using an UV detector without any extra software to correct for the loss due to degradation. This method can be effectively used for *in vitro* standard solutions as well as for quality control. However, this method cannot be applied to *in situ* perfusion studies, closed loop studies or *in vivo* pharmacokinetic studies as there exists the possibility that degradation products also get absorbed. Further, the degradation product 3F-RIF is one of the metabolites of RIF (Loos et al. 1987).

2.5. Dissolution study of a commercial RIF formulation

For dissolution test of various RIF containing dosage forms, media recommended in USP are 0.1 N HCl, simulated gastric fluid and 6.8 pH phosphate buffer (USP 26/2003). However as described earlier, RIF is unstable in acidic media. In the literature, release of RIF in 0.1 N HCl was calculated from the RIF peak alone and it was suggested that this media are not suitable for dissolution studies (Gharbo et al. 1989; Jindal et al. 1994; Shishoo et al. 1999). According to USP, the method of rifampicin analysis in acidic media is colorimetry at 475 nm and release needs to be calculated with respect to a reference vessel (USP 26/2003). As mentioned in section 3.2, the sum of RIF, 3F-RIF and HYD peaks almost represents total RIF content at the given point, which is also evident from Table 5. At 42 or 60 min, the ratio of sum RIF and total RIF is 99.5%. Thus, during dissolution studies total RIF that went into solution can also be determined by UV-Vis spectroscopy, which represents sum RIF. In addition, the release is calculated with respect to the absorbance values of a reference vessel that further minimizes the error involved due to decomposition.

To determine the release of RIF from commercial formulations, a dissolution study of four drug FDC tablets was done in 0.01 and 0.1 N HCl. Samples obtained were analysed both by HPLC and immediate analysis by colorimetry.

Table 5: Correction for RIF degradation in presence of INH at 0.1 N HCl

Time (min)	AUC values (× 10^{-6})				
	RIF	3F-RIF	HYD	Sum-RIF	Total RIF
0	12.06	0.04	0.07	12.17	12.17
21	11.45	0.23	0.45	12.13	12.16
42	10.87	0.44	0.80	12.11	12.18
63	10.37	0.52	1.22	12.11	12.17
84	9.95	0.54	1.62	12.12	12.17
105	9.37	0.68	2.07	12.11	12.18
126	8.98	0.70	2.44	12.12	12.17
147	8.62	0.74	2.77	12.12	12.17
168	8.22	0.80	3.09	12.11	12.16
189	7.77	0.90	3.45	12.12	12.17
% degradation	36.16	7.37	28.37	–	–

Total RIF was calculated using equation 2 and was found that same equation can be used for the RIF in presence of isoniazid in 0.1N HCl where degradation rate is high because of the increased decomposition at lower pH.
Note: All the AUC values are represented as (AUC × 10^{-6})

Table 6: % release of RIF calculated by different ways from dissolution study of four drug FDC tablet in 0.01 and 0.1 N HCl

Dissolution (% release) of four drug FDC tablet in 0.01 N HCl

Time (min)	HPLC-1	HPLC-2	HPLC-3	HPLC-4	Colorimetry
0	0.00	0.00	0.00	0.00	0.00
10	8.10	8.13	8.56	9.06	11.43
20	18.75	20.05	21.32	22.33	24.63
30	25.89	28.66	30.48	31.76	33.16
45	29.15	35.09	36.83	37.75	39.27

Dissolution (% release) of four drug FDC tablet in 0.1 N HCl

Time (min)	HPLC-1	HPLC-2	HPLC-3	HPLC-4	Colorimetry
0	0.00	0.00	0.00	0.00	0.00
10	43.35	66.80	63.41	62.59	61.88
20	50.52	70.13	72.66	73.01	73.84
30	38.79	73.28	78.28	79.08	79.91
45	35.16	78.62	80.78	82.48	81.37

Form HPLC data% release of RIF was calculated by various methods; HPLC-1: RIF peak compared to zero time RIF peak, HPLC-2: RIF peak compared to reference peak at that time point, HPLC-3: Summation of RIF, 3F-RIF and HYD peaks, HPLC-4: Total RIF after correction for degradation using equation 2. For colorimetric analysis % release was calculated by absorbance values of samples compared to absorbance of reference solution at that time point.
To generate a dissolution profile, total run time in case of HPLC analysis was 10 h. During this time, samples were simultaneously degrading when loaded in autosampler and hence, in HPLC-1 where degradation is not corrected for the calculation of% release, decrease in release was observed which was actually due to degradation occurred during chromatographic run

