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LC and LC-MS study of stress decomposition behaviour of isoniazid and establishment of validated stability-indicating assay method

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Abstract

Isoniazid was subjected to different ICH prescribed stress conditions of thermal stress, hydrolysis, oxidation and photolysis. The drug was stable to dry heat (50 and 60 °C). It showed extensive decomposition under hydrolytic conditions, while it was only moderately sensitive to oxidation stress. The solid drug turned intense yellow on exposure to light under accelerated conditions of temperature (40 °C) and humidity (75% RH). In total, three major degradation products were detected by LC. For establishment of stability-indicating assay, the reaction solutions in which different degradation products were formed were mixed, and the separation was optimized by varying the LC conditions. An acceptable separation was achieved using a C-18 column and a mobile phase comprising of water:acetonitrile (96:4, v/v), with flow rate and detection wavelength being 0.5 ml min^{-1} and 254 nm, respectively. The degradation products appeared at relative retention times (RR_T) of 0.71, 1.34 and 4.22. The validation studies established a linear response of the drug at concentrations between 50 and 1000 μg ml^{-1}. The mean values (±R.S.D.) of slope, intercept and correlation coefficient were 35,199 (±0.88), 114,310 (±4.70) and 0.9998 (±0.01), respectively. The mean R.S.D. values for intra- and inter-day precision were 0.24 and 0.90, respectively. The recovery of the drug ranged between 99.42 and 100.58%, when it was spiked to a mixture of solutions in which sufficient degradation was observed. The specificity was established through peak purity testing using a photodiode array detector. The method worked well on application to marketed formulation of isoniazid, and a fixed-dose combination containing isoniazid and ethambutol HCl. It was even extendable to LC-MS studies, which were carried out to identify the three degradation products. The *m/z* values of the peaks at RR_T 0.71 and RR_T 1.34 matched with isonicotinic acid and isonicotinamide, respectively. The product appearing at RR_T 4.22 was isolated using preparative LC-MS, and turned out to be a yellow compound that was identified as isonicotinic acid *N′*-(pyridyl-4-carbonyl)-hydrazide based on mass, FTIR and $^1H/^{13}C$ NMR spectral data. The same was indicated to be responsible for discolouration of isoniazid bulk drug substance and formulations, which is a familiar problem. The mechanism of formation of the said compound is outlined.

Keywords: Isoniazid; Stress testing; Stability-indicating assay; Validation; LC; LC-MS; Degradation products

1. Introduction

In our laboratory, we are carrying out extensive investigations on the stability behaviour of first-line anti-tuberculosis (TB) agents (*viz.*, rifampicin, isoniazid, pyrazinamide and ethambutol HCl), both when they are present alone or in fixed-dose combinations (FDCs) [1–3]. As a part of the same, we focused recently on stress studies and establishment of stability-indicating assay methods (SIAM) [4,5], following the principles enshrined in International Conference on Harmonization (ICH) drug stability test guideline Q1A(R2) [6]. Though ICH guidelines apply on new drugs, it was decided to extend them to old first-line anti-TB drugs, because of the increased insistence of International regulatory agencies these days on generation of data according to ICH requirements, even for generic drugs.

This paper presents the results of investigations on isoniazid, the oldest among the four first-line anti-TB drugs. Isoniazid

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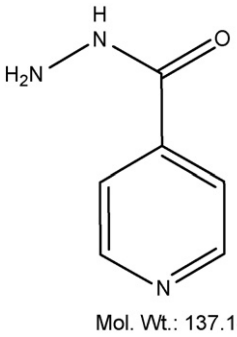


Fig. 1. Structure of isoniazid.

is chemically pyridine-4-carbohydrazide (Fig. 1). It is a white, odourless, crystalline powder that is freely soluble in water and melts between 170 and 174 °C [7,8]. As it was originated in 1950s, already there are lots of reports on stability behaviour of isoniazid in the literature. The stability of the drug has been investigated in various media, *viz*., aqueous solutions and plasma [9], frozen serum and plasma [10,11], solutions containing alcohol [12], sucrose and sorbitol [13]. There even exist a series of publications on the stability of isoniazid and its sodium methanesulfonate derivative [14–19]. In these, acid-catalyzed, base-catalyzed and oxidative degradation of isoniazid were investigated, and the evaluation of the degraded samples was done by initially passing them through ion exchange columns and subsequently analysis by UV. A report was later published on effect of chelating agents on the stability of injectable solutions of isoniazid [20]. Despite the above studies, a gap exists in the literature regarding systematic LC and LC-MS studies on the drug to establish a validated stability-indicating assay method through stress testing route, according to the requirements laid down by ICH.

