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Mechanistic explanation to the catalysis by pyrazinamide and ethambutol of reaction between rifampicin and isoniazid in anti-TB FDCs

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Abstract

Rifampicin and isoniazid are known to interact with each other in solid formulation environment to yield isonicotinyl hydrazone (HYD). In earlier studies, this reaction was indicated to be catalyzed by pyrazinamide and ethambutol hydrochloride, the two other co-drugs present in oral anti-tuberculosis fixed-dose combination (FDC) formulations. Accordingly, the present study was carried out to understand the catalytic role of pyrazinamide and ethambutol hydrochloride on the reaction between rifampicin and isoniazid. For the purpose, organic bases and amides similar in structure to pyrazinamide and ethambutol hydrochloride were combined individually with rifampicin and isoniazid. The compounds employed were pyrazine, piperdine, pyrollidine, pyridine, triethylamine, diisopropylethylamine, picolinamide, benzamide, ethylenediamine, ethanolamine, diethanolamine, and triethanolamine. An additional study was also carried out in the presence of free base of ethambutol. The mixtures were exposed to accelerated stability test condition of 40 °C/75% RH for 15 d. The nature of the products formed and the changes in relative concentrations of the drugs and products were followed by HPLC. The drugs showed different extent of degradation, yielding HYD, and in some cases degradation products of rifampicin. The results confirmed the catalytic role of pyrazinamide and ethambutol hydrochloride. The catalysis is postulated to involve intra-molecular proton transfer during transhydrazone formation process, entailing a tetrahedral mechanism.

Keywords: Mechanism; Catalysis; Rifampicin; Isoniazid; Isonicotinyl hydrazone; Pyrazinamide; Ethambutol hydrochloride; Anti-TB FDCs

1. Introduction

Tuberculosis (TB) is a global emergency, as declared by WHO [1]. An integral part of the strategy to fight the disease is use of quality anti-TB drugs, including fixed dose combinations (FDCs) containing two or more first-line drugs, viz., rifampicin, isoniazid, pyrazinamide and ethambutol hydrochloride [2]. The problems associated with quality of FDC products and identification of the quality assessment parameters are in current focus [3–5]. The two major problems associated with quality of FDCs are (i) loss of bioavailability of rifampicin upon administration [6–9] and (ii) instability of drugs within the formulation environment [10,11]. In both cases, the problem has been ascribed to the decomposition of rifampicin in the presence of isoniazid to isonicotinyl hydrazone (HYD).

The anti-TB FDCs are administered on an empty stomach, where the pH ranges from 1.4 to 2.1 [12]. In this pH range, rifampicin degrades in a facile manner in the presence of isoniazid to HYD [13]. This reaction has been ascribed to be responsible for the reduction of in vivo bioavailability of rifampicin from FDC products [6,8]. It has been postulated that below pH 2, rifampicin is converted to 3-formylrifamycin, which further reacts with isoniazid to form HYD [14].

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In recent studies, it had been established that the reaction between rifampicin and isoniazid to HYD occurs even in the solid formulation environment. This was found when FDC products were exposed to accelerated stability test conditions of temperature and humidity [10]. Further, it was observed that three- or four-drug combinations containing rifampicin and isoniazid along with pyrazinamide and/or ethambutol hydrochloride showed far more chemical instability than two-drug FDCs containing rifampicin and isoniazid [11]. As the main stability concern with anti-TB FDC products is the interaction between rifampicin and isoniazid, the observation that there was greater instability in three- or four-drug combinations lead to the conclusion that pyrazinamide and ethambutol hydrochloride perhaps were catalytic towards the reaction between rifampicin and isoniazid.

Accordingly, the present study was undertaken to confirm the catalysis of reaction between rifampicin and isoniazid by the co-drugs, pyrazinamide and ethambutol hydrochloride. For the purpose, studies were carried out in the presence of various organic bases and amides related in structure to the co-drugs. The mechanisms involved were also explored.

2. Materials and methods

2.1. Materials

Rifampicin, isoniazid, pyrazinamide, ethambutol hydrochloride and 25-desacetyl rifampicin (25-DAR) were gift samples from M/S Panacea Biotec Ltd., Lalru, India. Rifampicin-N-oxide and rifampicin quinone were obtained from Lupin Ltd., Aurangabad, India. 3-Formylrifamycin and HYD were synthesized by the method described earlier [14]. Piperidine, pyridine, benzamide, ethylinediamine and ethanolamine were procured from s.d. Fine-chem Ltd., Mumbai, India. Pyrollidine, triethylamine, diethanolamine, triethanolamine, sodium phosphate dibasic and orthophosphoric acid were purchased from Loba Chemie, Mumbai, India. Pyrazine and diisopropylethylamine were obtained from Lancaster Synthesis, Morecambo, England. Picolinic acid was procured from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Mexico City, Mexico) and Mallinckrodt Baker Inc. (Paris, KY, USA), respectively. Picolinamide and free base of ethambutol were prepared in-house. All other chemicals were laboratory or analytical grade, as required.