Release from HPLC analysis was calculated by four ways: RIF peak compared to zero time concentration (HPLC-1), RIF peak compared to concentration of reference peak (HPLC-2), sum of RIF, 3F-RIF and HYD peaks (HPLC-3) and total RIF corrected for degradation factor (HPLC-4). Table 6 shows the release calculated by various ways. The results obtained by HPLC-2, HPLC-3, HPLC-4 and colorimetry are comparable while that of HPLC-1 had less release due to degradation of RIF that was not corrected by any of the methods. This is further supported by f_2 values (taking HPLC-4 method of Total-RIF as reference) obtained for the comparison of dissolution profiles (Table 7). As evident from Table 7, f_2 values are less for the HPLC-1 method in both the dissolution media and hence do not give accurate results if used for the calculation of RIF release. On the other hand, profiles obtained with colorimetry are most similar to the HPLC-4 method based on the f_2 value close to 100. Thus, colorimetry with respect to a reference vessel gives accurate results and can be used for routine dissolution studies. Colorimetric detection provides a simple, rapid and cost-effective tool to determine quality of FDC formulations.

Table 7: Comparison of dissolution profiles obtained after different calculation methods by similarity factor (f_2)

Calculation method	f_2 values	
	0.01 N HCl	0.1 N HCl
HPLC-1	64.9	25.6
HPLC-2	81.2	70.0
HPLC-3	93.9	93.2
Colorimetry	84.9	94.7

Profile obtained by HPLC-4 method was considered as reference for the calculation of f_2 factor.
Degree of similarity between the profiles was determined from closeness of f_2 value to 100 whereas profiles were considered dissimilar if f_2 is below 50.
As described in the footnote of Table 6, RIF degradation in 0.1N HCl was more during the chromatographic run than degradation during dissolution study that has resulted in dissimilar dissolution profile as indicated by f_2 of 25.6. Whereas, profiles were similar when loss of RIF due to degradation was corrected (f_2 in the range of 50–100).
In 0.01N HCl, rate of degradation was less and hence HPLC-1 profile was similar to HPLC-4. However, as indicated by f_2 values, degree of closeness was less compared to HPLC-2, HPLC-3 and colorimetric method

2.6. Conclusions

This study explains the reasons for variability of *in vitro* RIF analysis that are reported in the literature. It was found that filter type, time of injection and interpretation of data are important factors that can affect analysis results of RIF from *in vitro* studies or quality control. We have provided a simple correction factor that can be applied to correct for decomposition of RIF thereby accounting for delay in injection and degradation products. In addition, this investigation has proved that dissolution samples of all types of FDCs can accurately be analysed for RIF by simple and convenient method like colorimetry, which is a recommended method of analysis for dissolution samples in acidic media for RIF alone, two drug and three drug FDC formulations in USP.

3. Experimental

3.1. Materials

RIF, INH, pyrazinamide and ethambutol hydrochloride were kindly supplied by Lupin Laboratories Ltd, India. HPLC grade acetonitrile and methanol were procured from J. T. Baker, USA). All other chemicals used were of analytical grade and procured from E. Merck, India. Filters were purchased from Millipore, India. Ultra pure water obtained by reverse osmosis (Elgastat, Elga Ltd, UK) was used in all the experiments. Always solutions were freshly prepared before start of the experiment.

3.2. Instruments

For analysis, a Waters HPLC system (Milford, MA, USA) consisting of a 600 E multisolvent delivery system, 717 plus autosampler and 2487 dual λ absorbance detector was used. MILLENNIUM^{32} software (version 3.05.01) was used for data acquisition and processing. Colorimetric analysis of RIF was done with a Beckman DU® 640i spectrophotometer (Fullerton, CA, USA). Other instruments include Electrolab dissolution tester (USP XXIII, Mumbai, India), Elgastat (Elga Ltd, Bucks, UK), electronic balance AG 245 (Greifensee, Switerzerland), Branson 3210 sonicator (The Hague, The Netherlands), Millipore syringe filtration assembly (Bangalore, India), Brand autopipettes from E. Merck (Mumbai, India) and microlitre syringes from Hamilton (Bonaduz, Switzerland).

3.3. Analysis of RIF alone or in combination with INH

RIF was analysed by a modified reverse phase HPLC method as specified in USP 26 for the assay of RIF, INH and pyrazinamide from a four drug FDC tablet. The column was reverse phase Symmetry C-18 (250 × 4.6 mm i.d., 4 μm) and the mobile phase was a gradient run of 10 mM pH 6.8

phosphate buffer and acetonitrile. At a flow rate of 1.5 ml/min and a run time of 20 min, the gradient was programmed as 99 : 1 phosphate buffer and acetonitrile for initial five min that had changed to 45 : 55 in the next minute maintained up to 16 min and back to the initial composition until 20 min. Detection wavelength was 238 nm. Retention times for INH, pyrazinamide and RIF were 2.45, 3.12 and 12.55 min, respectively. The method was validated for all the analytes. This method has the ability to resolve degradation products of RIF such as 3F-RIF and the RIF-INH interaction product HYD.

