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Short communication

Interference of isonicotinyl hydrazone in the microbiological analysis of rifampicin from anti-tuberculosis FDC products containing isoniazid

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Abstract

Microbiological assay is a sensitive method for the estimation of rifampicin (R). In the present study, interference due to isonicotinyl hydrazone (HYD), an interaction product of R and isoniazid (H), was checked during microbiological analysis of R, employing *Bacillus subtilis* and *Sarcina lutea*. The assays were done by disc diffusion method. Both R and HYD showed linear log response curves in the range of 0.01–10 μg. In the presence of HYD, R was overestimated when tested against *S. lutea* and underestimated in case of *B. subtilis*. The same extent and type of interference was observed on assay of a marketed anti-tuberculosis fixed-dose combination product, subjected to accelerated stability testing (40 °C/75% RH) for 1 month. This means that response of organisms used in microbiological assay of R might vary in the presence of HYD, with possibility of incorrect conclusions. Therefore, the study suggests that before a microbiological method involving a particular organism is extended to the determination of R in FDC formulations containing H, it should be tested for the influence of HYD and used only if non-interfering.

Keywords: Rifampicin; Isonicotinyl hydrazone; Interference; Microbiological assay

1. Introduction

Rifampicin (R) is an important anti-mycobacterial agent used in the treatment of tuberculosis and leprosy. Its analysis in formulations and in plasma samples is often carried out by microbiological assay methods involving organisms such as *Bacillus subtilis* [1–3], *Sarcina lutea* [4–9] and *Staphylococcus aureus* [10–12]. These methods are also used for analysis of R in the presence of other co-drugs, like isoniazid (H), pyrazinamide (Z) and ethambutol (E) [13–14]. A distinct advantage of these methods is their sensitivity [3–9].

When R is present along with H in fixed-dose combination (FDC) formulations, the two drugs interact with each other [15], resulting in formation of isonicotinyl hydrazone (HYD) [16–17]. Accordingly, the purpose of this study was to assess the interference of HYD in microbiological analysis of R using *B. subtilis* and *S. lutea*. The results are discussed in this communication.

2. Experimental

2.1. Materials

R and H were gift samples from M/S Panacea Biotec Ltd., Lalru, India. They were used as received (Assay: R = 99.20%; H = 99.72%). Their expiry dates were March 2005 and February 2006, respectively. HYD was prepared by a previously reported method [18] to a purity of 98.92%. A product containing 450 mg R, 300 mg H and 800 mg E (CONFEZ®-3, Batch No. 2001), manufactured by Deepin Pharmaceuticals

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Pvt. Ltd., Kalaria, India was purchased from the local market. *B. subtilis* MTCC 441 and *S. lutea* MTCC 1541 were obtained from Institute of Microbial Technology, Chandigarh, India. Mueller Hinton agar medium, antibiotic assay medium no. 1 and glass petri-plates (90 mm) were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Filter discs (ZEROHAZE grade A general purpose filters) were obtained from S.d. fine Chem Ltd., Mumbai, India. Buffer materials and all other chemicals were of analytical-reagent grade. HPLC grade acetonitrile and methanol were procured from J.T. Baker (Mexico City, Mexico) and Mallinckrodt Baker Inc. (Kentucky, USA), respectively. Ultra-pure water was obtained from an ELGA water purification unit (Elga Ltd., Bucks, England).

2.2. Equipment

The pH recordings were made on a research pH meter (MA 235, Mettler Toledo GmbH, Schwerzenbach, Switzerland). Other equipment used were stability chamber (KBF 720, WTB Binder, Tuttlingen, Germany), ultrasonic bath (Branson Ultra-sonic Corporation, Danbury, CT, USA), digital orbit shaker incubator (Neolab, Mumbai, India), stationary incubator (York Scientific Industries, Delhi, India), B.O.D. incubator (Mahajan Equipment Industries, Mumbai, India), analytical balance (AG 135, Mettler Toledo, Greifensee, Switzerland), shaking water bath (SWB20, Haake, Karlsruhe, Germany), and auto pipettes (Tripette, Merck KGaA, Darmstadt, Germany). The HPLC system consisted of a dual-piston-reciprocating pump (LC-10ATVP), UV–vis dual-wavelength detector (SPD-10AVP), an auto injector (SIL-10ADVP), an on-line degasser (DGU-14AM), and CLASS-VP software (all from Shimadzu, Kyoto, Japan).

