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Effect of Piperine on Pharmacokinetics of Rifampicin and Isoniazid: Development and Validation of High Performance Liquid Chromatography Method

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ABSTRACT

In the present investigation a method for simultaneous determination of rifampicin, isoniazid and piperine by liquid chromatography has been developed and optimized in terms of specificity, accuracy, precision, sensitivity and stability. The purpose of this investigation concerns the effect of piperine on the pharmacokinetic of rifampicin and isoniazid in rat model. Suspension of rifampicin, isoniazid and piperine were administered orally in Swiss albino rat and the drug concentration in plasma was estimated by high performance liquid chromatography. The chromatic separation of rifampicin, isoniazid and piperine were achieved by carrying out on a Zorbax Eclipse XDP C_{18} column (150 mm × 4.6 mm × 5 µm) using potassium dihydrogen phosphate (pH 4.5) and acetonitrile as the mobile phase (30:70) at a flow rate of 0.8 mL/min with detection at 270 nm. After oral administration the observed pharmacokinetic parameter of rifampicin along with piperine indicates significant enhancement in C_{max} and area under the curve (AUC), while in isoniazid with piperine shows reduced C_{max} and enhanced-AUC. The observed pharmacokinetics data after oral administration of rifampicin along with piperine indicate significant enhancement in C_{max} and AUC and thus bioavailability. The bioavailability of isoniazid was reduced when it was co-administered with piperine, it could be due to delay in gastric emptying time.

INTRODUCTION

Rifampicin (RIF) (Figure 1), a complex semi-synthetic macro cyclic antibiotic derived from *Streptomyces mediterranei*, is a member of the RIF class of antibiotics used for the treatment of tuberculosis and other infectious diseases (Maggi *et al.*, 1966). It is categorized as one of the first line antituberculous agent. Tuberculosis remains a major health public problem and is the single most deadly infectious disease. It kills approximately two million people each year (Gallieni *et al.*, 1999). RIF is chemically (12Z, 14E, 24E)-(2S, 16S, 17S, 18R, 19R, 20R, 21S, 22R, 23S)-1,2-dihydro-5, 6, 9, 17, 19-pentahydroxy, 23-methoxy-2, 4, 12, 16, 18, 20, 22-heptamethyl-8-(4-methylpiperazin-1 yliminomethyl)-1, 11-dioxo 2, 7 (epoxypentadeca-1, 11, 13-trienimino) naphtha [2,1-b] furan-21-yl acetate (Maryadele, 2006). It is official in Indian Pharmacopoeia (IP, 2010), British Pharmacopoeia (BP, 2010) and United State Pharmacopoeia (USP, 2005). The IP, BP and USP describe liquid chromatography and visible-spectrophotometry method for its estimation. Literature

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survey reveals high performance liquid chromatography (HPLC) (Panchagnula *et al.*, 1999), high performance thin layer chromatography (HPTLC) (Shishoo *et al.*, 2001) and visible spectrophotometry (Begum *et al.*, 2013) methods for determination of RIF in pharmaceutical dosage forms as well as in biological fluids. Literature survey also reveals spectrophotometric (Khuhawar and Rind, 1998), reverse phase high performance liquid chromatography (RP-HPLC) (Calleri *et al.* 2002) and visible spectrophotometry (Manna *et al.*, 2000; Ali *et al.*, 2007) methods for determination of RIF within combination drug.

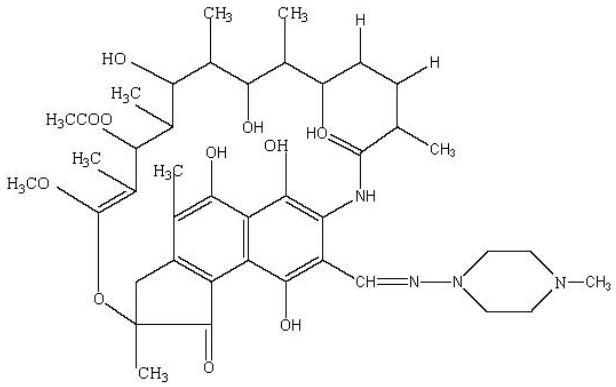

Fig. 1: Chemical structure of rifampicin.

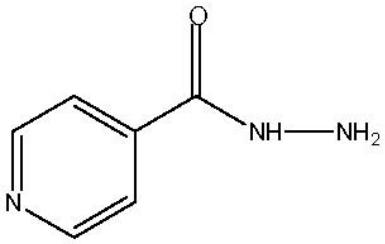

Fig. 2: Chemical structure of isoniazid.

Isoniazid (INH) (Figure 2), the hydrazide of isonicotinic acid is a synthetic analog of pyridoxine (Harvey and Champe, 2000). It is the first line antitubercular medication that never used on its own to treat active tuberculosis because resistance quickly develops (Shinkich, 2007). Thus, it is widely used together with RIF, ethambutol and pyrazinamide among others, for the chemotherapy of tuberculosis. Several methods for the determination and quantitation of isoniazid have been described. These include H-point standard addition method (Safari *et al.*, 2007), selective adsorption using a piezoelectric sensor (Yao *et al.* 1999), voltametric method (Wahdan, 2005), amperometric method (Quintino and Angnes, 2006), chromatographic methods (HPLC, GC and HPTLC) (Moussa, 2005; Carlina *et al.*, 1998; Khuhawara and Zardari, 2008), titrimetric methods (Tatarczak *et al.*, 2005), chemiluminisence (Juan *et al.*, 2004) and UV-spectrometric (Nagendra *et al.*, 2002).

