Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 377–383

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

LC–MS/TOF and UHPLC–MS/MS study of *in vivo* fate of rifamycin isonicotinyl hydrazone formed on oral co-administration of rifampicin and isoniazid

Bhagwat Prasad, Saranjit Singh*

Department of Pharmaceutical Analysis, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar 160 062, Punjab, India

ARTICLE INFO

Article history:
Received 20 April 2009
Received in revised form 30 June 2009
Accepted 15 July 2009
Available online 22 July 2009

Keywords:
Rifamycin isonicotinyl hydrazone
Rifampicin
Isoniazid
LC–MS/TOF
UHPLC–MS/MS

ABSTRACT

The formation and fate of 3-formylrifamycin isonicotinyl hydrazone (HYD) was investigated following oral co-administration of rifampicin (RIF) and isoniazid (INH) in Sprague–Dawley (SD) rats ($n = 5$) using advanced analytical modalities. The study was carried out with 20 and 5 mg/kg doses of RIF and INH, respectively. The plasma, urine and faeces samples were collected at different time points up to 48 h, which were qualitatively and quantitatively evaluated for the presence of HYD after proper sample preparation. For the same, initially liquid chromatography–mass spectrometry/time-of-flight (LC–MS/TOF) method was developed in electrospray ionization (ESI) positive mode, wherein separation was achieved on a C18 column (4.6 mm × 250 mm, 5 μm), using a volatile mobile phase in a gradient mode. The presence of HYD was confirmed by accurate mass study, spiking with the standard and UV–visible spectra matching. For quantitative evaluation of HYD, a selective and sensitive ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method was developed for all the three matrices. In this case, elution of HYD was achieved on a small C18 column (4.6 mm × 50 mm, 1.8 μm) using a short gradient method. The quantitation was done by selective reaction monitoring (SRM) in ESI positive mode. The validation parameters like linearity, accuracy, precision, selectivity, matrix effect, recovery and stability were assessed as per regulatory guidelines. The calibration range was established between 1 and 200 ng/ml, with $r^2 > 0.99$ in all the cases. The back calculated values for three quality control (QC) samples, and at lower limit of quantitation (LLOQ) were within 15 and 20%, respectively, of the nominal values. Similarly, the intra- and inter-day precisions were found within 15% at the four tested levels. The HYD was found to be stable for the duration of sample preparation and analysis in the controlled experimental conditions. The analysis of *in vivo* samples showed a significant extent of HYD in faeces, however, the interaction product was not found in plasma and urine. To verify the results, 5 mg/kg oral dose of HYD standard was given to rats separately, and its presence was studied in all the three matrices. Further, *in vitro* plasma stability of HYD was also carried out to explain its absence in plasma and urine, which showed ~55% disappearance of HYD in 2 h.

1. Introduction

Rifampicin (RIF) and isoniazid (INH) are front-line anti-tuberculosis (TB) drugs, which are usually prescribed to TB patients for a total period of 6 months. Initially, these are administered along with pyrazinamide (PYR) and ethambutol (ETB) for 2 months, followed by two drugs (RIF + INH) for next 4 months. These drugs are generally used in fixed dose-combination (FDC) formulations. The advantages of FDCs include prevention of mono-therapy and improvement in patient compliance. Unfortunately, combination use of these anti-TB drugs are associated with a few negative aspects [1–12], which include loss of bioavailability due to *in situ* interaction of RIF and INH, and also the stability problems [1–4,8,12–15].

In our laboratory, it was shown that RIF was converted to 3-formylrifamycin (3-RIF) under stomach acid conditions, which reacted with INH to form 3-formyl isonicotinyl hydrazone (HYD) (Fig. 1). In the process, RIF was lost to an extent of ~33% [16]. HYD was also reported to be formed during dissolution and stability test conditions [2,17–19]. Considering its importance, a limit has been prescribed for this interaction product in the monograph on the four-drug anti-TB combination in International Pharmacopoeia [20]. The formation of HYD is of serious concern because of its inactivity against mycobacterium both *in vitro* and *in vivo*. The *in vitro* activity of HYD against *Mycobacterium tuberculosis* H37Rv was 0.04 times less than that of RIF (MIC values 6.0 and 0.25 μg/ml, respectively). HYD was completely inactive on oral and intravenous administration of 4, 12 and 24 mg/kg to Laca mice infected with the same strain of mycobacterium [21].

There are plenty of literature reports, which show formation of hydrazones with endogenous carbonyl compounds, e.g., pyruvic

* Corresponding author. Tel.: +91 172 2214682; fax: +91 172 2214692.
E-mail address: ssingh@niper.ac.in (S. Singh).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.07.014