2.2. Instruments

The HPLC system consisted of a DGU-14A degasser module, FCV-10ALVP flow control valve, LC-10ATVP pump, SIL-10ADVP auto injector, CTO-10ASVP column oven, SPD-10AVP UV-visible dual wavelength detector, and SCL-10AVP system controller; data were acquired and processed using CLASS-VP software version 5.2 (all from Shimadzu, Kyoto, Japan). The separations were carried out on a Zorbax® C18 column (250 mm × 4.6 mm, particle size 5 μ) from Agilent Technologies (DE, USA). Infrared spectra were obtained on an Impact 410 IR Spectrometer (Nicolet, MD, USA). NMR spectra were recorded on an Avance DPX 300 Spectrometer (Bruker, Fallanded, Switzerland). Mass of the synthesized product was checked on a QP 5000 GC-MS system (Shimadzu, Kyoto, Japan). Water purification unit from Elga Ltd. (Bucks, England) was used to prepare HPLC grade water. A rotary evaporator (R-114, Buchi Labortechnik, Flawil, Switzerland) was employed for removal of solvents during synthesis of the products. The stability chamber used (KBF 720, WTB Binder, Tuttlingen, Germany) was able to control the temperature and humidity within ±1 °C and ±3% RH, respectively. Samples were weighed on a precision analytical balance (AG 135, Mettler Toledo, Greifensee, Switzerland). The melting points were recorded on an automatic apparatus (FP62, Mettler Toledo).

2.3. Synthesis of picolinamide

Picolinic acid (500 mg) was treated with thionyl chloride (2.5 ml) by heating under reflux for 3 h until the complete conversion to acid-chloride [15]. The mixture was cooled, diluted with methylene chloride (10 ml), and treated with ammonia (15 ml) under stirring for 1.5 h at room temperature. The mixture was diluted with methylene chloride (50 ml), washed with brine (2 ml × 10 ml) and dried (Na_2SO_4). The crude product was purified by column chromatography using methylene chloride:methanol (95:5, v/v) as eluent to yield piconilamide (401 mg, 80%). The melting point of the prepared compound was compared with the literature value. The structure was further confirmed with the help of IR, NMR and mass spectroscopy.

2.4. Preparation of free base of ethambutol

Ethambutol hydrochloride (2 g) was added to 5N aqueous NaOH (20 ml). The mixture was stirred for 10–15 min and extracted with methylene chloride (25 ml). The organic extract was dried (Na_2SO_4) and evaporated to dryness to afford quantitative amount of ethambutol as a free base. The crude product was recrystallised and characterized by determination of melting point, IR, NMR and mass spectroscopy.

2.5. Reaction of organic bases with combination of rifampicin and isoniazid

The catalysis of interaction between rifampicin and isoniazid by pyrazinamide and ethambutol hydrochloride and the mechanisms involved were explored through investigation of the behaviour of reaction between the former two drugs in the presence of organic bases and amides, related in structure to pyrazinamide and ethambutol hydrochloride. Fig. 1 provides structures of pyrazinamide, ethambutol hydrochloride and various compounds used in the study. For the reaction with