Piperine (PIP) (Figure 3) was discovered by Hans Christian Qrsted in 1819. He isolated it from the fruits of *Piper nigrum*, the source plant of both the black and white pepper grains. Piperine is an alkaloid found naturally in plants belonging to the Piperaceae family, such as *Piper nigrum L*, commonly known as black pepper and *Piper longum L*, known as long pepper.

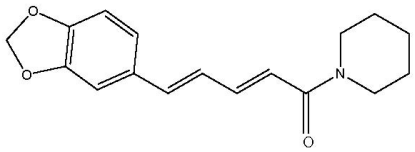

Fig. 3: Chemical structure of piperine.

PIP is chemically 1-5-(1,3-benzodioxol-5-yl)-1-oxo–2,4–pentadienyl piperidine (Sweetman 2005) is a natural alkaloids use as bioenhancer (Atal *et al.*, 1985). PIP is official in IP (2010), describes liquid chromatography method for its estimation (Wood *et al.*, 1988). Literature survey also reveals HPLC (Nagappan *et al.*, 2009), UV-spectrophotometry (Gupta and Jain, 2011) and HPTLC (Hamrapurkar *et al.*, 2011; Shanmugasundram *et al.*, 2008) method for determination of PIP. Literature survey also reveals HPLC (Pattanaik *et al.*, 2009) method for determination of PIP with other drug combination. The combined dosages forms of RIF and PIP along with INH are available in the market and used as anti-tubular drugs.

Bio-enhancer is such compounds simply enhance the bioavailability and bio-efficacy of particular drugs with which they are combined without any pharmacological activity of their own at the same dose level. PIP has increased the oral bioavailability of several drugs which includes antitubercular, antileprotic, antibiotics, NSAIDS, cardiovascular agents and central nervous agent (Jaakiramnan and Manavalan 2008; Hiwale *et al.*, 2002).

The aim of present method was to develop and validate the simple, fast, sensitive and precise RP-HPLC method for the assay of RIF, INH and PIP and also study the effect of PIP on the bioavailability of RIF and INH when accessed in Swiss albino rats.

MATERIALS AND METHODS

Materials

The standard API of RIF and INH was received from 'Exim' Pharm International, Mumbai, India. PIP was obtained from 'Tulsi Bioscience', Gujarat, India. Sodium carboxymethylcellulose, diethyl ether, EDTA, dextrose, chloroform of analytical grade was purchased from Qualigens (Fischer), Mumbai, India. Methanol and potassium dihydrogen orthophosphate of HPLC grade were procured from 'Exim' Pharm International, Mumbai, India. Ethylene diamine tetra acetic acid (EDTA) obtained from 'Sigma St. Lowis', MO, USA. Acetonitrile and dichloromethane were purchased from 'Merck', India. HPLC grade water was obtained from Millipore water purification system, USA.

Table 1: Quality control samples concentration of rifampicin, isoniazid and piperine.

Standard	Rifampicin			Isoniazid			Piperine		
	Strength (µg/mL)	Reference stock solution (mg/mL)	Dilution factor	Strength (µg/mL)	Reference stock solution (mg/mL)	Dilution factor	Strength (µg/mL)	Reference stock solution (mg/mL)	Dilution factor
QC STD 1	0.4	1	2500	0.4	1	2512.5	3	1	333.33
QC STD 2	250	1	4	75	1	13.4	250	1	4
QC STD 3	500	1	2	150	1	6.7	500	1	2

*QC STD- Quality Control Standard.

Apparatus

RP-HPLC determinations were performed on 'Shimadzu LC 2010-HT' with UV detector and 'Shimadzu LC Solution Software' 2010, equipped with auto sampler injector and a digital column oven. All weights were taken on electronic balance (Mettler Toledo, Switzerland) and others solvent evaporator (Thermo Electron Corporation, Milford, MA), centrifuge (Sigma St. Lowis MO, USA), sonicator (Banoelin Electronics, Berlin), digital pH meter (OAKTON) and vacuum filter (Millipore, USA) were used.

Quality control sample

The precision and accuracy parameters were ascertained in 3 different quality control samples, Table 1, ICH Q2R (1).

LQC (Lower quality control)

The lower quality control samples were prepared with RIF- 0.4 µg/mL, INH- 0.4 µg/mL and PIP- 3 µg/mL.

MQC (Middle quality control)

The middle quality control samples of RIF- 250 µg/mL, INH- 75 µg/mL and PIP- 250 µg/mL was prepared.

HQC (Higher quality control)

Higher quality control samples were prepared by RIF- 500 µg/mL, INH- 150 µg/mL and PIP- 500 µg/mL of concentrations.

Chromatographic conditions

Chromatographic separation was carried out at 40°C on a column Zorbax Eclipse XDP C_{18} (150 mm × 4.6 mm × 5 µm, GL Sciences, Inc. USA). The RIF, INH and PIP analyte were separated with a mobile phase consisting of 20 mM potassium dihydrogen orthophosphate (KH_2PO_4, pH = 4.5) and acetonitrile at 30:70 v/v ratio. The flow rate was 0.8 mL/min. and the eluted analyte was monitored by UV detection at 270 nm.

Animals

The *in vivo* study, on animal, was based on 'Breeding of and Experiments on Animals (Control and Supervision) Rules 1998' as amended by Government of India, Ministry of Environment and Forests (CPCSEA), with permission number 1149/ac/07/CPCSEA by Institutional Animal Ethical Committee (IAEC).

Albino Swiss rat (both sex), 9-14 weeks of age weight range 200 to 280g were taken as per demand after the prior permission of the animal ethical committee. They were given pellet diet (Ashirwad Industries, Chandigarh, India) and water *Ad libidum* during the course of experimentation. Light cycle was automatically controlled (on at 7 am and off at 7 pm) i.e. 12 hr light-dark cycle. The room temperature and relative humidity was maintained at 24 ± 2°C and 55 ± 10% respectively, that was maintained before the experimentation. After 3-5 days acclimatization in animal house, rat was taken for experiment purpose. All animals were exposed only once to every experiment.