2. Experimental

2.1. Materials

Isoniazid was supplied by M/S Panacea Biotec Limited, Lalru, Punjab, India, and used without further purification. Isonicotinic acid was procured from Acros Organics, NJ, USA. Sodium hydroxide and hydrochloric acid (both AR grade) were purchased from Ranbaxy Laboratories (SAS Nagar, Punjab, India) and LOBA Chemie Pvt. Ltd. (Mumbai, Maharashtra, India), respectively. Hydrogen peroxide was procured from S.D. Fine-Chem Ltd. (Boisar, Maharashtra, India). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Mexico City, Mexico). Ultra-pure water was obtained from a water purification unit (Elga Ltd., Wycombe, Bucks, UK).

2.2. Instrumentation

Precision water baths equipped with MV controller (Julabo, Seelbach, Germany) were used for degradation studies under acidic, alkaline and neutral conditions. Dri-Bath (Thermolyne, Iowa, USA) was used for thermal stress studies. Photodegradation was carried out in a photostability chamber (KBF 240, WTB Binder, Tuttlingen, Germany) equipped with a light bank consisting of two UV (OSRAM L73) and four fluorescent (OSRAM L20) lamps and capable of controlling temperature and humidity in the range of ±2 °C and ±5% RH, respectively. The light system complied with option 2 prescribed in the ICH guideline Q1B [21]. At any given time, UV energy at the point of placement of samples was ∼0.6 W/m^2 (tested with a calibrated radiometer, model 206, PRC Krochmann GmbH, Berlin, Germany) and visible illumination was ∼4500 lx (tested using a calibrated lux meter, model ELM 201, Escorp, New Delhi, India). The chamber was set at accelerated condition of 40 °C/75% RH during the studies. Other equipments used were an ultrasonic bath (3210, Branson Ultrasonic Corporation, Danbury, CT, USA), precision analytical balance (AG 135, Mettler Toledo, Greifensee, Switzerland), rotary evaporator (R-114, Buchi Labortechnik, Flawil, Switzerland), aspirator (Eyela A-3S, Tokyo Rikakai Co., Tokyo, Japan) and a freeze dryer (DW8-85, Heto Holten, AllerØd, Denmark).

The LC system consisted of an on-line degasser (DGU-14A), low-pressure gradient flow control valve (FCV-10AL$_{VP}$), solvent delivery module (LC-10AT$_{VP}$), auto injector (SIL-10AD$_{VP}$), column oven (CTO-10AS$_{VP}$), photodiode array (PDA) detector (SPD-M10A$_{VP}$), system controller (SCL-10A$_{VP}$) and CLASS-VP software, ver. 6.13 (all from Shimadzu, Kyoto, Japan). The chromatographic separations were carried out on a Zorbax® XDB eclipse C-18 (250 mm × 4.6 mm i.d., particle size 5 μm) column (Agilent Technologies, Wilmington, DE, USA).

LC-MS studies were carried out in negative electro spray ionization (ESI) mode on Bruker Daltonics micro TOF instrument (Bruker Daltonik GmbH, Bremen, Germany), which was controlled by software micrOTOF control ver. 2.0. LC part of the LC-MS comprised of Agilent 1100 series LC system (Agilent Technologies Inc., CA, USA), controlled by Hystar (ver. 3.1) software. Column used for LC-MS studies was same as that for LC. Preparative LC-MS system, used for isolation of one of the degradation products, included a 2767 sample manager, 2525 binary gradient module, control fluidic organizer, micromass ZQ quadrapole detector and data management software MassLynx ver. 4.0 (all from Waters Corporation, Milford, MA, USA). Semi-preparative column used for the preparative separations was XTerra® C-18 from Waters.