2.3. Preparation of solutions containing different ratios of R:HYD

Stock solutions containing 1 mg/ml solutions of R and HYD were prepared in methanol and mixed in different proportions to get R:HYD ratio between 100:0 to 0:100.

2.4. Degradation of a marketed product

The marketed FDC product was exposed to accelerated stability test conditions (40 °C/75% RH) in an unpackaged state for 1 month to induce reaction between R and H and formation of HYD.

2.5. Microbiological assays

Microbiological assay against *B. subtilis* was conducted using the procedure given by Saito and Tomioka [1]. A small quantity of *B. subtilis* culture was transferred to sterile nutrient broth. The same was shaken for 24 h at 250 rpm at 37 °C. The turbidity was matched with MacFarlane's standard (3×10^8 cells/ml) and 100 μl of this culture was transferred to fresh 10 ml nutrient broth. From this, 500 μl was inoculated into each autoclaved petri-plates containing Mueller Hinton agar medium and spreaded uniformly. A filter disc, which was autoclaved prior to use (6 mm), was placed on the medium in the centre of the plate and 5 μl of the test solution was dropped on this disc. One set of plates was run as negative controls, in which 5 μl blank solvent was applied on the disc, instead of the test solution. The plates were incubated for 24 h and zone of inhibition was measured as the diameter in three dimensions.

The method used for microbiological assay involving *S. lutea* was same as reported in literature [9]. The inoculum of *S. lutea* was added to sterile nutrient agar. It was shaken for 24 h at 250 rpm at 37 °C. The culture was harvested in saline and diluted to give transmittance of 25–30% at 530 nm. From this suspension, 1 ml was transferred to 100 ml of molten antibiotic assay medium no. 1. This medium was poured into autoclaved glass petri plates. The test samples were applied on 6 mm autoclaved filter discs, including negative controls, in a manner similar to the one discussed above for *B. subtilis*. Zone of inhibition was measured after 24 h incubation.

2.6. Validity of the microbiological assay results

The precision of the microbiological methods was established by carrying out the assay in quadruplicate for both the organisms. The whole set of analyses was also conducted in another laboratory by a different analyst to establish the robustness.

2.7. HPLC analyses

HPLC analyses were carried out on a Zorbax XDB C-18 (250 mm × 4.6 mm, 5 μm) column (Agilent Technologies, Wilmington, USA) using a validated gradient method reported earlier [19]. The mobile phase composed of acetonitrile and a buffer consisting of 0.01 M sodium dihydrogen orthophosphate (pH adjusted to 6.8 with dilute ortho phosphoric acid). The method was separative to R, H and HYD, allowing their simultaneous analysis.

2.8. Analysis of R from mixtures of R and HYD, and degraded marketed samples

The solutions containing different proportions of R and HYD (90:10 to 10:90) were analysed for R by microbiology using both the organisms. The degraded marketed product (n = 3) was analyzed for R microbiologically, and for R, H and HYD by HPLC.

3. Results and discussion

3.1. Standard plots of R and HYD

Fig. 1 shows standard plots for microbiological analysis of both R and HYD using *B. subtilis* and *S. lutea*. In the figure, all curves are linear, indicating that both R and HYD were