Dosing solutions

Dose for oral drug administration was prepared by transferring accurately weighed quantity in 0.2% (w/v) solution of sodium carboxymethyl cellulose, (Table 2).

Table 2: Detail of drug dose given to rat.

Drug	Dose
Rifampicin	10 mg/kg
Isoniazid	5 mg/kg
Rifampicin + Isoniazid	10 mg/kg + 5 mg/kg
Rifampicin + Piperine	10 mg/kg + 1 mg/kg
Isoniazid + Piperine	5 mg/kg + 1 mg/kg
Rifampicin + Isoniazid + Piperine	10 mg/kg + 5 mg/kg + 1 mg/kg

Administration route

Drug was administered via oral route with the help of rat cannula and syringe, corresponding to the body weight of respective animals.

Test groups

In the presence study six groups were selected having six rats in each group.

Group I: Rifampicin
Group II: Isoniazid
Group III: Rifampicin + isoniazid
Group IV: Rifampicin + piperine
Group V: Isoniazid + piperine
Group VI: Rifampicin + isoniazid + piperine

Sample collection and handling

Blood was obtained by excising retro-orbital plexus under anesthesia with diethyl ether at the time interval of 30 min, 1hr 30 min, 2 hrs, 4 hrs, 6 hrs, 9 hrs and 12 hrs. 10% EDTA solution (100 µL per tube) was used as an anticoagulant. Intraperitoneal injections of dextrose (250 µL) were given to rats after collection of each blood sample to minimize changes in volume of the central

compartment. Plasma was obtained after centrifugation of blood samples at 5000 rpm for 10 min.

Recovery of drug from the plasma matrix

A fixed amount of plasma (250 µL) was transferred in test tube and 4 mL chloroform was added to them. Each sample was vortexed on vortex mixer for 2 min. These extracted samples were centrifuged at 3000 rpm for 10 min, finally organic layer collected in pre-labeled test tubes. These tubes were evaporated to dryness by using solvent evaporator.

Analysis of samples

Residue left in each tube was reconstituted with mobile phase which was prepared in degassed mixture of buffer and acetonitrile in the ratio of 30:70 (v/v). The sample was mixed by vortex shaker for a minute. Then it was filtered through 0.22 µm syringe filters. All the samples were analyzed by using HPLC apparatus with UV detection.

Data analysis

After oral administration, the statistical analysis of observed pharmacokinetic parameters was determined by performing unpaired t-test (Graph Pad, Instat 3.0) to determine the level of significance between two groups. $P < 0.05$ was considered as the level of significance.

Pharmacokinetics

Concentration-time curve was established for RIF, INH, INH + RIF, RIF + PIP, INH + PIP and INH + RIF + PIP from the treated rat (group I to VI), and used for the determination of pharmacokinetic parameters such as peak plasma concentration (C_{max}), peak time (T_{max}), extent of absorption (AUC), half-life ($t_{1/2}$), and elimination rate constant (K_{el}) were determined by using software 'PK Solution', a non-compartmental analysis.

METHOD VALIDATION

The proposed method was validated according to the ICH guidelines (ICH Q2A 1995 and ICH Q2B 1996), in terms of linearity, specificity, suitability, accuracy, precision and stability.

Linearity and calibration curve

The linearity of the calibration curve was determined by regression equation and correlation coefficient (r^2). The calibration curve was constructed by plotting curve between drug concentration in plasma and average peak area of plasma samples (n = 3).

Range

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the results (e.g., percentage, parts per million) obtained by the analytical method.

Selectivity and specificity

The term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that provides response for a number of chemical studies that may or may not be distinguished from each other. If the response is distinguished from all other response, the method is said to be selective. Three different drug free plasma samples obtained from apparently selected animal rat were used to evaluate the selectivity of the developed method. This was done by investigating the potential interference of blank plasma peak in the drug peak area.

Accuracy and recovery

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. Accuracy can also described as the closeness of agreement between the value that is adopted, either as a conventional, true or accepted reference value and the value found.

The accuracy of the method was determined by selecting three (3) known concentrations of each analyte in plasma in the linearity range. The % recovery within day (intraday) and interday, with standard deviations (±SD) was calculated to know the accuracy of method.

Precision and reproducibility

The precision of a method is the extent to which the individual test results of multiple injections of a series of standard agree. The measured standard deviation can be subdivided into 3 categories: repeatability, intermediate precision and reproducibility.

Repeatability of the method was determined by calculating % RSD of the repeated six-determinates of same 100% concentration of analyte drug in plasma. The intermediate precision was determined by selecting six different drug plasma samples by another analyst. The precision of the method was calculated by taking samples (6 replicates each in 3 sets) on the same day and on another day by calculating % bias from nominal concentrations (quality control samples).

$$\% \text{ Bias} = [(\text{mean measured conc.} - \text{nominal conc.})/\text{nominal conc.}] \times 100.$$

Sensitivity

Limit of detection

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. LOD is calculated by measuring signal-to-noise (S/N) of the baseline and multiplying this value by 3.

Limit of quantitation

The limit of quantitation is the minimum injected amount that produces quantitative measurements in the target matrix with acceptable precision in chromatography, typically requiring peak heights 10 times higher than the baseline noise i.e. LOQ is determinate by measuring signal-to-noise (S/N) and multiplying this value by 10.

Stability

Many solutes readily decompose in plasma prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an

automatic sampler).