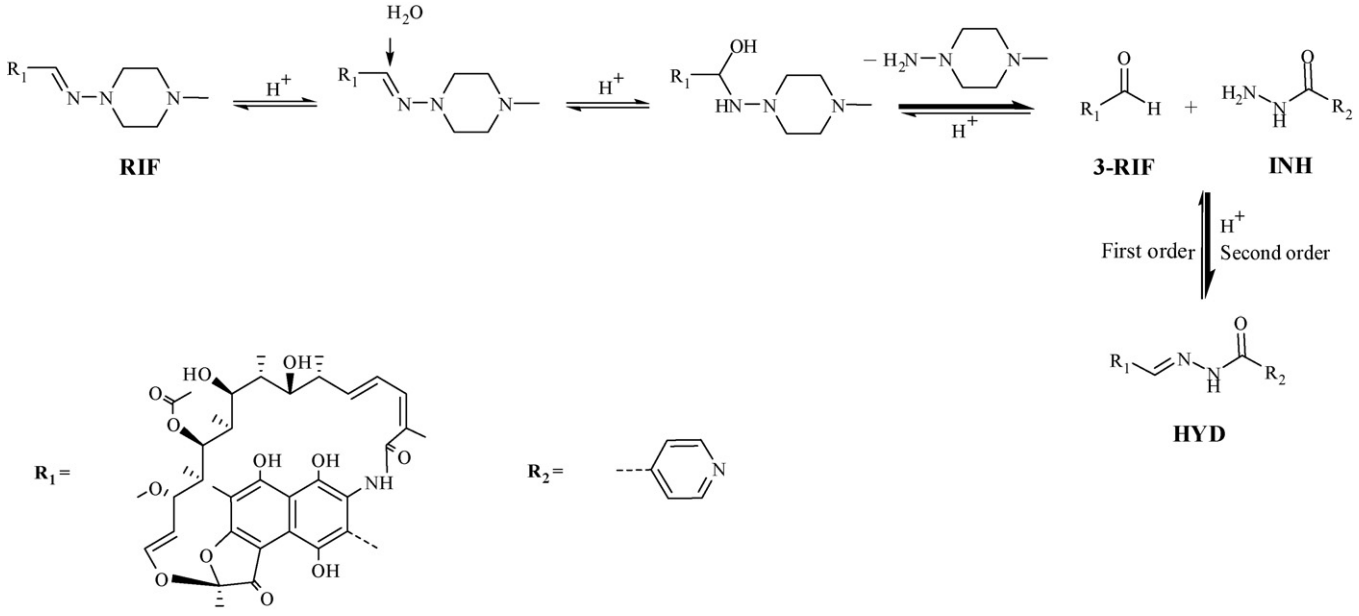


Fig. 1. Mechanism of formation and hydrolysis of HYD [16].

acid, α-ketoglutaric acid, etc. after oral administration of isoniazid [22–26]. These hydrazones have also been studied for their *in vivo* fate in many species, e.g., human, rat and dogs, and their presence is confirmed in the blood and urine. In addition, these hydrazones are known to hydrolyse in acidic pH (<4) and in biological matrices, e.g., blood, homogenates of stomach, intestine and liver [21,27].

In contrast, nothing has been investigated about the systemic exposure of HYD, after its *in situ* formation due to interaction of INH and 3-RIF in stomach. As this compound has completely different physico-chemical properties, i.e., poor water solubility and high partitioning coefficient, as compared to the hydrazones of INH with endogenous polar compounds, a dissimilar pharmacokinetics was anticipated for HYD. Hence, the purpose of this study was to investigate the *in vivo* fate of HYD in rats using modern qualitative and quantitative analytical tools, *viz*., liquid chromatography–mass spectrometry/time-of-flight (LC–MS/TOF) and ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS). The levels of HYD were monitored in plasma, urine and faeces after oral co-administration of RIF and INH. The results were substantiated by *in vitro* plasma stability study.

2. Materials and methods

2.1. Chemicals and reagents

RIF and INH were sourced from M/s Panacea Biotec Ltd. (Lalru, India). The internal standard (IS) ketoconazole was procured from Himedia Laboratories (Mumbai, India). Sprague–Dawley (SD) rats were procured from Central Animal Facility, NIPER (S.A.S. Nagar, India). HPLC grade acetonitrile (ACN) was procured from J.T. Baker (Phillipsburg, NJ, USA). HYD was synthesized in our laboratory. Pure water was obtained from a water purification unit (Elga Ltd., Bucks, England). All other chemicals were of analytical-reagent grade.

2.2. Animal studies

The *in vivo* studies were performed using adult SD rats (230–280 g). The animals were housed under controlled environmental conditions (temperature, 23 ± 2 °C; relative humidity (RH), 55 ± 5%; 12 h ligh/dark cycle), and, normal diet and water were freely available. The experiments were carried out according to institutional guidelines for the care and use of laboratory animals. The protocol was approved by the Institutional Animal Ethics Committee (IEAC/07/64). Before commencing the study, the rats ($n = 5$) were fasted for 12 h. The food and water were provided *ad libitum* after 2 h of the dosing. RIF and INH were co-formulated as water suspension using sodium carboxy methyl cellulose as a suspending agent, and immediately administered to rats ($n = 5$) orally at doses of 20 and 5 mg/kg, respectively. The blood samples (0.4–0.5 ml) were collected at different time points, i.e., predose, 0.25, 0.50, 1, 2, 4, 8, 12, 24 and 48 h, after which plasma was isolated immediately by centrifugation for 10 min at 9000 × *g*. Another set of animals ($n = 5$) were kept in metabolic cages for collection of urine and faeces. After initial acclimatization for 2 h in the cages, the animals were dosed similarly and the samples were collected from the metabolic cages before dosing, and 0–4, 4–8, 8–12, 12–24 and 24–48 h post dose. Additionally, pure HYD was administered orally (5 mg/kg) to rats ($n = 5$) separately, and blood, urine and faeces were collected at similar time points. The samples were collected in ice-bath and immediately transferred to −60 °C until analyses.

2.3. Sample preparation for analysis

During sample preparation, all the necessary steps were taken to avoid exposure of samples to ambient conditions for longer time, e.g., sample tubes were kept in ice-bath while processing, centrifugation was done at 4 °C, and prepared samples were immediately transferred to chilled autosampler at 4 °C or refrigerator set at 4 °C or less.