IR spectra were obtained on Impact 410 (Nicolet, MD, USA) FTIR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Avance DPX 300 spectrometer (Bruker, Fallanden, Switzerland).

2.3. Conduct of stress studies

The stress studies were carried out under the conditions of dry heat, hydrolysis, oxidation, and photolysis, as defined by ICH [6]. For thermal stress testing, the drug powder was sealed in glass ampoules and heated in dri-bath at 50 °C for 60 days and also at 60 °C for 15 days. Acid decomposition was carried out in

0.1N HCl at drug strength of 1 mg ml^{-1}. The studies were also performed in 1N acid, where the drug strength was increased to 10 mg ml^{-1} to compensate for dilution. These solutions were heated at 80 °C for 8 days. The studies in alkaline conditions were conducted similarly at a drug concentration of 1 mg ml^{-1} in 0.1N NaOH and 10 mg ml^{-1} in 1N NaOH. These solutions were also exposed to 80 °C for 8 days. Additionally, the drug solution in water at a drug strength of 1 mg ml^{-1} was heated at 80 °C for different time periods. The oxidative stress studies were conducted at drug strength of 1 mg ml^{-1} in 3% H_2O_2. The solution was stored at room temperature for 24 h. As sufficient decomposition was not observed, the drug was additionally exposed at a concentration of 10 mg ml^{-1} in 30% H_2O_2 at room temperature for upto 48 h. The photolytic studies were carried out in solid state by spreading a thin layer of drug in a petri-dish and exposing it directly to the combination of UV and florescent light in a photostability chamber set at accelerated conditions of temperature and humidity. A parallel set was kept in dark under similar conditions. Samples were withdrawn at different time periods up to 60 days.

2.4. Separation studies

First, the reaction solutions were individually subjected to LC studies, followed by a mixture of those solutions in which reasonable decomposition was observed. The studies were conducted using a mobile phase composed of water:acetonitrile. The separation was achieved by changing the mobile phase composition as well as the flow rate. The overall objective here was to develop a selective SIAM [4], using a buffer-free mobile phase in an isocratic mode, so that it was possible to transfer the method to LC-MS for characterization of the degradation products.

2.5. Validation of the method

Validation of the optimised LC method was done with respect to various parameters, as required under ICH guideline Q2(R1) [22]. To establish linearity and range, a stock solution of the drug was prepared at strength of 1 mg ml^{-1}, which was further diluted to prepare solutions in the drug concentration range of 50–1000 μg ml^{-1}. The solutions were injected in triplicate into the LC column, keeping the injection volume constant (10 μl). Precision of the method was studied by making six injections of three different concentrations, *viz.*, 100, 400, and 1000 μg ml^{-1} on the same day and the values of relative standard deviation (R.S.D.) were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision. Accuracy was evaluated by fortifying a mixture of degraded solutions with three known concentrations, *viz.*, 50, 200, and 500 μg ml^{-1} of the drug. The recovery of the added drug was determined. The specificity of the method was established through study of resolution factors of the drug peak from the nearest resolving peak, and also among all other peaks. Selectivity was confirmed through peak purity studies using a PDA detector.

2.6. Application of the developed method to stability samples

The developed method was used to analyze stability samples of a formulation containing isoniazid, and a FDC containing isoniazid and ethambutol HCl. The products were stored under accelerated conditions of temperature and humidity for 3 months before analysis.

2.7. Characterization of degradation product(s)

LC-MS studies were carried out to determine *m/z* values of the major degradation products formed under various stress test conditions. The obtained values were compared with the molecular weights of known degradation products of isoniazid. There was doubt on the identity of one of the products eluting late on LC, hence it was isolated using preparative LC-MS. The structure was confirmed by characterization through FTIR, and $^{1}H/^{13}C$ NMR spectral analyses.

3. Results and discussion

3.1. Degradation behaviour

In total, three degradation products were detected by LC on decomposition of the drug under various stress conditions. The retention times (R_T) and relative retention times (RR_T) of the drug and the products are listed in Table 1.