RIF from dissolution study of four drug FDC was also analysed by colorimetry at λ_{max} 475 nm. At this wavelength there was no interference with the other anti-TB drugs and excipients present in the formulation. The method was linear in the concentration range of 1–40 μg/ml in 0.1 N HCl and 0.01 N HCl ($R^2 > 0.999$). However, release of RIF from the dosage forms was calculated with respect to a reference vessel as specified by USP for the dissolution of RIF alone, 2 drug and 3 drug FDC formulations (USP 26, 2003).

3.4. Filter validation

For filter validation, five different types of filters were used (low protein binding polyvinylidene, glass fibre, polycarbonate, mixed cellulose esters and nylon filter) with 25 mm diameter. Except glass fibre, all the filters had a pore size of 0.45 μm whereas the glass fibre filter was pre-filter. For the purpose of determination of binding to the filters, RIF solution (1 mg/ml in 0.1 N HCl, 10 ml) was passed through each filter. After filtering the RIF solution, air was passed to remove the solution entrapped in the pores. The filters were air-dried and bound RIF was extracted in 10% methanol, which was then analysed by colorimetry and RIF binding results were expressed as μg/cm^2.

The effect of concentration of RIF solution on the binding was also studied using a nylon filter which showed the highest binding at 1 mg/ml. Eight concentrations of RIF solution were prepared in the range of 30 μg/ml to 4 mg/ml and 10 ml of this solution was passed through the nylon filter and the procedure was repeated as mentioned above.

3.5. Deriving the correlation between RIF and its degradation products

For the purpose of deriving the correlation between RIF and its degradation products in acidic medium, RIF solution was injected into HPLC over a period of time. All the peaks were integrated and its relation to decrease in RIF area was calculated.

3.5.1. RIF alone in 0.01 N HCl

RIF powder that would result in a 0.5 mg/ml solution in 0.01 N HCl was prepared immediately before the injection by sonicating it for 2 min and filtered through a 0.45 μm polycarbonate filter. The resultant solution was injected into HPLC (injection volume 50 μl) that was already kept equilibrated (please note that RIF solution that was injected into HPLC did not correspond to 0.5 mg/ml, as rate of solubilization of RIF in 0.01 N HCl is slow because of the wetting problem at this pH). Subsequently, the same solution was injected over a period of 11 h from the vial maintained at 25 °C in an autosampler. The first injection was considered as time zero injection and % degradation was calculated as decrease in RIF area corresponding to time zero peak.

3.5.2. RIF plus INH in 0.01 N HCl

RIF and INH corresponding to 0.5 mg/ml and 0.25 mg/ml solution (ratio of RIF and INH was taken according to WHO recommended dose) in 0.01 N HCl was prepared and processed similarly as mentioned for RIF alone.

3.5.3. RIF plus INH in 0.1 N HCl

The same experiment was repeated for RIF plus INH at 0.1 N HCl. Here, as the solution was formed almost instantaneously, the amount of RIF and INH were taken corresponding to 0.2 mg/ml and 0.1 mg/ml, respectively.

3.6. Dissolution study of commercial RIF formulation

An *in vitro* dissolution study was carried out on a marketed four drug FDC formulation in different dissolution media (0.01 N HCl and 0.1 N HCl) maintained at 37 ± 0.5 °C using an USP Type II apparatus (paddle) at 50 rpm. Samples (5 ml) were withdrawn at 10, 20, 30 and 45 min with replacement by fresh dissolution medium. For each dissolution test five tablets were used. The sixth vessel was used as reference vessel in which pure drugs equivalent to the amounts present in the formulation were dissolved (USP 2003). Samples collected at different time intervals were analysed by HPLC as well as immediate analysis by colorimetry at 475 nm after appropriate dilution. For colorimetric analysis, % release of RIF was calculated with respect to the reference vessel based on the labelled amount of RIF in the formulations. In case of HPLC, % release was calculated by different methods:

HPLC-1: RIF peak compared to zero time concentration
HPLC-2: RIF peak compared to concentration of reference peak
HPLC-3: Sum of RIF, 3F-RIF and HYD peaks
HPLC-4: Total RIF corrected for degradation factor

Dissolution profiles obtained by these five methods were compared by a model independent approach using the similarity factor (f_2) taking total RIF corrected for degradation factor (HPLC-4) as the reference (Moore 1996; FDA 1997). Dissolution profiles were considered similar when the f_2 value was greater than 50 (values range from 50–100).

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