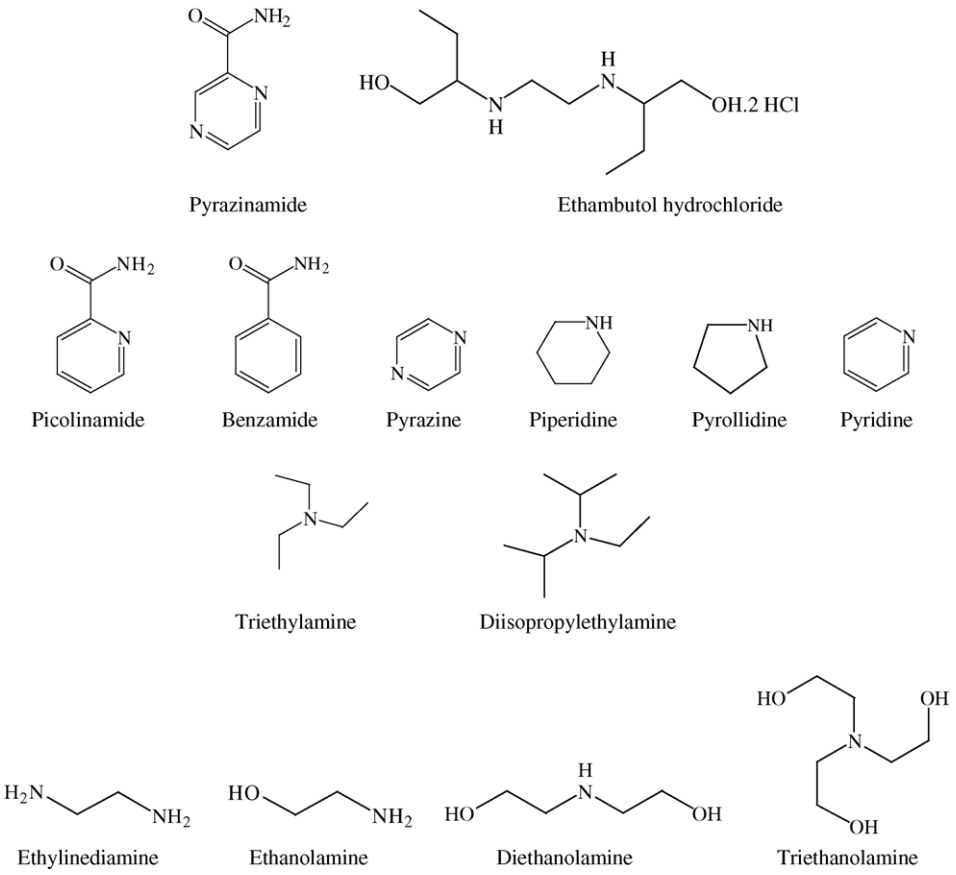


Fig. 1. Structures of pyrazinamide, ethambutol hydrochloride and various compounds used in the study.

organic bases, one equivalent each of rifampicin and isoniazid along with one equivalent of base were taken together. In case of benzamide, picolinamide and the free base of ethambutol, the amount of rifampicin and isoniazid were the same as to the doses contained in anti-TB FDC formulations prescribed by WHO [16]. While the amounts of benzamide and picolinamide were equivalent to pyrazinamide in FDCs, the free base of ethambutol was equivalent to ethambutol hydrochloride. Table 1 gives the quantities of various drugs, amides and bases employed for the reactions. The solid drugs and amides were directly weighed into 15 ml glass vials. In case of liquid bases, the drugs were first weighed and transferred to the

Table 1
Quantities of drugs and bases used in various combinations

Combination	Quantities in mixtures					
	Rifampicin (mg)	Isoniazid (mg)	Pyrazinamide (mg)	Ethambutol (mg)	Amide (mg)	Base (μl)
Rifampicin + isoniazid	150	75.0				
Rifampicin + isoniazid + pyrazinamide	150	75.0	400			
Rifampicin + isoniazid + ethambutol hydrochloride	150	75.0		275		
Rifampicin + isoniazid + pyrazine	100	16.7				9.80
Rifampicin + isoniazid + piperidine	100	16.7				10.3
Rifampicin + isoniazid + pyrrolidine	100	16.7				8.60
Rifampicin + isoniazid + pyridine	100	16.7				9.60
Rifampicin + isoniazid + triethylamine	100	16.7				8.70
Rifampicin + isoniazid + diisopropylethylamine	100	16.7				15.7
Rifampicin + isoniazid + benzamide	150	75.0			393	
Rifampicin + isoniazid + picolinamide	150	75.0			396	
Rifampicin + isoniazid + ethylinediamine	100	16.7				8.20
Rifampicin + isoniazid + ethanolamine	100	16.7				7.40
Rifampicin + isoniazid + diethanolamine	100	16.7				12.8
Rifampicin + isoniazid + triethanolamine	100	16.7				18.0
Rifampicin + isoniazid + free base of ethambutol	150	75.0		275		

vials and the base was dropped subsequently with the help of an auto pipette (Eppendorf AG, Hamburg, Germany). The open vials were charged to a stability chamber set at 40 °C and 75% RH for a total period of 15 d. All the studies were done in triplicate.

2.6. Analysis of samples

For analysis, the total contents of a vial were transferred to a 100 ml volumetric flask and 10 ml of methanol was added. The volume was made (q.s. to 100 ml) with a solution of water and methanol (1:1, v/v). Aliquot of the solution was filtered through a 0.2 μm nylon membrane and 5 μl was injected into the HPLC system. A previously reported validated RP-HPLC method [17] was used for the determination of rifampicin, isoniazid and pyrazinamide in the presence of degradation products.

3. Results

3.1. Characterization of picolinamide

Melting point: 110.5 °C (literature value: 110 °C) [18]; IR (KBr) cm^{-1}: 3437, 3276, 1682, 1588, 1391, 557, 506; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.58 (d, J = 4.6 Hz, 1H), 8.22–8.20 (d, J = 4.6 Hz, 1H), 7.89–7.83 (m, 1H), 7.48–7.43 (m, 1 H), 5.89 (bs, 2H); ^{13}C NMR (300 MHz) δ (ppm): 166.98, 149.53, 148.27, 137.26, 126.42, 122.40; EIMS m/z 122 (M^+).