The stability of analyte in plasma was investigated under the short-term and intermediate conditions. The short-term stability of analyte was investigated immediately and after 24 hrs at room and temperature at 10˚C. The intermediate study was planned for 1 month at low temperature. These conditions were;

(a) 24 hr storage of drug-plasma sample at room temperature (short term)

(b) 24 hr storage of drug-plasma sample at 10˚C in auto sampler (short term)

(c) 1 month storage of drug-plasma sample at −80˚C and (intermediate)

(d) 3 consecutive freeze-thaw cycles of drug-plasma sample from −80˚C to room temperature (intermediate).

After specified storage conditions, drug-plasma samples were analyzed by HPLC. The intra-assay and inter-assay accuracy (% recovery) of the method was determined at zero time and in successive intervals from mean measured concentrations and nominal concentrations.

RESULTS AND DISCUSSION

In the present study, the effect of PIP on pharmacokinetics of RIF and INH was explored. The study also explores the new liquid chromatographic method to analyze the RIF, INH, and PIP in plasma.

Due to better separation parameters of RP-HPLC technique, it has been frequently used in the analysis of biologically samples. To achieve the high assay value of drug the chromatographic condition were optimized in terms of mobile phase ratio, column temperature, flow rate and pH of inorganic mobile phase. As expected an increase of organic mobile phase from 10-25% resulted in decrease in retention time significantly and simultaneous increase in peak height. The temperature column in the range of 30-40˚C slightly influences retention parameters. Based on the results described above, we selected chromatographic condition as given in 2.4.

Analytical method

Linearity and range

The calibration curve of RIF (Figure 4), INH (Figure 5), and PIP (Figure 6), were found satisfactorily in the range of 0.156-640 µg/mL. The linear regression equation for the analyte were found Y = 21609X + 16628, Y = 23801X − 45739 and Y = 21609X + 16628. The linear regression data for above analyte were found good because of showing high linear regression coefficient (r2) that were the more than 0.999, proves the linearity of calibration curve over the high range, (the regression coefficient was demonstrated fit for purpose).

Selectivity and specificity

The developed method was found to specific because none of the plasma components was interfered with the chromatograph of RIF, INH or PIP. It proves the specificity of the developed method.

In an another test the separated and eluted sharp and symmetric peaks of RIF, INH and PIP was found with an average retention time of 7.59, 5.99 and 11.25 min. respectively. That proved the selectivity of the developed method.

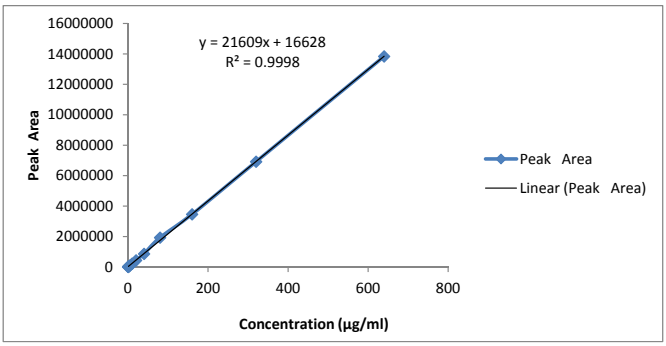

Fig. 4: Calibration plot of rifampicin.

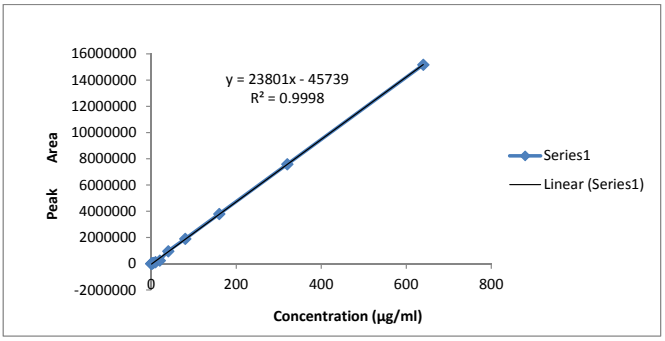

Fig. 5: Calibration plot of isoniazid.

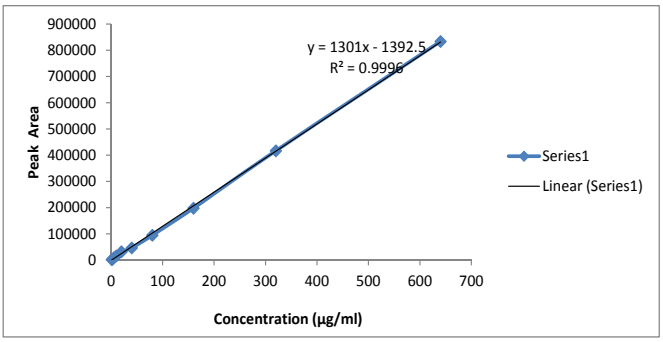

Fig. 6: Calibration plot of piperine.

Accuracy and recovery

Accuracy was determined by comparing the calculated concentration of the sample with the true concentration of rifampicin, isoniazid and piperine. The accuracy pertains to the extraction efficiency within the limit of variability. The accuracy of the method within inter and intraday was in ranged from 85 to 92.20%, that showed great back-calculation within the range (80-120% according to ICH guidelines), Table 3a and 3b.

Precision and reproducibility

Precision and reproducibility was given as inter and intra-day variations analyzed by three different concentration of rifampicin, isoniazid and piperine. The results were within the specified limit i.e., variation in recovery was around ±15%, that proves the considerable degree of precision and reproducibility, Table 4.

Table 3a: Intra-day accuracy data of rifampicin, isoniazid and piperine.