2.3.1. Sample pooling and pretreatment

For qualitative analyses, samples of all the five animals were pooled across different time points for individual matrices, separately. However, samples from each animal were pooled across different time points, individually for different matrices. Faeces samples were pretreated to slurry with addition of three volumes of water (w/w), while urine and plasma samples were subjected to sample preparation without any pretreatment.

2.3.2. Sample preparation for qualitative studies

For qualitative studies, the sample preparation method involved protein precipitation, freeze-liquid and solid-phase extraction (SPE) approaches. For the same, 9 ml ACN was added to 3 ml of the samples, i.e., plasma, urine and faeces slurry, in a 15 ml centrifuge tube. All the samples were then vortexed gently and centrifuged for 10 min at 9000 × *g*. The supernatants were subjected to freezing at −20 °C for 30 min till both ACN and aqueous layers became immiscible, and lower water layer was frozen. The upper ACN layer was collected in another set of tubes and the lower aqueous layer was subjected to SPE using HLB® cartridges (Waters, USA). The SPE procedure involved cartridge conditioning with 1 ml methanol followed by 2 ml water, loading of 1 ml samples, washing with 1 ml water and elution of the analytes with 0.5 ml ACN. Finally, samples recovered from SPE were pooled with the previously collected ACN layers and the mixtures were dried at controlled temperature (40 °C) under nitrogen purging for 5 min using a nitrogen evaporator (Turbovap, Caliper, USA). Samples were reconstituted in 40 μl diluent (ACN:H_2O, 70:30). To evaluate stability of HYD during sample preparation, a known concentration of standard HYD was spiked into respective blank matrices and treated similarly before analyses.

2.3.3. Sample preparation for quantitative studies

A much simpler sample preparation method was used for quantitative determination of HYD concentration in all the three types of samples. 200 μl of plasma, urine or faeces slurry was taken in a micro-centrifuge tube and 600 μl of ACN solution of IS (25 ng/ml) was added. All the samples were then vortexed gently and centrifuged for 10 min at 9000 × *g*. Supernatant was collected and used for analysis directly.

2.4. Qualitative LC–MS–DAD/TOF studies

2.4.1. Analytical method

Initial identification studies were performed on a liquid chromatograph equipped with diode array and time-of-flight mass detectors (LC–DAD–MS/TOF), which comprised of 1100 series HPLC system from Agilent Technologies (Waldbronn, Germany) and MicrOTOF-Q spectrometer from Bruker Daltonics (Bremen, Germany). The two were operated using combined software comprising of Hystar (version 3.1) and MicrOTOF Control (version 2.0). The LC–MS/TOF parameters used were: end plate offset voltage, 500 V; capillary voltage, 4500 V; nebulizer pressure, 1.2 bar; dry gas flow, 6 l/min; dry temperature, 200 °C; collision energy, 18 eV/*z*; transfer time, 100 μs and collision RF, 400 Vpp.

The separation was achieved on a Zorbax C18 column (250 mm × 4.6 mm, 5 μm) purchased from Agilent Technologies (Wilmington, Delaware, USA). The mobile phase composed of ACN and 10 mM ammonium acetate was used in a gradient mode (Table 1). After passage through DAD, only one-fourth of the LC flow was allowed to enter into the mass source using a splitter. The flow rate, detection wavelength, and column oven temperature were 0.8 ml/min, 254 nm and 25 °C, respectively. On-line mass calibration was done in all the cases using calibration standard supplied by the vendor.

2.4.2. Presence of HYD in biological matrices

10 μl of the prepared samples (Section 2.3.2) were injected into LC–MS/TOF and chromatograms of all the *in vivo* samples were studied for the presence of HYD by comparing elution behaviour against the standard. HYD peak detected in the extracted ion chromatograms (EICs) was confirmed by spiking with its standard, by UV–vis spectra matching, accurate mass data and MS/MS fragmentation studies.

Table 1
Different time programs used in LC–MS/TOF and UHPLC–MS/MS analysis of HYD.

Program	Time (min)	Event
LC–MS/TOF		
Gradient program (% ACN)	0.0–2.0	4
	2.0–27.0	4 → 30
	27.0–42.0	30 → 40
	42.0–55.0	40 → 60
	55.0–65.0	60 → 80
	65.0–70.0	80
	70.0–80.0	80 → 4
	80.0–90.0	4
UHPLC–MS/MS		
Gradient program (% ACN)	0.0–0.7	30
	0.7–1.8	30 → 58
	1.8–2.7	58 → 60
	2.7–2.9	60 → 72
	2.9–3.9	72
	3.9–4.2	72 → 30
	4.2–6.0	30
Segments program	0.0–4.0	SRM monitoring of HYD
	4.0–6.0	SRM monitoring of IS
Divert valve program	0.0–2.0	Waste
	2.0–5.5	MS source
	5.5–6.0	Waste

2.5. Quantitative UHPLC–MS/MS studies

2.5.1. Analytical method development

Quantitation of HYD was performed using an ultra high performance liquid chromatography (UHPLC) Accella™ (Thermo Electron Corp., San Jose, USA) system having quaternary pump and temperature controlled autosampler connected with linear ion trap mass spectrometer (LTQ XL™, Thermo Electron Corp.) via an electrospray interface. Instrument control and data collection were done with the help of Xcalibur (Version 2.0.7 SP1) and LCquan (Version 2.5.6) software (Thermo Electron Corp.).