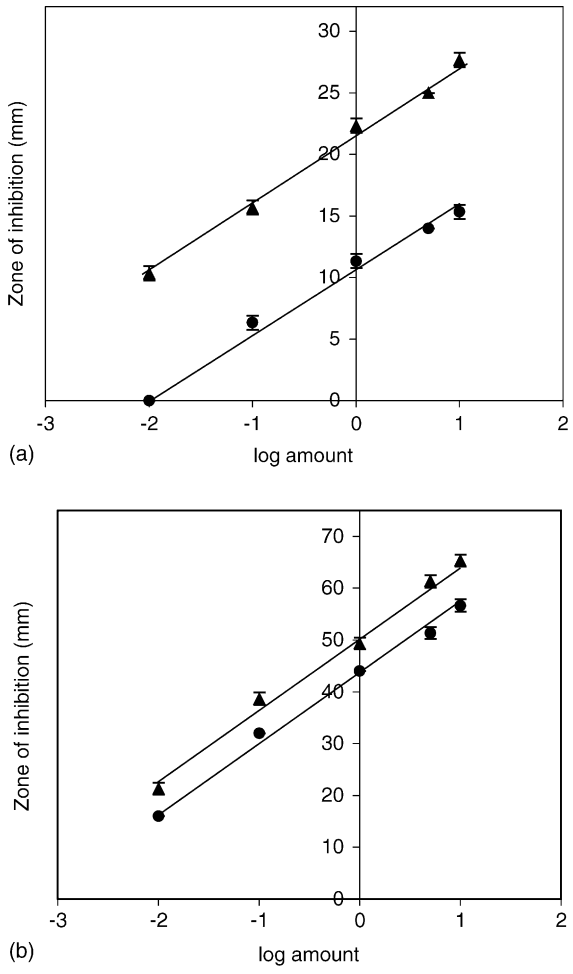

Fig. 1. Standard plots of R (▲) and HYD (●) against *B. subtilis* (a) and *S. lutea* (b).

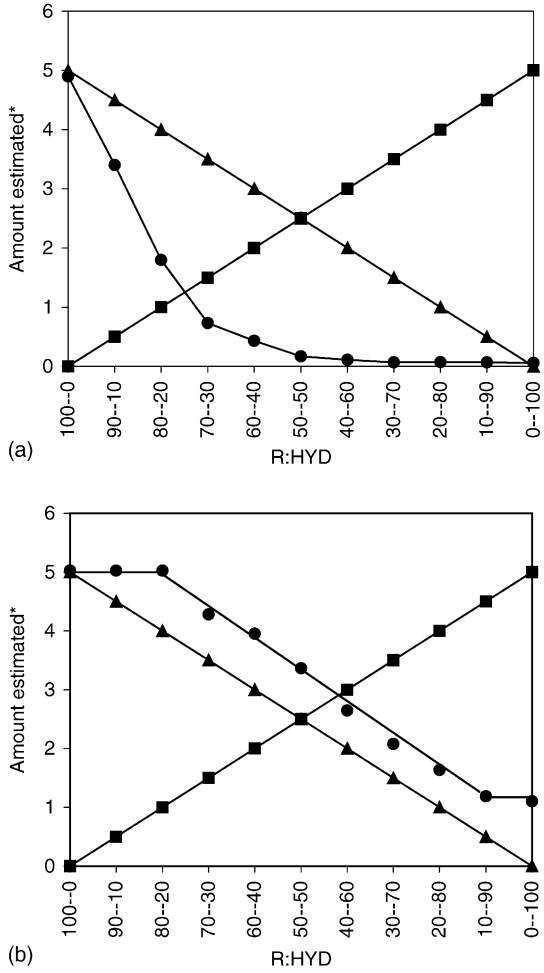

Fig. 2. Interference of HYD in estimation of R by microbiological assay using *B. subtilis* (a) and *S. lutea* (b). *Key*: (▲) R alone; (■) HYD alone; (●) R in the presence of HYD (* in μg).

active against the two organisms. The sensitivity of organisms to R was more in case of *S. lutea* than *B. subtilis*, which is in agreement with the literature reports [20]. As evident, HYD possessed lesser activity than R in both organisms, though it was more active to *S. lutea* than *B. subtilis*.

3.2. Validation of the microbiological methods

Table 1 shows the results of the zone of inhibition of R and HYD against both the organisms along with S.D. and R.S.D. values. It indicates that the method was precise, as R.S.D. values are <5% in all the determinations. Table 2 shows the slope, intercept and R^2 values of standard plots for both R and HYD established separately in two different laboratories by separate analysts. It is clear from the data that the slope and intercept values were almost same, despite that estimations were done by separate analysts in different laboratories.