Drug	Nominal conc. (µg/mL)	†Intra-day accuracy			% Accuracy
		Set 1	Set 2	Set 3	Average
RIF	0.4	0.35 ± 0.03	0.33 ± 0.02	0.37 ± 0.04	87.50
	250	221 ± 4.65	235 ± 6.93	229 ± 5.34	91.30
	500	471 ± 9.03	463 ± 10.84	449 ± 10.44	92.20
INH	0.4	0.37 ± 0.01	0.39 ± 0.01	0.34 ± 0.02	90.66
	75	62 ± 2.13	69 ± 2.54	64 ± 2.46	86.65
	150	141 ± 5.33	121 ± 5.87	128 ± 6.12	86.66
PIP	3	2.88 ± 0.12	2.76 ± 0.15	2.53 ± 0.18	90.77
	250	236 ± 7.30	227 ± 7.15	223 ± 7.52	91.46
	500	458 ± 10.43	473 ± 12.22	453 ± 11.36	92.20

†Average of three (n = 3), RIF- Rifampicin, INH- Isoniazid, PIP- Piperine.

Table 3b: Inter day accuracy data of rifampicin, isoniazid and piperine.

Drug	Nominal conc. (µg/mL)	Inter day accuracy			% Accuracy
		Set 1	Set 2	Set 3	Average
RIF	0.4	0.33 ± 0.02	0.35 ± 0.01	0.34 ± 0.02	85.00
	250	225 ± 5.94	231 ± 6.21	230 ± 6.32	91.63
	500	462 ± 10.54	452 ± 11.73	443 ± 10.52	90.46
INH	0.4	0.34 ± 0.01	0.35 ± 0.01	0.33 ± 0.01	85.16
	75	61 ± 2.79	65 ± 2.86	69 ± 3.14	86.65
	150	133 ± 6.15	129 ± 6.91	121 ± 6.88	85.00
PIP	3	2.74 ± 0.17	2.79 ± 0.23	2.57 ± 0.19	91.90
	250	229 ± 7.97	221 ± 8.20	219 ± 8.11	89.20
	500	447 ± 11.31	463 ± 13.20	449 ± 14.3	89.60

†Average of three (n = 3), RIF- Rifampicin, INH- Isoniazid, PIP- Piperine.

Table 4: Inter and intra-day precision data of rifampicin, isoniazid and piperine.

Drug	Nominal conc. (µg/mL)	Intra-day precision				Inter day precision			
		*Percentage variation			% Precision (†Average)	Percentage variation			% Precision (†Average)
		Set 1	Set 2	Set 3		Set 1	Set 2	Set 3	
RIF	0.4	8.57	6.06	9.18	7.93%	4.24	2.85	6.17	4.42%
	250	2.10	2.94	2.33	2.45%	2.64	2.68	2.74	2.68%
	500	1.90	2.30	2.32	2.16%	2.28	2.59	2.37	2.41%
INH	0.4	2.75	4.60	3.82	3.72%	2.90	3.40	3.31	3.20%
	75	3.43	3.60	3.84	3.62%	4.50	4.40	4.50	4.41%
	150	3.78	4.85	4.78	4.50%	4.62	5.35	5.68	5.20%
PIP	3	4.00	5.43	7.10	5.51%	6.20	8.20	7.30	7.20%
	250	3.09	3.10	3.37	3.18%	3.48	3.71	3.70	3.63%
	500	2.27	2.58	2.50	2.45%	2.53	2.86	3.18	2.85%

†Average of three (n = 3), *Percentage variation- It is based on difference between standard nominal percentage conc. (100%) and test sample (set 1, 2 and 3), RIF- Rifampicin, INH- Isoniazid, PIP- Piperine.

Sensitivity

The sensitivity of the analytical technique was expressed as the limit of quantification, which is the minimum concentration of drug that can be quantitatively determined with a peak height to base line ratio of at least 10:1, and the limit of detection (LOD) as peak height to base line ratio of 3:1, Table 7. It concludes that the developed method was sufficient sensitive to identify and quantify the drug rifampicin, isoniazid and piperine.

Stability studies

The stability of analyte in plasma was investigated by LQC, MQC and HQC samples. The recovery of the analyte at time zero (initial concentration) was assumed 100%. The stability of analyte RIF, INH and PIP in ex-vivo at 0, 24 hr and 10˚C, −80˚C in plasma was tested. Under room temperature and at auto-sample site the residual percentage of analyte RIF, INH and PIP were not more than 15% (i.e. % recovery was more than 85%) indicating no stability problem, Table 5a.

In the intermediate stability the results corresponds to those obtained after zero time and 1 month, the recovery percentage was not less than 85% (i.e. residual percentage not more than 15%) proves stability of the analyte drug RIF, INH and PIP in plasma, Table 6b.

Table 5: Limit of detection and limit of quantitation of rifampicin, isoniazid and piperine.

Drug	LOD (µg/mL)	LOQ (µg/mL)
RIF	0.852	2.500
INH	0.689	2.102
PIP	0.756	2.224

RIF- Rifampicin, INH- Isoniazid, PIP- Piperine.

Application of method in study of pharmacokinetics

The above developed HPLC method was utilized to study the effect of PIP on pharmacokinetics of RIF and INH. In this view, six groups of albino rat, each having six rats, was taken to know the effect of PIP on drugs with reference to pharmacokinetical parameters. The drugs were given to each group of animal as given in Table 2. For validation purposes blank plasma were collected before the dose administration.

The different pharmacokinetic parameters of RIF and INH were given in Table 7 that tells about the high peak plasma concentration (C_{max}), peak time (T_{max}), extent of absorption (AUC), half-life ($t_{1/2}$), and low elimination rate constant (K_{el}) of RIF and INH respectively.

Table 6a: Stability studies of rifampicin, isoniazid and piperine at 24h room temperature and storage at 10°C in auto sampler.