For quantitative studies, ketoconazole was selected as an IS because of similar partitioning (cLog P) profile to HYD. A rapid gradient UHPLC method was developed to achieve satisfactory separation of HYD, IS and matrix components employing a short C18 (4.6 mm × 50 mm, 1.8 μm) column (Agilent Technologies, Wilmington, Delaware, USA) using a similar volatile mobile phase, as discussed in Section 2.4.1. The flow rate was 500 μl/min. The data for both HYD and IS were acquired in positive SRM mode using ESI/MS/MS parameters, optimized for monitoring the most abundant fragments of HYD and IS. The optimized parameters for MS/MS studies were: ionization spray voltage, 5 kV; spray current, 5 μA; sheath gas flow rate, 25 l/min; auxilliary gas flow rate, 10 l/min, capillary voltage, 50 V; capillary temperature, 325 °C; tube lens, 55 V and collision energies for HYD and IS, 25 and 30 eV, respectively. Ion transitions used for monitoring response were *m*/*z* 845 → *m*/*z* 813 for HYD and *m*/*z* 531 → *m*/*z* 489 for IS.

2.5.2. Method validation

The method was validated by assessing linearity, accuracy, precision, selectivity, matrix effect, recovery and stability in plasma, urine and faeces according to the US-FDA guideline [28].

2.5.2.1. Preparation of working solutions for calibration curve standards and quality control (QC) samples. Stock standard solutions of HYD and IS (1 mg/ml each) were prepared in ACN:water (70:30, v/v). Further dilution of the same was done to get a secondary stock solution of 100 μg/ml. This solution was then used to prepare a series of eight HYD working standards of 20–4000 ng/ml. The IS solution contained 25 ng/ml of ketoconazole in ACN.

2.5.2.2. Preparation of calibration curve standards and QC samples in biological matrices. Blank plasma, urine and faeces slurry (water:faeces = 3:1) were spiked with different concentrations of working standard solutions (Section 2.5.2.1.) to give respective sets of calibration curve standards and QC samples. The calibration curves for the biological matrices were constructed with eight standards of HYD at 1, 3, 8, 16, 40, 80, 160 and 200 ng/ml in plasma, urine and faeces slurry. Among these, QC samples were selected at three concentrations, i.e., 3, 80 and 160 ng/ml. Validation experiments were carried out at LLOQ and QC concentration levels.

2.5.2.3. Linearity. Linearity of the matrix-matched calibration curves was established in all the matrices by calculation of the coefficient of determination (r^2) on 3 different days.

2.5.2.4. Accuracy and precision. Accuracy and precision of the methods were tested by analysis of individually prepared standard samples at LLOQ and all the QC levels in plasma, urine and faeces. Accuracy was evaluated by back calculating concentrations of HYD at the four tested levels and expressed as % nominal concentration. Intra-day precision was performed in a single day by repeating analysis for five times, while, inter-day precision was carried out by analysing LLOQ and QC samples for 3 consecutive days. The precision values were expressed as % R.S.D.

2.5.2.5. Selectivity, matrix effect and recovery. In all the 3 matrices, 6 blank samples were analysed, and the absence of interfering peaks in the retention time window of HYD was checked. Matrix effect was evaluated at LLOQ level ($n = 6$), where peak areas of the neat standards were compared to those of samples in which the extracted matrices were spiked. Similarly, the overall recoveries were also determined at four levels, i.e., at LLOQ and three QC levels by comparing peak areas of the neat standards with the area of processed samples.

2.5.2.6. Stability of HYD in spiked biological matrices. A battery of stability studies were performed at low and high QC levels. Benchtop and autosampler stabilities were evaluated for 2 and 24 h, respectively, while freeze–thaw stability was checked up to three cycles.

2.6. Quantitative analysis of HYD in biological matrices

Plasma, urine and faeces samples, collected after administration of RIF and INH combination and HYD alone, were processed using the simple protein precipitation method discussed in Section 2.3.3. The analyses of samples were done using the UHPLC–MS/MS method discussed in Section 2.5.1.

2.7. In vitro plasma stability of HYD

1 ml HYD-free plasma was pre-incubated at 37 °C for 10 min, which was then spiked with HYD stock solution to get a final concentration of 5 μM ($n = 3$). The samples were then incubated at 37 °C, and 200 μl incubates were sampled out at different time points, i.e., 0, 30, 60, 90 and 120 min. The same were transferred into the micro-centrifuge tubes containing 600 μl of ACN solution of IS. The solutions were vortexed for 30 s and centrifuged immediately, as described in Section 2.2. The supernatant was analysed using UHPLC–MS/MS method and stability was expressed as a fall in the concentration with time.

