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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING HPTLC METHOD FOR ANALYSIS OF ANTITUBERCULAR DRUGS

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SUMMARY

A simple, selective, precise, and stability-indicating high-performance thin-layer chromatographic (HPTLC) method has been established and validated for analysis of isoniazid and rifampicin both as the bulk drugs and in formulations. The compounds were separated on aluminum-backed silica gel 60 F_{254} plates with *n*-hexane–2-propanol–acetone–ammonia–formic acid, 3:3.8:2.8:0.3:0.1 (*v*/*v*) as mobile phase. This system was found to give compact spots for isoniazid and rifampicin (R_F values 0.59 ± 0.02 and 0.73 ± 0.04, respectively). Densitometric analysis of isoniazid and rifampicin was performed at 254 nm. Regression analysis data for the calibration plots were indicative of good linear relationships between response and concentration over the range 100–700 ng per spot. The correlation coefficients, r^2, were 0.994 and 0.997 for isoniazid and rifampicin respectively. The values of slope and intercept of the calibration plots were 3.755 ± 0.22 and 3099.1 ± 51.21, respectively, for isoniazid and 4.0957 ± 0.25 and 3567.6 ± 61.11, respectively, for rifampicin. The method was validated for precision, recovery, and robustness. The limits of detection and quantification were 20 ± 0.51 and 60 ± 1.05 ng, respectively, for isoniazid and 25 ± 0.63 and 75 ± 1.12 ng, respectively, for rifampicin. Isoniazid and rifampicin were subjected to acid, base, peroxide, and UV-induced degradation. In stability tests the drugs were susceptible to acid and basic hydrolysis, oxidation and photodegradation. Statistical analysis proved the method is repeatable, selective, and accurate for estimation of isoniazid and rifampicin. Because the method could effectively separate the drugs from their degradation products, it can be used as a stability-indicating method.

INTRODUCTION

Isoniazid (isonicotinic acid hydrazide; INH), a whitish crystalline powder, and rifampicin (3-[[(4-methyl-1-piperazinyl)imino]methyl]-5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[*N*-(4-ethyl-1-piperazinyl) formimidoyl]-2,7-epoxypentadeca[1,11,13]trienimino)naphtha[2,1-*b*]furan-1,11(2*H*)-dione 21-acetate; RIF), which is reddish in colour, are among the most important antitubercular drugs. They are used in combination therapy for treatment of the tuberculosis caused by *Mycobacterium tuberculosis* [1,2].

A literature survey reveals that RIF degrades rapidly in the presence of acid, which means that at acidic pH in the stomach its bioavailability is low [3–5]. A variety of methods, for example ultraviolet spectroscopy (UV) [6–9], high-performance liquid chromatography (HPLC) [10–13], and high-performance thin-layer chromatography (HPTLC) [14], are available for the analysis of INH and RIF in combination therapy. Only one HPTLC method is available for analysis of RIF and INH in the bulk drug and in the pharmaceutical dosage form, and it is not a stability-indicating method [14]. The International Conference on Hormonization (ICH) guideline entitled "Stability Testing of New Drug Substances and Products" requires stress testing to be conducted to assess the inherent stability of the active substance [15]. Tests of susceptibility to oxidation, hydrolysis, and photolytic degradation are required. An ideal stability-indicating method is one that quantifies the drug and resolves its degradation products [16]. HPTLC is becoming a routine analytical technique because of advantages [17–19] which include the small amount of mobile phase required, the speed of the method, and the possibility of analysis of several samples simultaneously (i.e. on the same plate), unlike HPLC. It thus reduces analysis time and cost per analysis. HPTLC does not, moreover, suffer from pH restrictions – mobile phases of pH 8 and above can be used, in contrast with HPLC. Cloudy samples and suspensions can also be analysed directly by HPTLC. Automatic sample application is possible in HPTLC and repeated scanning can be performed on the same plate, so scanning conditions can be changed.

The objective of this work was to develop and validate an accurate, specific, precise, repeatable, and stability-indicating method for determination of INH and RIH in combination dosage forms, in the presence of their degradation products, as stipulated by the ICH guidelines.

EXPERIMENTAL

Chemicals

Isoniazid and rifampicin were obtained as gifts from Sunij Pharma, Ahmedabad, India. Chloroform, formic acid, and ammonia were purchased from S.D. Fine Chemicals, Mumbai, India. Acetone and 2-propanol were purchased from Merck, Mumbai, India. Methanol was purchased from Spectrochem, Mumbai, India, and *n*-hexane from Thomas Baker, Mumbai, India. All chemicals and reagents were of analytical reagent (AR) grade.