The degradation behaviour of the drug in individual stress conditions is outlined below:

3.1.1. Thermal stress

The exposure of the solid drug to 50 °C for 60 days and 60 °C for 15 days did not result in significant decomposition. It indicated that isoniazid was stable to dry heat.

3.1.2. Hydrolysis

The drug degraded gradually with time on heating at 80 °C in 0.1N HCl, forming a single major peak at RR_T 0.71. Similar behaviour was observed in 1N HCl, but the rate of decomposition was faster and more than 50% drug was degraded within 4 days. Overall, the rate of hydrolysis in acid was higher as compared to that in other hydrolytic conditions (water or alkali).

Upon heating the drug in water at 80 °C for 2 days, ~16% fall in the drug peak area was observed. After 6 days, >40% drug was degraded. The major peak in this case too appeared at RR_T 0.71. Another small peak appeared at RR_T 1.34.

Table 1
Retention time and relative retention times of various peaks

Peak	Retention time (R_T)	Relative retention time (RR_T)
I	4.89	0.71
Isoniazid	6.58	1.00
II	8.91	1.34
III	28.82	4.22

The reaction in 0.1 M NaOH at 80 °C was mild and only ~9% drug was degraded in 4 days. Similar studies in 1N NaOH resulted in ~22% loss of the drug in 4 days at 80 °C. The degradation of drug was associated with appearance of the peaks at RR_T 0.71 (major) and 1.34 (minor).

3.1.3. Oxidation

The drug was stable to 3% hydrogen peroxide at room temperature and no significant degradation was observed. However, 19% decomposition occurred in 30% hydrogen peroxide, resulting in products resolving again at RR_T 0.71 and 1.34.

3.1.4. Photolysis

The LC profiles of light exposed drug samples in acid, water and alkali were similar to those in the dark, indicating that light had no particular influence on the drug in solution. On the other hand, the solid drug turned yellow on exposure to ICH dose of light, and became brown on prolonged exposure. The chromatogram showed a small peak at RR_T 0.71, and a new peak at RR_T 4.22, which was not observed in any of the previous studies.

3.2. Development and optimization of the stability-indicating method

The resolution of degradation products from the drug was influenced by concentration of acetonitrile in the mobile phase. The peaks merged when acetonitrile concentration was increased, while there was improvement in resolution with the decrease in percentage of the organic modifier. Similarly, there was merging of peaks at a mobile phase flow rate of 1.0 ml min^{-1}, but better separation was achieved on decreasing the same. Finally, acceptable separations with reasonable peak shapes and peak purity were achieved by using water:acetonitrile in the ratio of 96:4% (v/v) and flow rate of 0.5 ml min^{-1}. The injection volume and detection wavelength were 10 μl and 254 nm, respectively. A typical chromatogram is shown in Fig. 2.

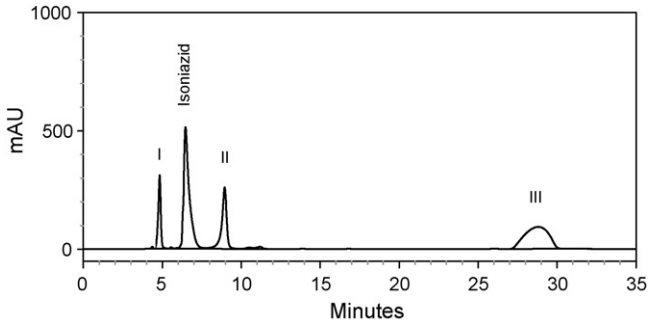


Fig. 2. Chromatograms showing separation of drug and the three degradation products. Key: I (RR_T 0.71): isonicotinic acid, formed in all types of hydrolytic and photolytic conditions; II (RR_T 1.34): isonicotinamide, formed in water under dark and oxidative conditions; III (RR_T 4.22): isonicotinic acid *N′*-(pyridyl-4-carbonyl)-hydrazide, formed on exposure of solid drug under light.