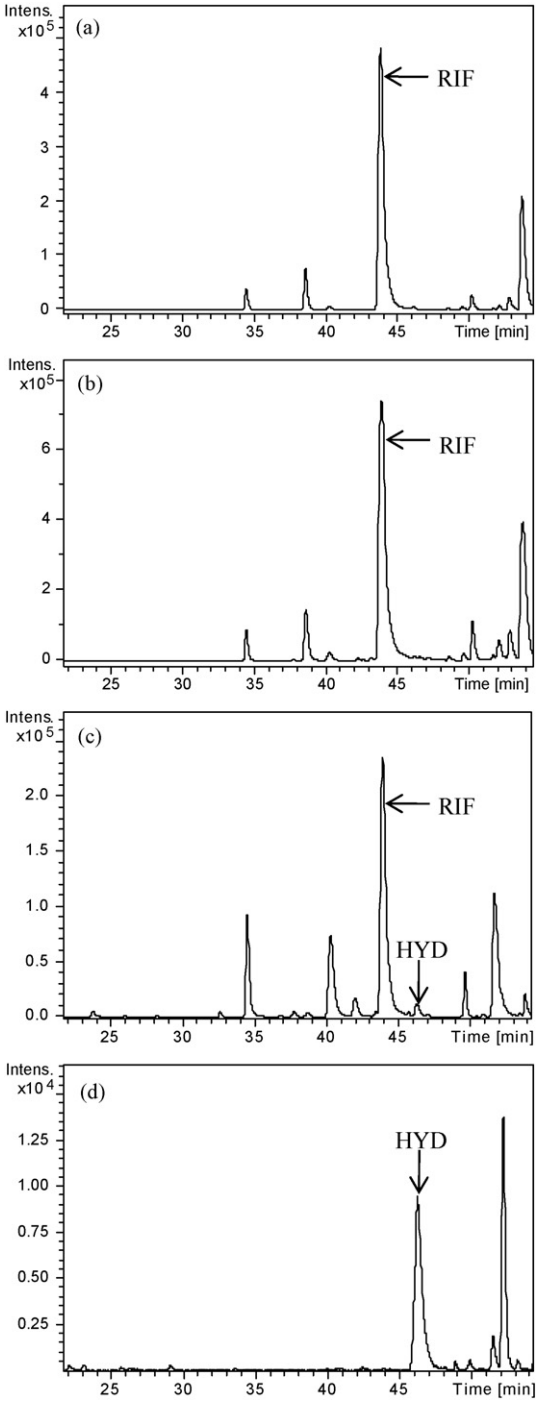


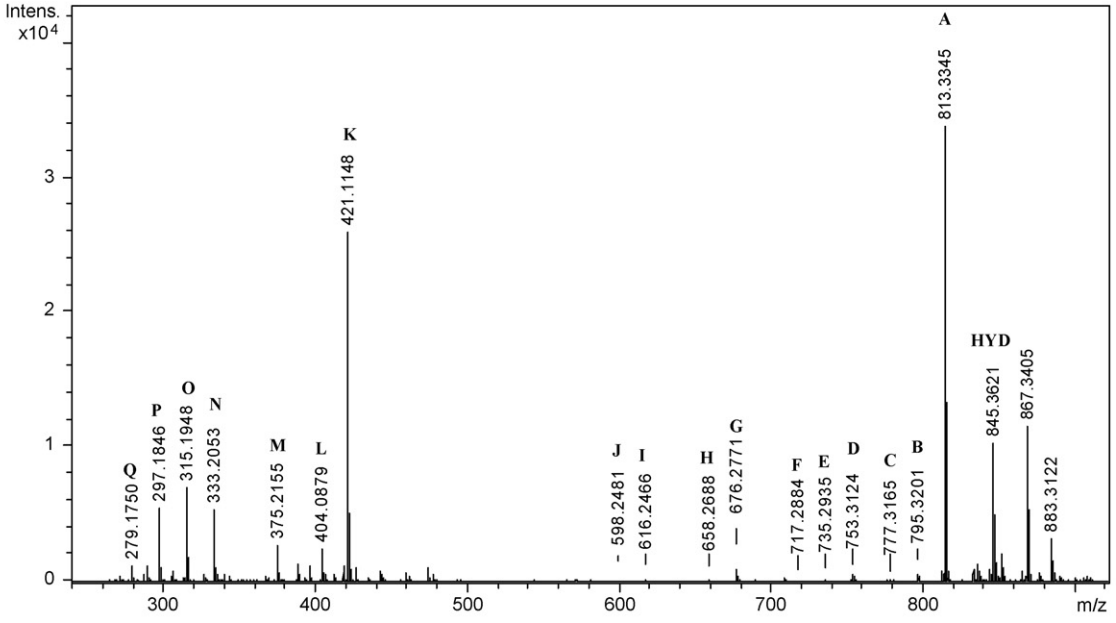
Fig. 2. LC–MS/TOF profiles of plasma, urine and faeces samples (a, b and c, respectively) collected after co-administration of RIF and INH. LC–MS/TOF profile of faeces sample after administration of pure HYD (d).

3. Result and discussion

3.1. Presence of HYD in in vivo matrices

The stability of HYD under the conditions of sample handling and processing was established to be >93% in all the spiked matrices. Fig. 2a–c shows LC–MS/TOF ion chromatograms of plasma, urine and faeces pooled samples collected after administration of the combination of RIF and INH. The peak of HYD, which was verified through its accurate mass of 845.3621 (Fig. 3), spiking with the standard and UV–visible spectral matching with reported spectrum

(a)



(b)

Code	Mass error (ppm)	Molecular formula
HYD	2.01	$C_{44}H_{53}N_4O_{13}^+$
A	0.49	$C_{43}H_{49}N_4O_{12}^+$
B	4.40	$C_{43}H_{47}N_4O_{11}^+$
C	4.50	$C_{43}H_{45}N_4O_{10}^+$
D	0.80	$C_{41}H_{45}N_4O_{10}^+$
E	12.24	$C_{41}H_{43}N_4O_9^+$
F	4.88	$C_{41}H_{41}N_4O_8^+$
G	2.81	$C_{37}H_{42}NO_{11}^+$
H	6.23	$C_{37}H_{40}NO_{10}^+$
I	12.77	$C_{35}H_{38}NO_9^+$
J	7.69	$C_{35}H_{36}NO_8^+$
K	1.19	$C_{21}H_{17}N_4O_6^+$
L	0.49	$C_{21}H_{14}N_3O_6^+$
M	2.93	$C_{22}H_{31}O_5^+$
N	2.22	$C_{20}H_{29}O_4^+$
O	2.13)	$C_{20}H_{27}O_3^+$
P	1.04	$C_{20}H_{25}O_2^+$
Q	2.51	$C_{20}H_{23}O^+$

(c)