Chromatography

Chromatography was performed on 20 cm × 20 cm aluminium-backed plates coated with 0.2 mm layers of silica gel 60 F_{254} (E. Merck, Germany). Samples were applied to the plates as 4 mm bands, 5 mm apart, by means of a Camag (Switzerland) Linomat V sample applicator fitted with a Camag microlitre syringe. A constant application rate of 150 nL s^{-1} was used. Linear ascending development of the plates to a distance of 80 mm was performed with *n*-hexane–2-propanol–acetone–ammonia–formic acid, 3:3.8:2.8:0.3:0.1 (%, *v*/*v*), as mobile phase in a twin-trough glass chamber previously saturated with mobile phase vapour for 10 min at room temperature (25°C). (This saturation time had previously been found to be optimum.) After development the plate was scanned at 254 nm by means of a Camag TLC scanner in absorbance mode, using the deuterium lamp. The slit dimensions were 4 mm × 0.1 mm and the scanning speed was 20 mm s^{-1}.

Method Development

A variety of mobile phases were investigated to establish a suitable HPTLC method for analysis of RIF and INH in bulk drug and tablet dosage form. These included *n*-hexane–2-propanol, 5:5 (%, *v*/*v*), *n*-hexane–2-propanol–acetone, 4:4:2 (%, *v*/*v*), *n*-hexane–2-propanol–acetone, 3:3:4 (%, *v*/*v*), *n*-hexane–2-propanol–acetone–ammonia, 3:3:3:1 (%, *v*/*v*), and *n*-hexane–2-propanol–acetone–ammonia–formic acid, 3:3.8:2.8:0.3:0.1 (%, *v*/*v*). The suitability of the mobile phase was decided by study of the sensitivity of the assay, the time required for analysis, and the use of readily available solvents.

Calibration Plots for INH and RIF

A stock solution of isoniazid and rifampicin (100 μg mL^{-1}) was prepared in chloroform–methanol, 50:50 (% *v*/*v*). Different volumes of the

stock solution (1, 2, 3, 4, 5, 6, and 7 μL, equivalent to 100, 200, 300, 400, 500, 600, and 700 ng INH and RIF) were applied, in duplicate, to a TLC plate, and after development peak height and peak area data and drug concentration data were treated by linear least-squares regression to determine linearity.

Method Validation

Precision

In accordance with ICH recommendations precision was determined at two levels, i.e. repeatability and intermediate precision. Repeatability of sample application was determined as intraday variation whereas intermediate precision was determined by measuring inter-day variation for triplicate determination of INH and RIF at three different concentrations (200, 300, and 600 ng per spot).

Robustness

Small changes in mobile phase composition were introduced and the effect on the results was examined.

Limits of Detection (LOD) and Quantification (LOQ)

INH and RIF at concentrations in the lower part of the linear range of the calibration plot were used to determine limit of detection (LOD) and limit of quantification (LOQ). They were determined from the slope of the calibration plot and standard deviation (*SD*) of the blank sample by use of the equations:

$$\text{LOD} = 3.3 \times SD/S \text{ and } \text{LOQ} = 10 \times SD/S$$

where *SD* is the standard deviation of the blank response and *S* is the slope of the calibration plot.

Recovery

Preanalyzed samples were spiked with 50, 100, and 150% extra INH and RIF standards and then analysed in triplicate to check recovery from the formulations of the drug at different levels.

Analysis of INH and RIF in Formulations

To determine the INH and RIF content of tablets (label claim 750 mg per tablet of which 300 mg is INH and 450 mg is RIF) a tablet was

powdered and powder equivalent to 10 mg drugs was weighed. The drugs were extracted from the powder with methanol and chloroform (50:50, *v*/*v*). To ensure complete extraction of the drug it was sonicated for 20 min. The volume was then diluted to 100 mL and the solution (200 ng per spot) was applied to the TLC plate followed by development and scanning as described above. The analysis was repeated in triplicate.

Forced Degradation of INH and RIF

Acid and Base-Induced Degradation

Isoniazid (50 mg) and rifampicin (50 mg) were separately dissolved in 50 mL 0.1 M HCl and in 0.1 M NaOH. These solutions were heated under reflux for 1 h at 75°C and then applied to a TLC plate (200 ng per spot). Chromatography was performed as described above.