3.3. Validation of the developed stability-indicating method

The data obtained from linearity studies are given in Table 2. The response of the drug was strictly linear in the concentration range between 50 and 1000 μg ml^{-1}. The mean (±%R.S.D.) values of slope, intercept and correlation coefficient were 35,199 (±0.88), 114,310 (±4.70) and 0.9998 (±0.01), respectively.

The %R.S.D. values for intra-day and inter-day precision studies (Table 3) were <0.5% and <1.1%, respectively, confirming that the method was sufficiently precise. Good separation was achieved even when the procedure was repeated by

Table 2
Linearity data obtained on three different days

Regression parameters	Day 1	Day 2	Day 3	Mean ± S.D. (%R.S.D.)
Slope	35,515	34,895	35,187	35,199 ± 310.17 (0.88)
Intercept	120,315	109,940	112,675	114,310 ± 5377.27 (4.70)
R^2	0.9999	0.9997	0.9998	0.9998 ± 0.0001 (0.01)

Table 3
Reproducibility and precision data obtained during intra-day ($n = 6$) and inter-day ($n = 3$) studies

Actual concentration (μg ml^{-1})	Intra-day measured concentration (μg ml^{-1}) ± S.D.; %R.S.D.	Inter-day measured concentration (μg ml^{-1}) ± S.D.; %R.S.D.
100	98.864 ± 0.357; 0.361	98.581 ± 0.684; 0.699
400	403.256 ± 1.151; 0.285	403.917 ± 3.999; 0.990
1000	1001.849 ± 0.731; 0.073	992.644 ± 9.971; 1.004

Table 4
Recovery studies ($n = 3$)

Actual concentration (μg ml^{-1})	Measured concentration (μg ml^{-1}) ± S.D.; %R.S.D.	Recovery (%)
50	49.71 ± 0.274; 0.552	99.42
200	203.00 ± 1.615; 0.795	100.58
500	501.18 ± 1.361; 0.271	100.12

Table 5
Peak purity studies

Peak (RR_T)	Peak purity threshold	Peak purity index
I (0.71)	0.99967	0.99981
Isoniazid (1.00)	0.99986	0.99999
II (1.34)	0.99999	1.00000
III (4.22)	0.98562	0.99124

a different person, thus confirming the reproducibility of the method.

As shown from the data in Table 4, good recoveries were made at the added concentration of 50, 200 and 500 $\mu g\,ml^{-1}$, with the mean recovery being 100.04%.

Fig. 2 shows that the method was selective to the drug as well as the degradation products. The resolution factor for the drug peak was >3 from the nearest resolving peak. Also, the peaks were pure, which was proved from the single point purity threshold and purity index values, given in Table 5.

3.4. Applicability of the developed method to stability samples

The developed method was found to apply even to real stability samples, which was verified through successful analyses of single-drug formulation containing isoniazid, and a FDC containing isoniazid and ethambutol HCl, both of which had been stored at accelerated conditions of temperature (40 °C) and humidity (75% RH) for 3 months. Although minor decomposition was observed, the degradation products were still discernible and well separated. The latter appeared at RR_T 0.71 and RR_T 4.22. In general, degradation was more in the FDC formulation than isoniazid-alone tablets. Indirectly, it was also established that the developed method could even be used for FDC formulations containing isoniazid along with ethambutol HCl, without interference from the latter due to lack of UV absorbance at the wavelength of analysis (254 nm).

3.5. Characterization of degradation products

Mass chromatograms in the negative electron spray ionization (ESI) mode for the drug and degradation products are shown in Fig. 3. Evidently, the *m/z* value of 136 for isoniazid corresponded to its molecular weight of 137, thus validating the output of the mass spectrometer. The *m/z* values obtained for the degradation products resolving at RR_T 0.71, 1.34 and 4.22 in the same run were 122, 121 and 241, respectively. These were compared to the molecular weights of known degradation products of isoniazid (Fig. 4).