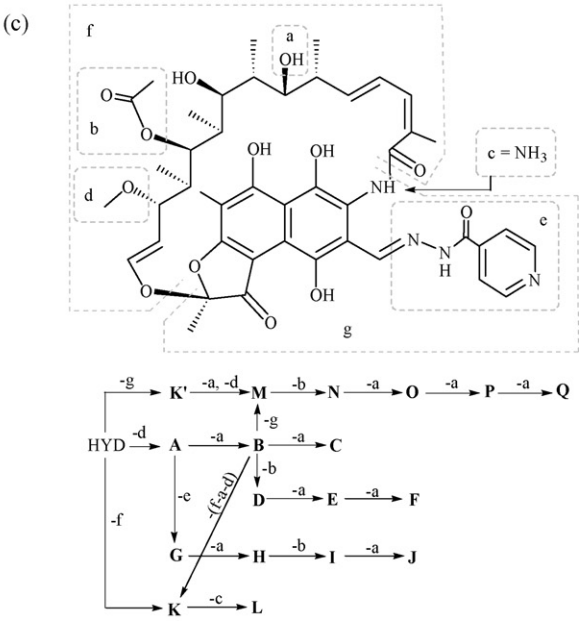


Fig. 3. MS/TOF spectra of HYD peak observed in faeces after co-administration of RIF and INH (a). Accurate mass data and derived molecular formulae for the observed fragments (b). Proposed fragmentation pathway of HYD (c), where, A–Q represent different fragments obtained in LC–MS/TOF. Letters a, b, c, d, e, f and g represent leaving moieties, i.e., H_2O, CH_2CO, NH_3, CH_3OH, hydrazone part, aliphatic part and aromatic moiety of the structure, respectively (indicated by dotted lines in the figure).

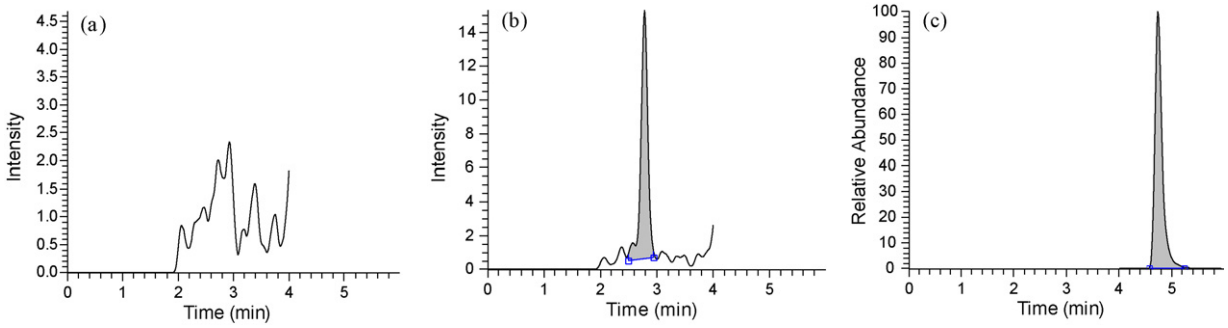


Fig. 4. Representative UHPLC–MS/MS chromatograms of blank (a), LLOQ standard (b) and IS (c) in faeces.

[15], was observed next to the RIF peak at 46.2 min (RRT, 1.05). It was present only in faeces, and was absent in plasma and urine. Again, it was only observed in faeces on administration of pure HYD, as shown in Fig. 2d.

3.2. Fragmentation pattern of HYD

The comprehensive fragmentation pattern of HYD, which is yet unreported, was delineated based on chemical formulae derived from accurate mass values for various fragments (Fig. 3). Initially, HYD (*m*/*z* 845) underwent ionization into an intense fragment of *m*/*z* 813 (**A**) on neutral loss of CH_3OH. There was consecutive water loss from **A** to yield fragments of *m*/*z* 795 (**B**) and *m*/*z* 777 (**C**). Loss of CH_2CO moiety from **B** resulted in a fragment of *m*/*z* 753 (**D**), which also consecutively lost two water molecules to form ions of *m*/*z* 735 (**E**) and *m*/*z* 717 (**F**). **A** could also undergo parallel C–N cleavage to eliminate hydrazone moiety, leaving back fragment of *m*/*z* 676 (**G**). Ionization of **G** followed the scheme **G** → 658 (**H**) → *m*/*z* 616 (**I**) → *m*/*z* 598 (**J**) upon loss of water, –CH_2CO and water, respectively. Further, ring cleavage took place to separate aromatic and aliphatic sides of the structure, resulting in fragments of *m*/*z* 421 (**K**) and *m*/*z* 375 (**M**), respectively. For formation of the latter, ring cleavage resulted in an intermediate fragment **K′**, which on spontaneous loss of water and CH_3OH moieties yielded the fragment **M**. Moreover, **K** and **M** could be formed from fragment **B** on direct ring cleavage. The aromatic fragment **K** lost ammonia on further fragmentation and resulted in **L** (*m*/*z* 404), while **M** formed fragment of *m*/*z* 333 (**N**) on loss of CH_2CO moiety. Further ionization of **N** on sequential loss of water followed the scheme: **N** → *m*/*z* 315 (**O**) → *m*/*z* 297 (**P**) → *m*/*z* 279 (**Q**).