3.2. Characterization of free base of ethambutol

Melting point: 88.6 °C (literature value: 87.5–88.8 °C) [19]; IR (KBr) cm^{-1}: 3271, 3153, 2959, 2873, 1460, 1353, 1144, 1092, 986, 882; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 3.61 (dd, J = 3.5 Hz, 0.9 Hz, 2H), 3.44 (dd, J = 7.2 Hz, 0.8 Hz, 3H), 2.84–2.72 (m, 3H), 2.68–2.64 (m, 2H), 2.52 (m, 2H), 1.53–1.33 (m, 4H), 0.91 (t, J = 7.4 Hz, 6H); ^{13}C NMR (300 MHz) δ (ppm): 63.16, 60.49, 46.63, 24.10, 10.42; EIMS m/z 205 (M^+).

3.3. Data for degradation of rifampicin and isoniazid and extent of the formation of degradation products in the presence of co-drugs and organic bases

Table 2 gives the data for the loss of rifampicin and isoniazid on exposure of various mixtures to accelerated stability test conditions for 15 d. It also lists the percent area values of major degradation products formed. The corresponding HPLC chromatograms for a few selected cases are shown in Fig. 2.

4. Discussion

4.1. Degradation behaviour of rifampicin and isoniazid in the mixtures containing co-drugs

The comparison of the data in Table 2 for the binary mixture of rifampicin and isoniazid against the ternary mixtures containing pyrazinamide or ethambutol hydrochloride indicates a higher extent of decomposition in the presence of the latter two drugs. This confirms that pyrazinamide and ethambutol hydrochloride catalyze the reaction between rifampicin and isoniazid, which was indicated in our previous study [11].

It is further shown that ethambutol hydrochloride influenced the reaction between rifampicin and isoniazid to a greater extent than pyrazinamide. This can be attributed to hygroscopicity and acidic nature of ethambutol hydrochlo-

Table 2
Percent degradation of rifampicin and isoniazid and area percent of major degradation products in various reaction mixtures

Combination	Degradation (%)		Peak area of major degradation products (%)			
	R	H	HYD	25-DAR	R-NO	RQ
Rifampicin + isoniazid	1.3	1.0	1.0	–	–	–
Rifampicin + isoniazid + pyrazinamide	10.4	5.2	9.6	–	–	–
Rifampicin + isoniazid + ethambutol hydrochloride	52.0	15.6	35.1	–	–	–
Rifampicin + isoniazid + pyrazine	47.5	21.7	4.2	3.2	1.2	–
Rifampicin + isoniazid + piperidine	16.4	10.6	5.1	0.8	0.2	–
Rifampicin + isoniazid + pyrrolidine	20.7	12.2	7.0	2.7	1.3	0.4
Rifampicin + isoniazid + pyridine	16.7	10.9	5.4	2.2	0.8	–
Rifampicin + isoniazid + triethylamine[a]	22.4	15.4	10.6	3.9	2.3	0.8
Rifampicin + isoniazid + diisopropyl ethylamine	81.3	69.6	70.2	0.9	–	–
Rifampicin + isoniazid + benzamide	3.8	3.2	2.9	–	–	–
Rifampicin + isoniazid + picolinamide	85.4	76.1	71.3	–	–	–
Rifampicin + isoniazid + ethylinediamine[a]	24.2	14.3	13.1	4.7	4.1	–
Rifampicin + isoniazid + ethanolamine	19.5	16.2	8.5	3.8	1.5	–
Rifampicin + isoniazid + diethanolamine	42.6	25.7	15.6	5.1	1.8	–
Rifampicin + isoniazid + triethanolamine	60.5	46.8	32.5	3.9	0.9	–
Rifampicin + isoniazid + free base of ethambutol[a]	71.3	63.1	20.2	10.2	2.8	3.9

Key: HYD, isonicotinyl hydrazone; 25-DAR, 25-desacetyl rifampicin; R-NO, rifampicin-N-oxide; RQ, rifampicin quinone.
[a] Unidentified products were formed in addition to HYD, 25-DAR, R-NO and RQ.