Hydrogen Peroxide (H_2O_2)-Induced Degradation

Hydrogen peroxide (30% (*v*/*v*), 25 mL) was added to separate solutions of INH and RIF (2 mg mL^{-1}, 25 mL). The solutions obtained (200 ng per spot) were applied to TLC plates in triplicate and chromatography was performed as described above.

Photochemical Degradation

INH and RIF (50 mg of each) were separately dissolved in methanol–chloroform (50:50, *v*/*v*; 50 mL) and exposed to direct sunlight and UV at 254 nm in a UV chamber for 24 h. The solutions obtained were applied (200 ng per spot) to TLC plates and chromatography was performed as described above.

RESULT AND DISCUSSION

Optimisation of the Method

TLC procedure was optimized to develop a stability-indicating assay. The mobile phase *n*-hexane–2-propanol–acetone–ammonia–formic acid, 3:3.8:2.8:0.3:0.1, resolved INH and RIF with R_F values of 0.59 ± 0.02 and 0.73 ± 0.04, respectively (Fig. 1). Well defined spots (and peaks) were obtained when the chamber was saturated with mobile phase vapour for 15 min at room temperature (25°C).

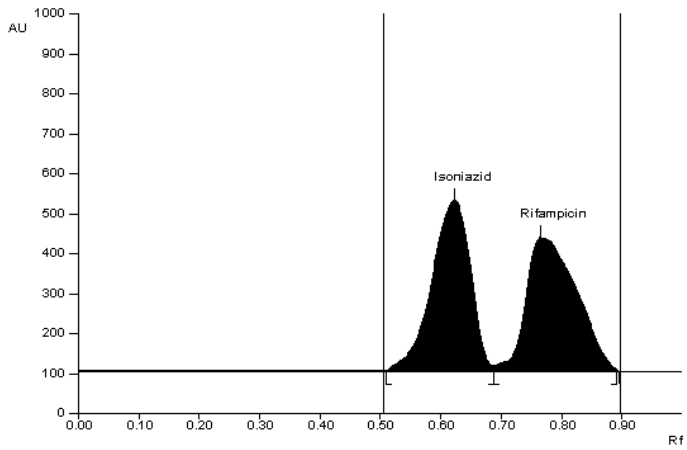

Fig. 1

HPTLC chromatogram of isoniazid and rifampicin

Calibration Plots

Linear regression data for the calibration plots for INH and RIF ($n = 3$) were indicative of a good linear relationship between peak area response and concentration over the range 100–700 ng per spot (Table I). There was no significant difference between the slopes of the calibration plots.

Table I

Calibration plot data for INH and RIF ($n = 3$)

Concn (ng per spot)	Mean area ± *SD* (INH)	Mean area ± *SD* (RIF)
100	3511.0 ± 59.83	3991.4 ± 14.00
200	3899.5 ± 15.60	4423.0 ± 35.23
300	4169.3 ± 21.27	4795.3 ± 20.64
400	4501.0 ± 33.81	5134.6 ± 13.45
500	5023.6 ± 19.84	5567.6 ± 21.93
600	5312.1 ± 15.91	6078.2 ± 18.84
700	5787.2 ± 58.38	6453.9 ± 35.23

Validation of the Method

Linearity

The linear range for INH and RIF solutions was 100–700 ng per spot, as shown in Table I. The linear regression equations were $y = 3.755x + 3099.1$,

regression coefficient 0.994, for INH and $y = 4.0957x + 3567.6$, regression coefficient 0.997, for RIF (Table II).

Table II

Linear regression data for calibration plots for INH and RIF ($n = 3$)

Data	INH	RIF
Linearity range (ng per spot)	100–700	100–700
Regression equation	$y = 3.755x + 3099.1$	$y = 4.095x + 3567.6$
Correlation coefficient	0.994	0.997
Slope ± *SD*	3.755 ± 0.22	4.095 ± 0.25
Intercept ± *SD*	3099.1 ± 51.21	3567.6 ± 61.11
Slope without intercept ± *SD*	3.854 ± 0.24	4.193 ± 0.27
Standard error of slope	0.127	0.144
Standard error of intercept	29.56	35.28
Bias of intercept	−0.0351	−0.0432

Precision

The repeatability (intraday precision) of sample application was determined as intraday variation whereas the intermediate precision was determined by measuring inter-day variation for determination of INH and RIF at three different concentrations (200, 300, and 600 ng per spot), in triplicate. Results from determination of repeatability and intermediate precision, as % *RSD*, are shown in Table III. The low values of % *RSD* are indicative of the high repeatability of the method.