Table 1
Mean zones of inhibition and precision of the analyses

Amount (μg)	Mean zone of inhibition (mm) ± S.D. (%R.S.D.) (n = 4)			
	B. subtilis		*S. lutea*	
	R	HYD	R	HYD
0.01	11.3 ± 0.5 (4.4)	–[a]	21.8 ± 0.5 (2.3)	16.0 ± 0.0 (0.0)
0.1	14.8 ± 0.5 (3.4)	10.0 ± 0.0 (0.0)	38.5 ± 1.0 (2.6)	32.0 ± 0.0 (0.0)
1.0	20.8 ± 0.9 (4.6)	12.3 ± 0.5 (4.1)	49.0 ± 1.2 (2.4)	44.0 ± 0.0 (0.0)
5.0	27.0 ± 0.8 (4.9)	14.8 ± 0.5 (3.4)	61.0 ± 1.2 (1.9)	51.5 ± 1.0 (1.9)
10.0	33.3 ± 0.9 (2.9)	17.5 ± 0.6 (3.3)	65.0 ± 1.2 (1.8)	56.5 ± 1.0 (1.8)

[a] No zone of inhibition observed at the given concentration.

Table 2
Robustness of microbiological assays

Organisms employed	Laboratory/analyst	R			HYD		
		Slope	Intercept	R^2	Slope	Intercept	R^2
B. subtilis	I	6.691 (0.448)[a]	22.591 (0.549)	0.992 (0.007)	5.322 (0.097)	12.085 (0.380)	0.979 (0.002)
	II	5.985 (0.151)	21.858 (0.077)	0.991 (0.004)	4.914 (0.214)	11.279 (0.206)	0.978 (0.013)
S. lutea	I	14.193 (0.404)	50.693 (0.779)	0.993 (0.002)	13.095 (0.296)	43.408 (0.403)	0.994 (0.002)
	II	14.178 (0.953)	50.389 (1.307)	0.989 (0.003)	13.325 (0.457)	43.018 (0.982)	0.991 (0.005)

[a] Values in the parentheses are S.D. of $n = 4$.

Table 3
Area % values of R, H and HYD obtained on HPLC analyses of marketed FDC product before and after storage

	Area % ± S.D. ($n = 3$)		
	R	H	HYD
Initial	63.65 ± 2.54	24.84 ± 2.79	11.46 ± 0.15
After storage[a]	24.46 ± 0.53	15.69 ± 1.61	59.65 ± 1.29

[a] 40 °C/75% RH for 1 month.

3.3. Analysis of R in the presence of different proportions of HYD

Fig. 2 shows the curves for analysis of R and HYD alone and when they were present together in different proportions. The plots show opposite effect, clearly indicating that R was underestimated when tested against *B. subtilis* (Fig. 2a) and was overestimated in case of *S. lutea* (Fig. 2b).

3.4. Analysis of degraded samples

Table 3 gives the results of HPLC analyses of the marketed FDC product for R, H and HYD, before and after storage under accelerated conditions. It indicated that the area % of R and H decreased in the degraded samples due to the involvement of the two drugs in the formation of HYD, with corresponding rise in the value of HYD.

The comparison of the results of analyses of the marketed FDC product by microbiology and HPLC is given in Table 4. It showed that R was underestimated to an extent of ∼85.0% in assay involving *B. subtilis* and overestimated by 36.9% when *S. lutea* was used. Thus this also confirmed the over- and underestimation of R in the presence of HYD by the microbiological method, involving *S. lutea* and *B. subtilis*, respectively.

Table 4
Comparison between microbiological and HPLC analyses of marketed FDC formulation

Analytical methods	Percentage of R in marketed sample		Percentage under/over estimation with respect to HPLC analysis[a]	
	Initial	After storage[b]	Initial	After Storage
HPLC	93.11	30.44	–	–
B. subtilis	72.22	4.55	−22.44	−85.01
S. lutea	109.11	41.66	+17.18	+36.85

[a] Negative values mean underestimation, and positive values mean overestimation.
[b] 40 °C/75% RH for 1 month.

4. Conclusion

The finding of this study is that R can be overestimated when tested against *S. lutea* and it can be underestimated against *B. subtilis* in the presence of HYD. It means any microbiological method, before it is extended to analysis of R in the presence of H, should be tested for interference due to HYD and used only if proved to be non-interfering. In conclusion, this study suggests that as much possible, microbiological assays should be avoided for estimation of R from FDC formulations containing H, and instead a specific technique like HPLC should be preferred.

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