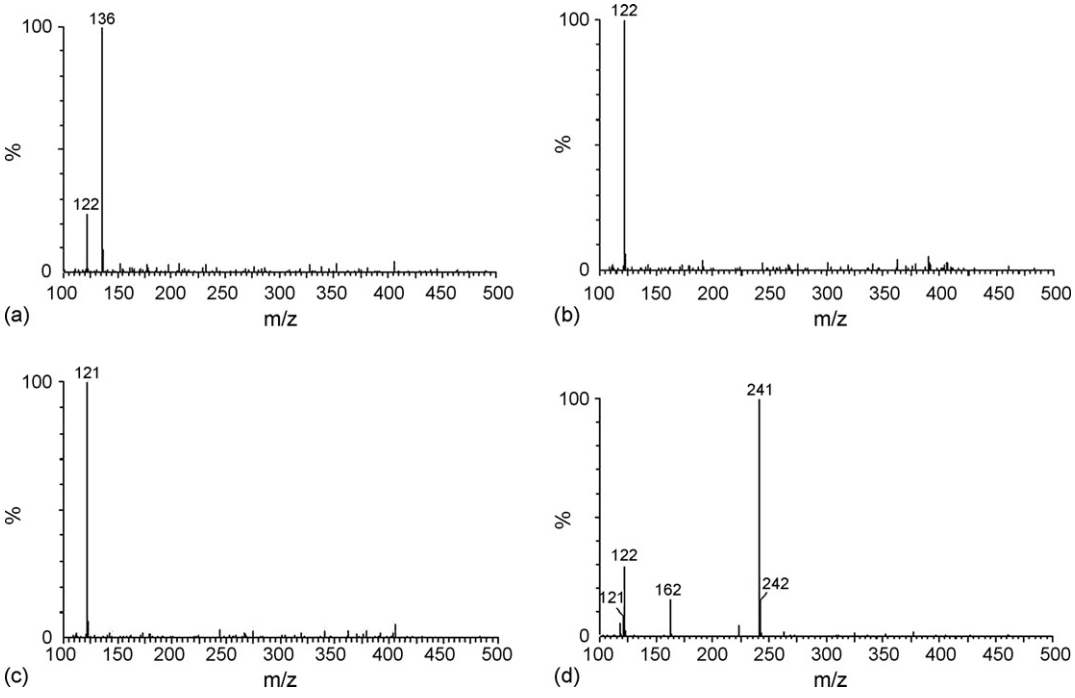


Fig. 3. Mass profiles for isoniazid (a) and the three degradation products appearing at RR_T 0.71 (b), RR_T 1.34 (c) and RR_T 4.22 (d).

Table 6
Spectral data for the isolated compound and isoniazid

Spectral technique	Isolated compound	Isoniazid
m/z (ESI)	241	136
IR (cm^{-1})	3432 (NH); 1682 and 1660 (C=O)	3308 (NH); 3113 (NH_2); 1666 (C=O); 1633 (NH deformation)
^1H NMR (CD_3OD, 300 MHz); δ (ppm)	8.73 (d, $J = 5.2$ Hz); 7.85 (d, $J = 5.2$ Hz)	8.68 (d, $J = 6.1$ Hz); 7.75 (d, $J = 6.1$ Hz)
^{13}C NMR (CD_3OD, 60 MHz); δ (ppm)	120.99; 139.77; 149.04; 164.76	122.19; 142.00; 150.37; 166.36