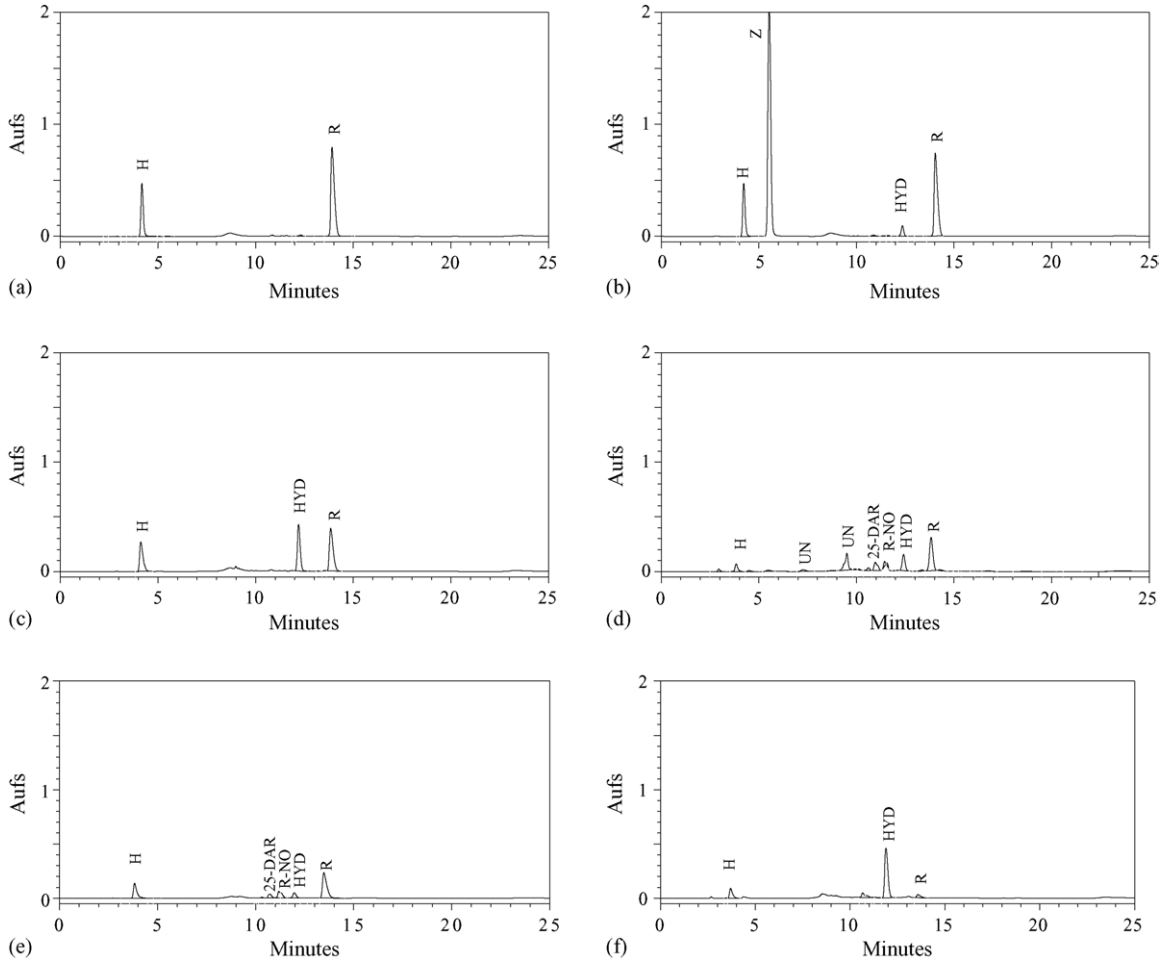


Fig. 2. Chromatograms showing formation of different degradation products after storage of mixture of R + H (a), R + H + Z (b), R + H + E (c), R + H + free base of E (d), R + H + diethanolamine (e) and R + H + diisopropylethylamine (f) after storage at 40 °C and 75% relative humidity for 15 d. Key: R, rifampicin; H, isoniazid; Z, pyrazinamide; E, ethambutol hydrochloride; HYD, isonicotinyl hydrazone; 25-DAR, 25-desacetyl rifampicin; R-NO, rifampicin-N-oxide; UN, unknown product.

ride [10,11]. The solid mixtures containing ethambutol hydrochloride turned into liquid after some time, thus allowing better interaction between the components. On the other hand, the mixtures containing pyrazinamide remained solid throughout. However, the data in Table 2 reveal that there was no difference in the nature of the product formed. With both the co-drugs, HYD was the major degradation product.

4.2. Degradation behaviour of rifampicin and isoniazid in the presence of organic bases and amides

The data in Table 2 depict that degradation of rifampicin and isoniazid occurred with all investigated organic bases and amides. More important is that HYD was formed as the main product in all the cases, along with other known degradation products of rifampicin.