Table III

Precision of the method ($n = 3$)

Concn (ng per spot)	Repeatability (intraday precision)			Intermediate precision (interday)		
	Mean area ± *SD*	Standard error	*RSD* (%)	Mean area ± *SD*	Standard error	*RSD* (%)
INH						
200	3892.98 ± 6.90	2.81	0.17	3727.28 ± 49.22	20.09	1.32
300	4199.83 ± 11.17	6.44	0.26	4118.13 ± 12.21	7.04	0.29
600	5328.76 ± 10.30	4.20	0.19	5276.32 ± 16.52	6.74	0.31
RIF						
200	4452.93 ± 16.88	6.89	0.37	4461.21 ± 19.63	8.01	0.44
300	4815.3 ± 21.34	12.32	0.44	4745.6 ± 18.55	10.71	0.39
600	6154.76 ± 23.60	9.63	0.38	6059.71 ± 21.14	8.63	0.34

Robustness

The low values of % *RSD* obtained after introducing small changes in mobile phase composition and volume (Table IV) were indicative of the robustness of the method. There was no significant variation of the slopes of the calibration plots.

Table IV

Robustness of the method for INH and RIF (n = 3)

Amount (ng per spot)	Change in mobile phase composition (% *RSD*)	When 15 mL mobile phase was used (% *RSD*)
200	0.71	0.38
300	0.32	0.51
600	0.54	0.23

Limits of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ were determined from the slope of the lowest part of the calibration plot. The LOD and LOQ were 20 ± 0.51 and 60 ± 1.05 ng, respectively, for INH and 25 ± 0.63 and 75 ± 1.12 ng, respectively, for RIF, which indicates the sensitivity of the method is adequate.

Recovery

The method was used for estimation of INH and RIF in pharmaceutical dosage forms after spiking with 50, 100, and 150% additional drug. The recovery was 100.10–100.48% (Table V), which indicated the accuracy of the method was adequate.

Table V

Results from recovery studies (n = 3)

Excess drug added (%)	Total theoretical content (mg)	Individual theoretical content (mg)		Recovery (%)		*RSD* (%)		Standard error	
		INH	RIF	INH	RIF	INH	RIF	INH	RIF
50	75	30	45	100.10	100.08	0.25	0.21	8.35	7.57
100	100	40	60	100.36	100.29	0.26	0.34	11.89	15.98
150	125	50	75	100.48	100.37	0.20	0.15	11.43	9.26

Analysis of INH and RIF in Formulation

Spots at R_F 0.59 ± 0.02 for INH and 0.73 ± 0.04 for RIF were observed in the chromatogram obtained from INH and RIF extracted from tablets. There was no interference from excipients commonly present in the tablets. The INH and RIF content were found to be 99.28–99.53% and 98.99–99.74%, respectively, of the label claim. The low value of % *RSD* indicated the method was suitable for routine analysis of the INH and RIF in pharmaceutical dosage forms.

Forced Degradation of INH and RIF

The chromatograms obtained from samples degraded by treatment with acid, base, hydrogen peroxide, sunlight, and UV light contained well-separated spots of the pure drugs and some additional peaks at different R_F values. It is apparent from Figs 2–6 that the spots of the degradation pro-