Drug	Nominal conc. (µg/mL)	†Recovery (µg) after storage at room temperature			†Recovery (µg) after storage in auto-sampler at 10°C		
		0 hr	24 hr	% Stability††	0 hr	24 hr	% Stability††
	0.4	0.35 ± 0.03	0.33 ± 0.02	91.42%	0.35 ± 0.03	0.31 ± 0.02	88.50%
RIF	250	228.30 ± 4.65	225 ± 6.93	98.50%	228.30 ± 4.65	215 ± 5.94	94.17%
	500	461 ± 9.03	456 ±10.84	98.91%	461 ± 9.03	453 ± 10.54	98.26%
	0.4	0.37 ± 0.01	0.36 ± 0.02	92.20%	0.37 ± 0.01	0.36 ± 0.01	98.07%
INH	75	65 ± 2.13	62 ± 2.54	95.30%	65 ± 2.13	61 ± 2.79	93.84%
	150	130 ± 5.33	125 ± 5.87	96.15%	130 ± 5.33	126 ± 6.15	96.92%
	3	2.72 ± 0.12	2.65 ± 0.15	97.40%	2.72 ± 0.12	2.67 ± 0.17	98.16%
PIP	250	228.60 ± 7.30	227 ± 7.15	99.30%	228.60 ± 7.30	221 ± 7.97	96.6%
	500	461 ± 10.43	457 ± 12.22	99.2%	461 ± 10.43	455 ± 11.31	98.69%

†Average of three (n = 3), ††After 24 hr of time % stability, RIF- Rifampicin, INH- Isoniazid, PIP- Piperine. Note: At '0' hr (initial concentration) the recovery was assumed to be 100%.

Table 6b: Stability studies of rifampicin, isoniazid and piperine when stored at −80°C.

Drug	Nominal conc. (µg/mL)	†Recovery (µg) after storage (−80°C)		
		0 month	1 month	% Stability††
	0.4	0.35 ± 0.03	0.33 ± 0.02	94.20%
RIF	250	228.30 ± 4.65	227 ± 6.93	99.40%
	500	461 ± 9.03	456 ± 10.84	98.91%
	0.4	0.37 ± 0.01	0.36 ± 0.02	97.70%
INH	75	65 ± 2.13	60 ± 2.54	92.30%
	150	130 ± 5.33	121 ± 5.87	93.00%
	3	2.72 ± 0.12	2.65 ± 0.15	97.40%
PIP	250	228.60 ± 7.30	227 ± 7.15	99.30%
	500	461 ± 10.43	453 ± 12.22	98.20%

†Average of three (n = 3), ††After one month % stability, RIF- Rifampicin, INH- Isoniazid, PIP- Piperine. Note: At '0' month (initial concentration) the recovery was assumed to be 100%.

Table 6c: Stability studies of rifampicin, isoniazid and piperine after 3 freeze-thaw cycles when stored at −80°C.

Drug	Nominal conc. (µg/mL)	†Recovery (µg) after freeze-thaw cycles				
		Cycle 0	Cycle 1	Cycle 2	Cycle 3	% ¥Stability
	0.4	0.35 ± 0.03	0.33 ± 0.02	0.31 ± 0.01	0.32 ± 0.02	91.40%
RIF	250	228.30 ± 4.65	225 ± 5.94	221 ± 6.21	222 ± 6.32	97.53%
	500	461 ± 9.03	459 ± 10.54	452 ± 11.73	453 ± 10.52	98.62%
	0.4	0.37 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.33 ± 0.01	93.84%
INH	75	65 ± 2.13	61 ± 2.79	62 ± 2.86	59 ± 3.14	96.41%
	150	130 ± 5.33	123 ± 6.15	129 ± 6.91	121 ± 6.88	95.64%
	3	2.72 ± 0.12	2.71 ± 0.17	2.68 ± 0.23	2.57 ± 0.19	97.50%
PIP	250	228.60 ± 7.30	225 ± 7.97	221 ± 8.20	225 ± 8.11	97.80%
	500	461 ± 10.43	457 ± 11.31	455 ± 13.28	449 ± 14.30	98.40%

†Average of three (n = 3), ¥Average of cycle 0, 1, 2 and 3, RIF- Rifampicin, INH- Isoniazid, PIP- Piperine. Note: At '0' cycle (initial concentration) the recovery was assumed to be 100%.

Pharmacokinetics of rifampicin and isoniazid (combined administration-dose)

When the RIF and INH was given as combined administration-dose, the mean plasma concentrations of RIF with INH were lower as compared to RIF alone, at all time points (Figure9). The difference in AUC was found to be statistically significant ($p < 0.001$) when RIF was administered alone or in combination with INH. Further the C_{max} was also found to be reduced when it was administered in combination with INH. Relative bioavailability of RIF was found to be 67.19%, Table 8.

Table 7: Pharmacokinetics parameters of rifampicin and isoniazid (combined administration-dose).

Pharmacokinetic parameters	††Rifampicin		††Isoniazid	
	Alone	Combined administered dose	Alone	Combined administered dose
C_{max} (µg/mL)	8.85 ± 1.65	6.49 ± 1.75	8.05 ± 4.75	8.11 ± 1.82
Biological half life (h)	2.80 ± 0.42	2.04 ± 0.42	1.04 ± 0.22	1.02 ± 0.18
†Area under curve ($AUC_{0 to \infty}$)	40.36 ± 3.59	27.12 ± 4.59	24.77 ± 3.59	27.03 ± 3.88
T_{max} (h)	2.12 ± 0.48	2.20 ± 0.63	0.50 ± 0.04	0.50 ± 0.08
K_{el} (h^{-1})	0.24 ± 0.02	0.33 ± 0.04	0.66 ± 0.07	0.67 ± 0.09

†Unit of area under curve (µg.h/mL), ††Average of six (n = 6).