In the presence of nucleophilic bases related in structure to pyrazinamide, e.g., pyridine, pyrrolidine, piperdine and pyrazine, there was higher decomposition of rifampicin (16.7–47.5%) and isoniazid (10.9–21.7%) than the combination containing rifampicin, isoniazid and pyrazinamide. Triethylamine also showed decomposition in the same ranges. The reactions yielded HYD along with decomposition products of rifampicin, viz., 25-DAR, rifampicin quinone and rifampicin-N-oxide [20]. In comparison, a large extent of loss of rifampicin (>80%) and isoniazid (∼70%) occurred in case of diisopropylethylamine, the least nucleophilic but strongest base among those tested. In this case, the reaction yielded HYD almost quantitatively.

With benzamide and picolinamide, the two amides more closely related in structure to pyrazinamide, the results in Table 2 show that, as observed in the presence of pyrazinamide, the loss of rifampicin was accompanied by the formation of HYD alone and no other products. The degradation of rifampicin with benzamide was expectedly less (3.8%) than that with pyrazinamide (10.4%), but was unexpectedly far higher (85.4%) in the presence of picolinamide. With picolinamide also, the loss of rifampicin should have been in the same order as that of pyrazinamide, due to

the closest structural similarity. The higher drug loss is best explained through the observation that mixtures containing picolinamide became liquid with time, allowing greater interaction between rifampicin and isoniazid. This was perhaps due to hygroscopic nature of picolinamide. There was no such physical change in the presence of benzamide.

In case of ethambutol related amines, viz., ethylinediamine, ethanolamine, diethanolamine and triethanolamine, the degradation of rifampicin and isoniazid was in the order of 20–60% and 14–47%, respectively, which was similar to the extents shown by nucleophilic bases related to pyrazinamide. In the case of these amines also, the reaction yielded other degradation products along with HYD.

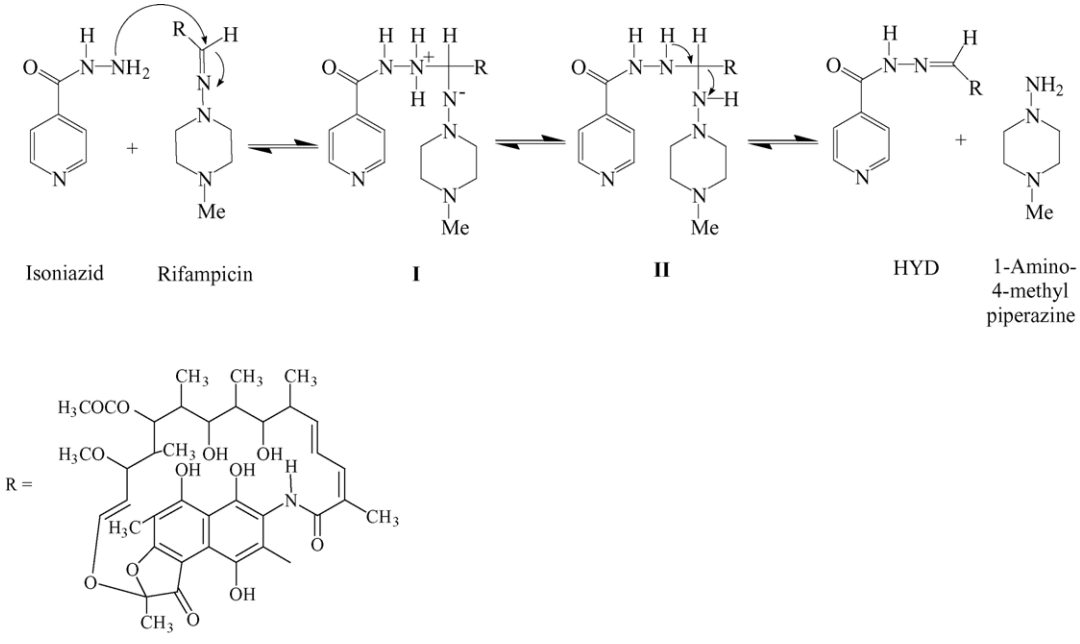
Extensive degradation of rifampicin and isoniazid to HYD and other products occurred even with free base of ethambutol, but the behaviour here was much different from the hydrochloride salt. The salt resulted in a loss of 52% rifampicin and 15.6% of isoniazid, whereas the loss of the two drugs was 71.3% and 63.1%, respectively, in the presence of free base. In the presence of ethambutol hydrochloride, HYD was the major product along with minor quantities of 3-formylrifamycin, whereas the free base yielded much lesser quantity of HYD despite higher loss of the drugs and there was no evidence of formation of 3-formylrifamycin.