3.3. UHPLC–MS/MS method development and validation

3.3.1. Method development and optimization

A short gradient method (Table 1) was optimized so that sufficient separation could be obtained among matrix components, HYD and IS. Initially, 70% buffer was used to quickly elute the matrix components, while retaining the non-polar analyte and IS in the column. The ACN concentration was then gradually increased to elute analyte and IS free from matrix components, without affecting separation and peak shapes. The flow was diverted to waste both before and after elution of peaks of interest, to prevent entrance of any matrix components in the MS source. HYD and IS were separated efficiently, therefore, two segments were used rather than using two scan events, which resulted in increased sensitivity. Different time programs used in the analysis are shown in Table 1. Figs. 4a–c depict representative UHPLC–MS/MS chromatograms of blank, HYD and IS, respectively in faeces sample.

3.3.2. Method validation

3.3.2.1. Linearity. Good linearity was established across the studied range of 1–200 ng/ml for HYD in different biological matrices. The linearity equations for HYD in plasma, urine and faeces were $y = 0.0062x + 0.0040$ ($r^2 = 0.9990$), $y = 0.0064x + 0.0109$ ($r^2 = 0.9994$), and $y = 0.0045x + 0.0043$ ($r^2 = 0.9983$), respectively.

3.3.2.2. Accuracy and precision. The method was found accurate as back calculated concentrations at LLOQ and three QC levels were within the 15 and 20% of the nominal values, respectively. The intra- and inter-day precisions expressed as % R.S.D. were lower than 15% at LLOQ and all tested QC levels.

3.3.2.3. Selectivity, matrix effect and recovery. Figs. 4a and b show typical UHPLC–MS/MS chromatograms of HYD representing blank and LLOQ levels in faeces sample, respectively. The signal to noise (S/N) ratio was found to be >5 in faeces sample at LLOQ. Similar results were obtained in samples of plasma and urine.

The recovery was more than 87% in all the cases, including LLOQ. There was no significant difference between peak area of neat LLOQ standard and area of the peak observed in sample where extracted matrix spiked LLOQ standard was used. This also revealed that matrix effect was insignificant due to any co-eluting components.

3.3.2.4. Stability of HYD in spiked biological matrices. HYD was found within 15% of initial concentration at both low and high QC levels, when prepared in controlled conditions. As the total time of sample preparation was <30 min, and all the precautions were taken to avoid unnecessary exposure of samples to ambient conditions, HYD was proven stable during normal sample handling and processing.

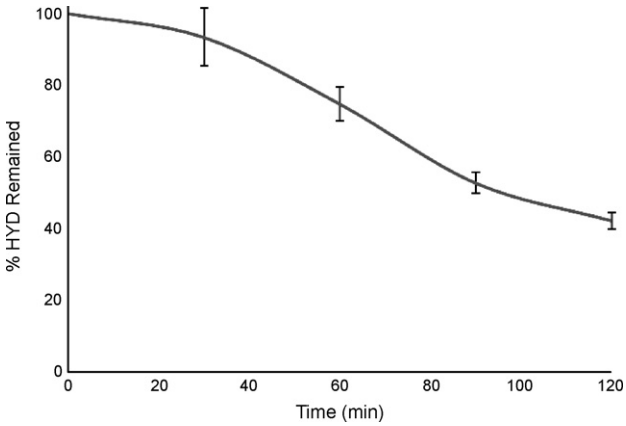


Fig. 5. *In vitro* plasma stability profile of HYD at 37 °C.

3.4. Quantitation of HYD in plasma, urine and faeces

HYD was not found in plasma and urine. It was found in the concentration of 32.4 ± 6.5 ng/ml in faeces slurry on *in situ* formation, and 61.3 ± 9.2 ng/ml after administration of pure HYD. The *in vitro* plasma stability of HYD is shown in Fig. 5, which depicted that ~55% of the compound disappeared after 2 h at 37 °C. This may be a part reason for the absence of HYD in plasma and urine, after getting formed *in situ* in stomach.

3.5. Relevance of the study with respect to bioavailability of RIF and INH

In the stomach, interaction between INH and the fraction of RIF that has been converted into 3-RIF can give rise to therapeutically inactive HYD. As HYD is poorly water soluble and prone to hydrolysis, its absorption is less. This phenomenon can have a negative impact on the bioavailability of the two anti-TB drugs, producing lower than expected blood levels and therapeutic efficacy. Hopefully, studies conducted in the near future will measure blood levels of each drug at different time points after combined administration. The data from such study will allow us to quantify this phenomenon.

4. Conclusion

A very sensitive and selective UHPLC–MS/MS method was developed and validated for the determination of HYD in rat plasma, urine and faeces. This method was employed to determine *in vivo* fate of HYD after oral co-administration of RIF and INH, and also pure HYD. In both situations, HYD was mainly found in faeces, and was practically absent in plasma and urine. *In vitro* studies

revealed that HYD was hydrolysed in plasma, which also explained its absence in urine.

Acknowledgement

Authors gratefully acknowledge the financial assistance provided by Bristol Myers Squibb, Bangalore, India.