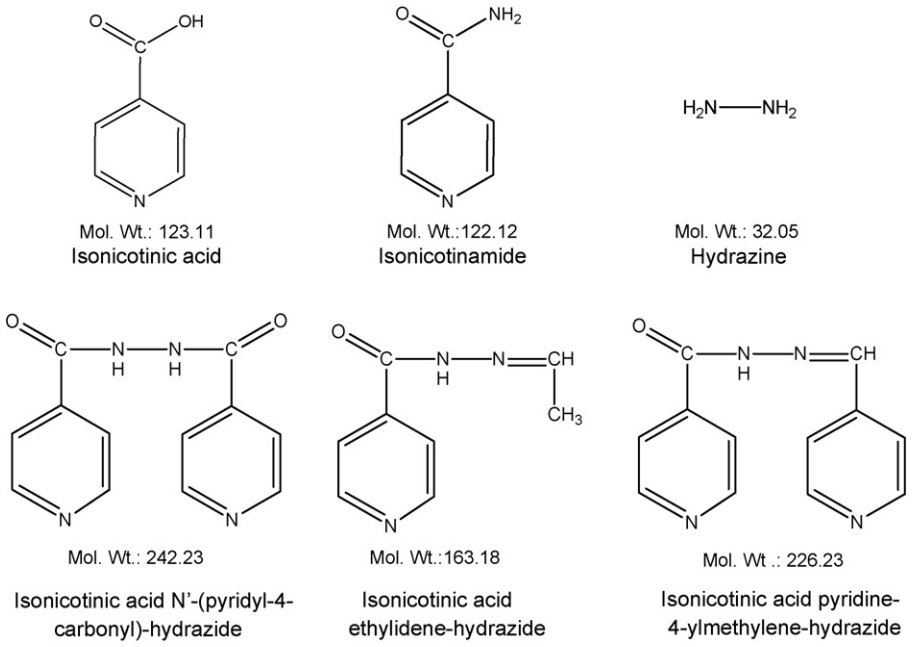
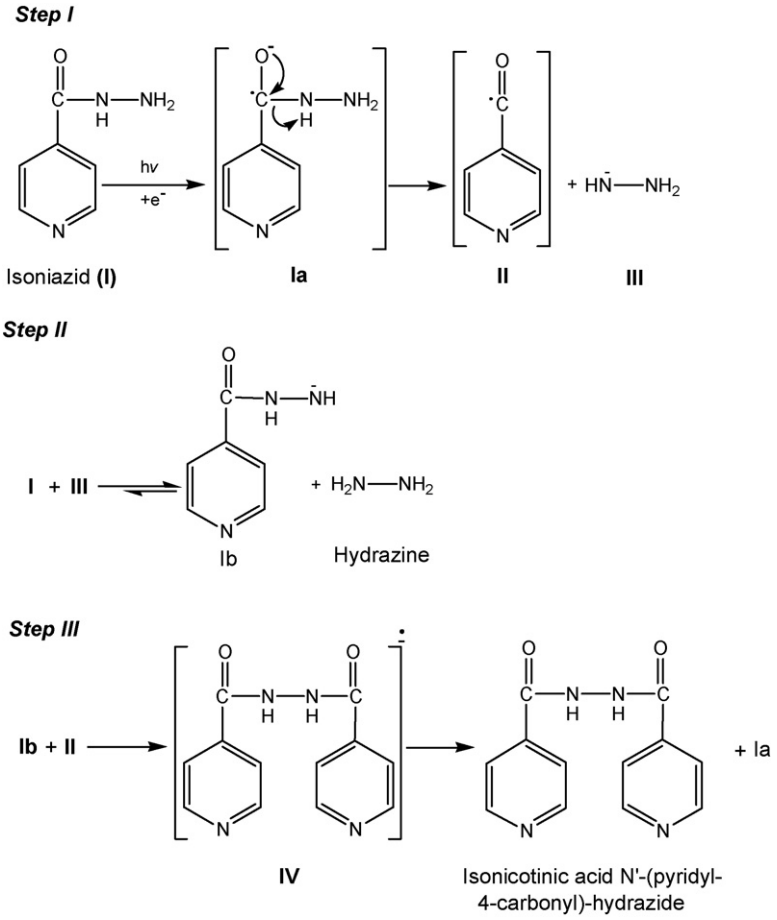


Fig. 4. Structures of known degradation products of isoniazid [7,8,12,14–19]. Key: Mol. Wt.: molecular weight.



Scheme 1. Mechanism of formation of isonicotinic acid *N′*-(pyridine-4-carbonyl)-hydrazide from isoniazid on exposure of solid drug to light.

The *m/z* value of 122 of the peak at RR_T 0.71 corresponded to isonicotinic acid (molecular weight 123). The formation of this degradation product was subsequently confirmed by spiking with the standard on LC. The other product at RR_T 1.34, which had *m/z* value of 121, was indicated to be isonicotinamide (molecular weight 122). Despite that its standard was not available, a fair affirmation of the formation of this compound was provided from a literature report [7], which showed that isonicotinamide is produced along with isonicotinic acid under hydrolytic and oxidative conditions. The degradation of isoniazid to isonicotinic acid and isonicotinamide were supposed to result in hydrazine and ammonia, respectively, but the latter were not detected on LC or LC-MS due to their non-chromophoric nature and because their molecular weights were below the lower mass limit of LC-MS used in the study.