Table 8: Pharmacokinetics parameters of rifampicin and piperine (combined administration dose).

Pharmacokinetics parameters	††Rifampicin	††Rifampicin + Piperine
C_{max} (µg/mL)	8.85 ± 1.75	13.63 ± 1.82
Biological half life (h)	2.80 ± 0.42	3.09 ± 0.28
†Area under curve ($AUC_{0 to \infty}$)	40.36 ± 3.59	58.03 ± 3.88
T_{max} (h)	2.20 ± 0.63	2.42 ± 0.78
K_{el} (h^{-1})	0.24 ± 0.02	0.23 ± 0.09

Table 9: Pharmacokinetics parameters of isoniazid and piperine (combined administration dose).

Pharmacokinetics Parameters	††Isoniazid	††Isoniazid + Piperine
C_{max} (µg/mL)	8.40 ± 0.47	5.33 ± 1.02
Biological half life (h)	1.40 ± 0.38	1.20 ± 0.26
Area under curve, ($AUC_{0 to \infty}$)	24.77 ± 4.59	13.36 ± 3.78
T_{max} (h)	0.50 ± 0.08	0.50 ± 0.05
K_{el} (h^{-1})	0.66 ± 0.22	0.66 ± 0.09

†Unit of area under curve (µg.h/mL), ††Average of six (n = 6).

Table 10: Pharmacokinetics parameters of rifampicin, isoniazid and piperine (combined administration dose).

Pharmacokinetics parameters	††Isoniazid		††Rifampicin	
	Alone	Combined administered dose	Alone	Combined administered dose
C_{max} (µg/mL)	8.05 ± 4.75	5.35 ± 2.75	8.85 ± 1.65	13.63 ± 0.82
Biological half life (h)	1.04 ± 0.22	1.05 ± 0.42	2.80 ± 0.42	3.09 ± 0.28
†Area under curve ($AUC_{0-\infty}$)	24.77 ± 3.59	13.36 ± 2.59	40.36 ± 3.59	58.03 ± 3.78
T_{max} (h)	0.50 ± 0.04	2.20 ± 0.63	2.12 ± 0.48	2.42 ± 0.78
K_{el} (h^{-1})	0.66 ± 0.07	0.66 ± 0.12	0.24 ± 0.02	0.22 ± 0.09

†Unit of area under curve (µg.h/mL), ††Average of six (n = 6).

Pharmacokinetics of rifampicin and piperine (combined administration-dose)

The mean plasma concentrations of RIF were found to be higher with PIP as compared to the administration of RIF alone (Figure 10). The difference was statistically significant at 0.5, 1, 1.5, 2 and 4 hr. Table 9 compares various pharmacokinetic parameters of RIF when it was administered alone and in combination with PIP. Further C_{max} of RIF was found to be significantly higher when administered along with PIP ($p < 0.001$). Relative bioavailability of INH was found to be 141.7% when it was co-administered with PIP.

Pharmacokinetics of isoniazid and piperine (combined administration dose)

The mean plasma concentrations of INH was found to be lower with PIP as compared to the administration of INH alone (Figure 12). The difference was statistically significant at 0.5, 1, 1.5, 2 and 4 hr. Table 10, compares various pharmacokinetic parameters of INH when it was administered alone and in combination with PIP. Further C_{max} of INH was found to be significantly lower when administered along with PIP ($p < 0.001$). Relative bioavailability of INH was found to be 53.9% when it was co-administered with PIP.

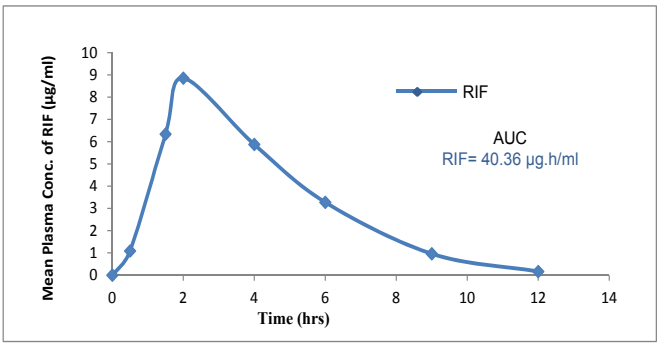

Fig. 7: Mean plasma concentration of rifampicin administered a dose of 10 mg/kg by oral route.

Pharmacokinetics of rifampicin, isoniazid and piperine (combined administration dose)

Plasma concentration was reduced when INH was administered in combination with PIP ($p < 0.001$). Further plasma concentration of RIF was enhanced when it was co-administered with PIP ($p < 0.001$). However Cmax of RIF when administered with PIP was found to be significantly enhanced ($p < 0.001$) when compare to RIF alone. Relative bioavailability of RIF was found to be 141.7%. C_{max} of INH was found to be reduced when it was

co-administered with PIP ($p < 0.001$). Relative bioavailability of INH was observed to be 53.9% when it was co-administered with PIP, Table 11.

CONCLUSION

In this study an effective RP-HPLC method was developed for determination of RIF, INH and PIP with high accuracy and precision. Thus, it can be concluded that the proposed method was sufficiently sensitive, reproducible and specific for analysis of RIF, INH and PIP in biological samples or plasma. The proposed method was also validated by evaluating different parameter according to ICH guidelines like specificity, sensitivity, accuracy, precision, and stability of the analyte.