References

[1] H. Bhutani, T.T. Mariappan, S. Singh, The physical and chemical stability of anti-tuberculosis fixed-dose combination products under accelerated climatic conditions, Int. J. Tuberc. Lung Dis. 8 (2004) 1073–1080.
[2] H. Bhutani, T.T. Mariappan, S. Singh, An explanation for the physical instability of a marketed fixed dose combination (FDC) formulation containing isoniazid and ethambutol and proposed solutions, Drug Dev. Ind. Pharm. 30 (2004) 667–672.
[3] H. Bhutani, Ph.D. Thesis. National Institute of Pharmaceutical Education and Research, S.A.S. Nagar, India, (2006) pp. 57–73.
[4] K.F. Laserson, A.S. Kenyon, T.A. Kenyon, T. Layloff, N.J. Binkin, Substandard tuberculosis drugs on the global market and their simple detection, Int. J. Tuberc. Lung Dis. 5 (2001) 448–454.
[5] N.A. Sabri, H.Y. El-Zorba, In vitro and in vivo effects of isoniazid on stability and pharmacokinetics of rifampicin, Egypt J. Pharm. Sci. 44 (2003) 19–38.
[6] K. Rookkapan, V. Chongsuvivatwong, S. Kasiwong, S. Pariyawatee, Y. Kasetcharoen, P. Pungrassami, Deteriorated tuberculosis drugs and management system problems in lower southern Thailand, Int. J. Tuberc. Lung Dis. 9 (2005) 654–660.
[7] G. Acocella, Human bioavailability studies, Bull. Int. Union Tuberc. Lung Dis. 64 (1989) 38–40.
[8] H. Bhutani, S. Singh, K.C. Jindal, A.K. Chakraborti, Mechanistic explanation to the catalysis by pyrazinamide and ethambutol of reaction between rifampicin and isoniazid in anti-TB FDCs, J. Pharm. Biomed. Anal. 39 (2005) 892–899.
[9] V. Kumar, B. Prasad, S. Singh, Pharmaceutical issues in the development of a polypill for the treatment of cardiovascular diseases, Drug Discov. Today Ther. Strat. 5 (2008) 63–71.
[10] G. Pillai, P.B. Fourie, N. Padayatchi, P.C. Onyebujoh, H. McIlleron, P.J. Smith, G. Gabriels, Recent bioequivalence studies on fixed-dose combination antituberculosis drug fomulations available on the global market, Int. J. Tuberc. Lung Dis. 3 (1999) S309–S316.
[11] C.J. Shishoo, S.A. Shah, I.S. Rathod, S.S. Salave, M.J. Vora, Impaired bioavailability of rifampicin in presence of isoniazid from fixed dosed combination (FDC) formulation, Int. J. Pharm. 228 (2001) 53–67.
[12] S. Singh, T.T. Mariappan, R. Sankar, N. Sarda, B. Singh, A critical review of the probable reasons for the poor/variable bioavailability of rifampicin from antitubercular fixed-dose combination (FDC) products, and the likely solutions to the problem, Int. J. Pharm. 228 (2001) 5–17.
[13] S. Singh, B. Mohan, A pilot stability study on four-drug fixed-dose combination anti-tuberculosis products, Int. J. Tuberc. Lung Dis. 7 (2003) 298–303.
[14] T.T. Mariappan, S. Singh, Regional gastrointestinal permeability of rifampicin and isoniazid (alone and their combination) in the rat, Int. J. Tuberc. Lung Dis. 7 (2003) 797–803.
[15] T.T. Mariappan, K.C. Jindal, S. Singh, Overestimation of rifampicin during colorimetric analysis of anti-tuberculosis products containing isoniazid due to formation of isonicotinyl hydrazone, J. Pharm. Biomed. Anal. 36 (2004) 905–908.
[16] S. Singh, T.T. Mariappan, N. Sarda, S. Kumar, A.K. Chakraborti, The reason for an increase in decomposition of rifampicin in the presence of isoniazid under acid conditions, Pharm. Pharmacol. Commun. 6 (2000) 405–410.
[17] K.C. Jindal, R.S. Chaudhary, A.K. Singla, S.S. Gangwal, S. Khanna, Dissolution test method for rifampicin–isoniazid fixed dose formulations, J. Pharm. Biomed. Anal. 12 (1994) 493–497.
[18] H.I. Seifart, D.P. Parkin, P.R. Donald, Stability of rifampin, isoniazid and pyrazinamide in suspensions used for the treatment of tuberculosis in children, Pediatr. Infect. Dis. 10 (1991) 827–831.
[19] C.J. Shishoo, S.A. Shah, I.S. Rathod, S.S. Savale, J.S. Kotecha, P.B. Shah, Stability of rifampicin in dissolution medium in presence of isoniazid, Int. J. Pharm. 190 (1999) 109–123.
[20] http://www.who.int/phint/en/p/docf/ (accessed on: 20.04.09).
[21] T.T. Mariappan, Ph.D. Thesis. National Institute of Pharmaceutical Education and Research, S.A.S. Nagar, India, (2004) pp. 127–146.
[22] D.W. Russell, Low circulating levels of acid-labile hydrazones after oral administration of isonicotinic acid hydrazide, Clin. Chim. Acta 41 (1972) 163–168.
[23] J.A. Timbrell, J.M. Wright, Studies on the effects of isoniazid on acetylhydrazine metabolism in vivo and in vitro, Drug Metab. Dispos. 7 (1979) 237–240.
[24] J.M. Wright, J.A. Timbrell, Factors affecting the metabolism of [14C]acetylhydrazine in rats, Drug Metab. Dispos. 6 (1978) 561–566.
[25] J.H. Peters, M. Gill, V.E. Hayes, The influence of D-glucose and p-aminosalicylic acid on the metabolism of isoniazid, Arch. Int. Pharmacodyn. Ther. 159 (1966) 340–352.
[26] G.A. Ellard, P.T. Gammon, S.M. Wallace, The determination of isoniazid and its metabolites acetylisoniazid, monoacetylhydrazine, isonicotinic acid and isonicotinylglycine in serum and urine, Biochem. J. 126 (1972) 449–458.
[27] P. Kovarikova, Z. Mrkvickova, J. Klimes, Investigation of the stability of aromatic hydrazones in plasma and related biological material, J. Pharm. Biomed. Anal. 47 (2008) 360–370.
[28] Anonymous. Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Rockville, MD, 2001.