In a similar manner, the mass comparison indicated that the peak at RR_T 4.22 (*m/z* 241) could be isonicotinic acid *N′*-(pyridine-4-carbonyl)-hydrazide (molecular weight 242), which was reported in the literature to be formed on exposure of alcoholic drug solution to light [12]. As there was no earlier report on the formation of this product on exposure of solid drug to light, studies were carried out to verify its structure though isolation and spectral studies.

The spectral data (IR and $^1H/^{13}C$ NMR) for the isolated compound are listed in Table 6, along with similar data for isoniazid. In case of the drug, the bands at 3308, 3113 and 1666 cm^{-1} correspond to the NH, NH_2 and C=O stretching vibrations, and the band of 1633 cm^{-1} arises due to NH_2 deformation [7]. In conformance with the postulated hydrazide structure, the IR spectrum of the isolated compound had no band of NH_2 deformation. Also, the NH stretching vibration at 3308 cm^{-1} shifted to higher frequency (3432 cm^{-1}), due to the attachment of NH groups in the new structure to two C=O groups, for which twin frequency bands appeared at 1682 and 1660 cm^{-1} in the IR of the isolated compound. The NMR data also supported the latter, as indicated by the difference in chemical shift values of the aromatic portion, and ^1H NMR *J* values.

3.6. Mechanism of degradation of isoniazid under light conditions to yield isonicotinic acid N′-(pyridine-4-carbonyl)-hydrazide

The above discussed formation of isonicotinic acid *N′*-(pyridine-4-carbonyl)-hydrazide on exposure of isoniazid to light in solid state can also be explained mechanistically. The conversion is postulated to follow a $S_{RN}1$ type radical mediated chain reaction [23], involving atleast three steps (Scheme 1). The first step entails transfer of an electron to isoniazid (I) in the presence of light, leading to formation of a radical anion (Ia). The latter is assumed to disintegrate through a unimolecular process into a 4-pyridoyl radical (II) and hydrazine anion (III). In the second step, III would abstract a proton from the NH_2 group of I to form an anion of isoniazid (Ib) and hydrazine. The nucleophilic attack by Ib on II in the third step leads further to radical anion IV that finally transfers an electron to I to produce the end product. In the process, radical anion Ia is released, which continues to propagate the chain reaction.

4. Conclusions

It was possible in this study to develop a stability-indicating LC assay method for isoniazid by subjecting the drug to ICH recommended stress conditions. The drug and degradation products got well separated from each other in an isocratic mode using a reversed-phase C18 column and mobile phase composed of water:acetonitrile (96:4, v/v). The flow rate and detection wavelength were 0.5 ml min^{-1} and 254 nm, respectively. The method proved to be simple, accurate, precise, specific and selective. It was easily transferable to LC-MS. Also, it could be successfully employed for analysis of the drug and degradation products in the marketed products stored for 3 months under accelerated conditions of temperature and humidity.

The stress studies and subsequent LC-MS analyses showed that the drug was decomposed to previously known degradation products, *viz*., isonicotinic acid and isonicotinamide under hydrolytic and oxidative conditions. An added finding was that the solid drug was practically stable to dry heat. Also, the drug discoloured under light due to formation of a yellow compound, isonicotinic acid *N′*-(pyridine-4-carbonyl)-hydrazide, which is not previously reported to occur in solid state. The product was characterized through MS, IR and NMR spectral studies. The same may be responsible for discolouration of isoniazid bulk drug substance and formulations, which is a familiar problem of industry. The mechanistic explanation to formation of this product is postulated.

It may be pertinent to add here that the stability-indicating method proposed in this paper may be used for the establishment of mass balance, provided consideration is given to the formation of equimolar quantities of hydrazine or ammonia during degradation of the drug to isonicotinic acid and isonicotinamide, respectively.

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