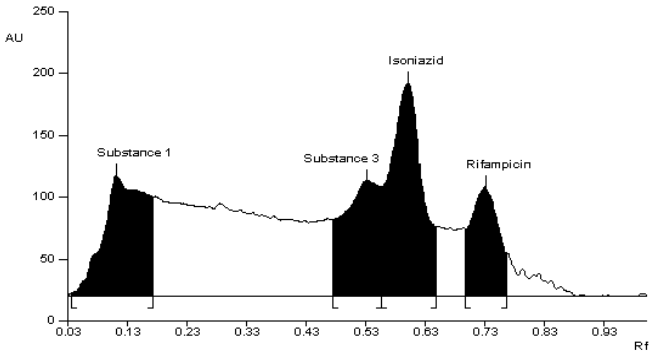

Fig. 2

Peaks obtained after acidic stress of isoniazid and rifampicin

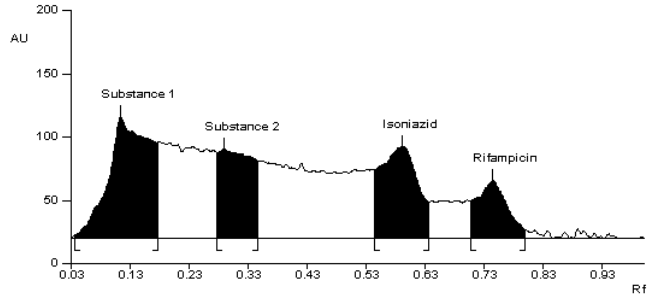

Fig. 3

Peaks obtained after basic stress of isoniazid and rifampicin

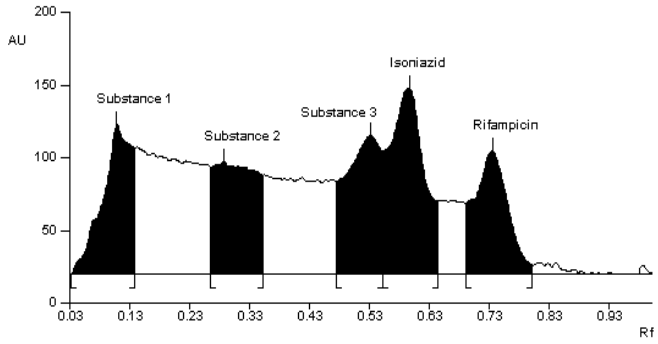

Fig. 4

Peaks obtained after peroxide stress of isoniazid and rifampicin

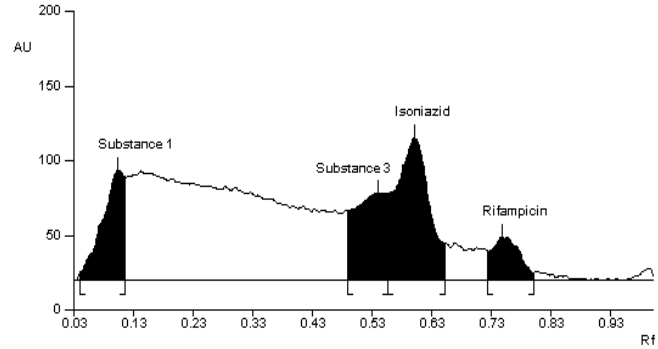

Fig. 5

Peaks obtained after UV degradation of isoniazid and rifampicin

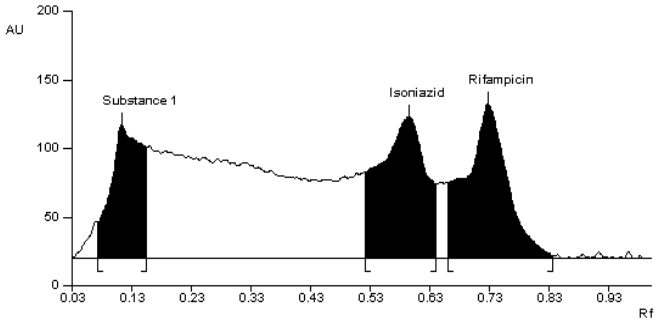

Fig. 6

Peaks obtained after photolytic stress of isoniazid and rifampicin

ducts were well resolved from those of the drugs. The peaks of INH and RIF were not significantly shifted in the presence of the degradation peaks, which indicated the stability-indicating nature of the method. The R_F values of the degradation products are listed in Table VI.

Table VI

R_F values of degradation products obtained from different stress conditions

Stress conditions	Number of degradation products (R_F values)	INH (R_F)	RIF (R_F)
Acid (0.1 M HCl)	2 (0.11, 0.53)	0.60	0.73
Base (0.1 M NaOH)	2 (0.11, 0.29)	0.59	0.74
Peroxide (30% H_2O_2)	3 (0.11, 0.28, 0.53)	0.59	0.73
UV degradation	2 (0.10, 0.54)	0.60	0.74
Photolytic degradation (day light)	1 (0.11)	0.59	0.72

CONCLUSION

This HPTLC method is precise, specific, accurate, and stability-indicating. Statistical analysis proved the method is repeatable and selective for the analysis of isoniazid and rifampicin as bulk drug and in pharmaceutical formulations. The method can be used to determine the purity of the drug obtained from different sources by detecting related impurities. It may be extended to determination of the degradation kinetics of isoniazid and rifampicin in biological fluids. Because the method separates the drug from its degradation products, it can be used as a stability-indicating method.

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