4.3. Postulation of the mechanism of reaction between rifampicin and isoniazid in the formulation environment

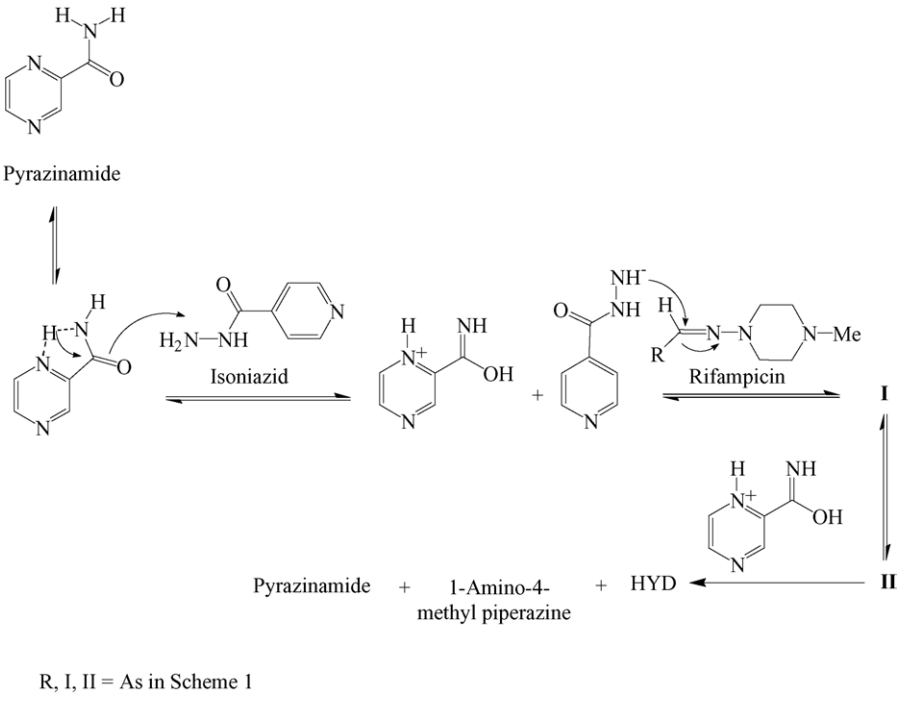
It is indicated in literature that even under non-acidic formulation conditions, possibility exists of direct reaction between rifampicin and isoniazid yielding HYD as the product [21]. This direct interaction can be best explained through a transhydrazone formation, *via* nucleophilic attack on the imine group of rifampicin by the amino group of isoniazid, following a tetrahedral mechanism (Scheme 1). Due to the inherent reversibility of such reactions [22], the overall decomposition leading to HYD should depend upon: (i) the relative nucleophilic property of the amino group of isoniazid and that of 1-amino-4-methyl piperazine and (ii) the relative leaving group ability of the terminal amide anion of isoniazid and the corresponding anion of 1-amino-4-methyl piperazine. The presence of the nitrogen lone pair adjacent to the amino group of 1-amino-4-methyl piperazine makes it α-nucleophile [23], with a stronger nucleophilic property as compared to that of isoniazid. On the other hand, the carbonyl group in isoniazid resonatically withdraws the lone pair of electron from the nitrogen atom adjacent to the amino group and makes the corresponding amide anion a better leaving group, compared to the amide anion of 1-amino-4-methyl piperazine. Therefore, the overall equilibrium should not favor decomposition (transhydrazone formation) under non-acidic condition. The lack of appreciable decomposition of rifampicin and isoniazid in the solid state provides rationale to this, as only 1–1.3% decomposition of both the drugs was observed in their binary mixture (Table 2).

4.4. Explanation to the catalysis by pyrazinamide

In the light of the above, it was felt that the decomposition of rifampicin and isoniazid in a fixed-dose combination of rifampicin, isoniazid and pyrazinamide was not the result of a mere direct transhydrazone formation involving rifampicin and isoniazid, rather pyrazinamide played a crucial role in catalyzing the decomposition. Accordingly, it is proposed that the increased rate of decomposition in the presence of pyrazinamide is due to base catalyzed transhydrazone for-



Scheme 1. Postulated mechanism of formation of HYD on direct interaction between rifampicin and isoniazid.