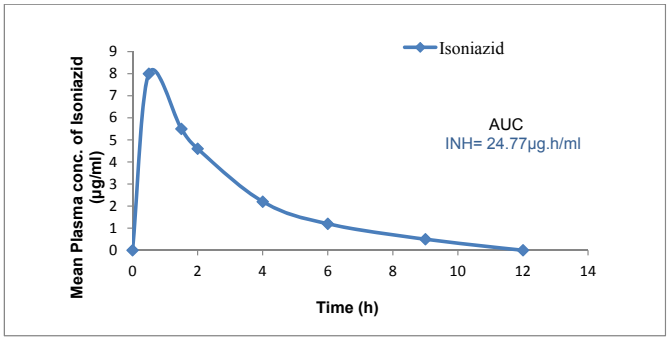

Fig. 8: Mean plasma concentration of isoniazid administered a dose of 5 mg/kg by oral route.

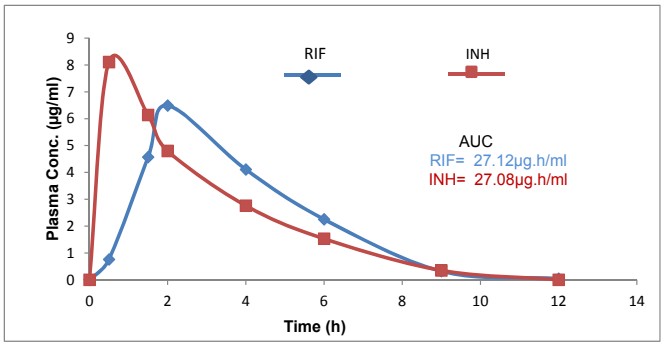

Fig. 9: Mean plasma concentration of rifampicin administered a dose of 10 mg/kg and isoniazid 5 mg/kg by oral route (combined administration dose).

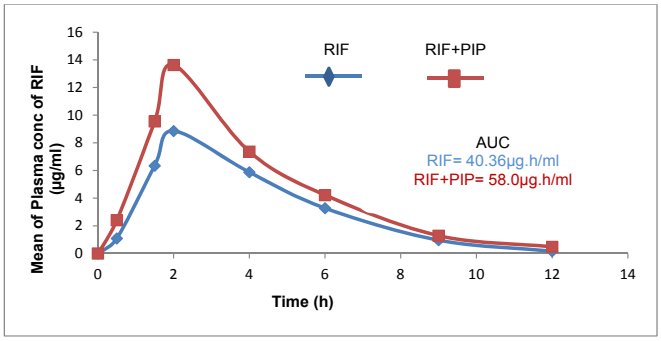

Fig. 10: Mean plasma concentration of rifampicin administered a dose of 10 mg/kg and piperine 1 mg/kg by oral route (combined administration dose).

Pharmacokinetic profile indicates that the PIP enhanced the rate and extent of absorption of RIF ($P < 0.001$). The observed pharmacokinetic data after oral administration of RIF along with PIP indicate a significant enhancement in C_{max} (8.85 ± 1.75 to 13.62 ± 1.82) and AUC (40.36 ± 3.59 to 58.03 ± 3.88). However, the plasma elimination half life were found to be similar which is likely due to the reduced gastrointestinal absorption of RIF caused by INH and degradation of RIF and degradation of RIF in presence of INH. Unfortunately, the oral bioavailability of INH was reduced when it was co-administered with PIP. The observed pharmacokinetic profile found to be reduced C_{max} (8.4 ± 0.47 to 5.328 ± 113.36 ± 3.78.02) and AUC (24.77 ± 4.59 to 58.03 ± 3.78) and biological half life (1.04 ± 0.1 h). The possible reason for decline may be the PIP antagonizes effects of acetylcholine on isolated ileum result of delay in gastric empty rate and delay the exposure of INH to vast absorptive surface of the small intestine.

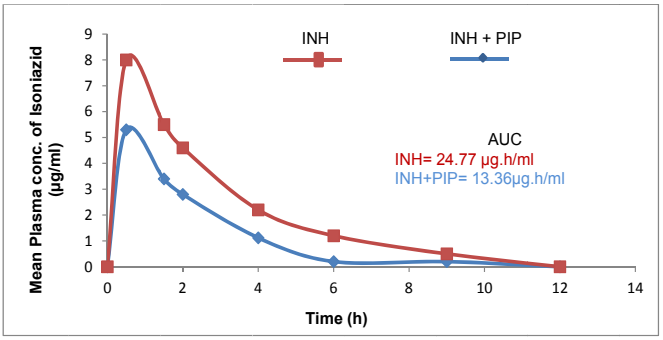

Fig. 11: Mean plasma concentration of isoniazid administered a dose of 5 mg/kg and piperine 1 mg/kg by oral route (combined administration dose).

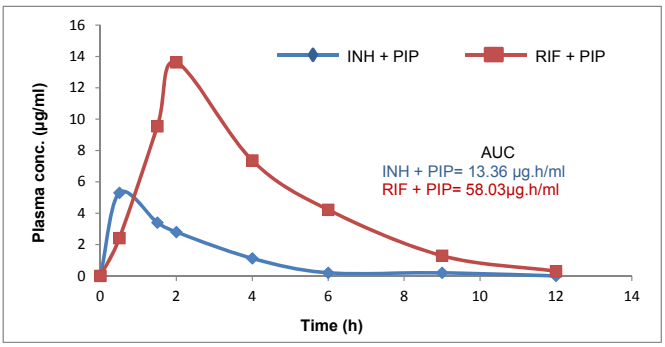

Fig. 12: Mean plasma concentration of rifampicin administered a dose of 10 mg/kg, isoniazid 5 mg/kg and piperine 1 mg/kg by oral route (combined administration dose).

DECLARATION OF INTEREST

The authors confirm that this article contents and writing of the paper has no conflicts of interest.

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