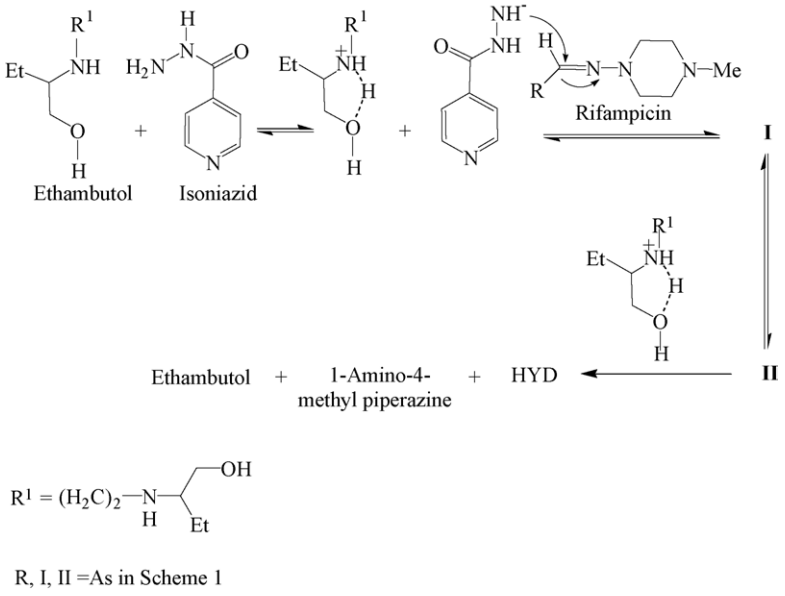
Scheme 2. Mechanistic explanation to the catalytic effect of pyrazinamide on the direct interaction between rifampicin and isoniazid.

mation (Scheme 2), in which pyrazinamide acts as a proton scavenger via a five membered ring transition state involving intra-molecular hydrogen bond formation between one of the hydrogens of $CONH_2$ and the adjacent ring nitrogen.

The same is proved by catalysis of the decomposition, leading mostly to the formation of HYD, albeit to varied extents, in the presence of nucleophilic bases (Table 2). The higher loss of rifampicin and isoniazid in mixtures containing pyridine, pyrazine, pyrollidine and piperidine, compared to that of pyrazinamide, confirms that the transhydrazone formation catalyzed by pyrazinamide is not mediated by the basic property of the N-4. The same is even supported by maximum decomposition with a clean formation of HYD in the presence of diisopropylethylamine, which had the strongest basic property amongst the investigated bases. No side product formation took place in this case, as diisopropylethylamine is a poor nucleophile due to its bulkier nature. The occurrence of intra-molecular proton transfer, through the five-membered ring transition state of pyrazinamide, is further demonstrated by the absence of appreciable acceleration of decomposition in the presence of benzamide. However, no support is provided here from the study with picolinamide, because incidentally it showed higher rate of decomposition than even pyrazinamide, due to its hygroscopic nature and liquefaction of the mixture.

4.5. Mechanism for the catalysis by ethambutol

Scheme 3 gives the plausible mechanism for the catalysis of the interaction between rifampicin and isoniazid in the presence of ethambutol, either as a free base or hydrochloride salt. The reaction in this case is postulated to follow the similar path, as described in Scheme 2 for pyrazinamide, because 1,2-amino alcohol or ethylenediamine moiety in ethambutol can also serve as a proton scavenger. Rather one ethambutol molecule can abstract proton simultaneously from two isoniazid molecules and this perhaps also contributes to the enhanced catalytic effect in comparison to pyrazinamide. The catalytic role of ethambutol is well supported by the data in Table 2 for ethylenediamine, ethanolamine, diethanolamine and triethanolamine, where also sufficient decomposition of the drugs took place, forming HYD, in particular. The formation of other products, such as 25-DAR and rifampicin-N-oxide in addition to HYD in case of ethylenediamine, ethanolamine, and diethanolamine can be attributed to the nucleophilic property of these bases. The data for triethanolamine shows that the reaction between rifampicin and isoniazid in its presence resulted in a relatively cleaner formation of HYD, which may be due to the fact that triethanolamine is a sterically hindered base, and no reaction is possible through nucleophilic attack on rifampicin other than the formation of HYD. The prevalence of base catalyzed route of decomposition is supported by specific degradation study of rifampicin and isoniazid in the presence of free base of ethambutol. The comparison of data for the ethambutol free base with the mixture containing hydrochloride salt (Table 2) shows that in the presence of the former, higher decomposition of rifampicin and isoniazid occurred, while the formation of HYD was lesser, because lot other products were generated simultaneously in the alkaline environment (Fig. 2d).



Scheme 3. Mechanistic explanation to the catalytic effect of ethambutol on the direct interaction between rifampicin and isoniazid.

5. Conclusions

The study of interaction of rifampicin with isoniazid in the presence of various organic bases and amides shows that the reaction leading to HYD even occurs under non-acidic (rather alkaline) environment created by the investigated compounds. It thus lends support to the formation of HYD on storage of solid anti-TB FDC formulations under accelerated stability test conditions. The fact that some bases were able to convert rifampicin and isoniazid quantitatively to HYD, confirms the possibility of catalysis of the reaction by pyrazinamide and ethambutol hydrochloride. It is postulated that pyrazinamide and ethambutol hydrochloride exhibit catalytic role through involvement of intra-molecular proton transfer during reaction between rifampicin with isoniazid, which is conceived to occur through a base-catalyzed transhydrazone formation process entailing a tetrahedral mechanism.

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