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Bioequivalence of isoniazid in a two drug fixed dose combination and in a single drug dosage form

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To increase the patient compliance and reduce the risk of drug resistant strains, WHO and IUATLD recommend the use of Fixed Dose Combination (FDC) tablets as a routine therapeutic regimen in Directly Observed Treatment Shortcourse (DOTS). But the main issue in the use of FDC is the quality of the formulation. At present WHO and IUATLD suggest the bioequivalence assessment of only rifampicin from FDC compared to separate formulations. For the therapeutic effectiveness all the components of the FDCs should be bioavailable at tissue site. Also, the primary and acquired resistance rate of isoniazid is much higher compared to other anti-tubercular drugs. Hence, a comparative bioavailability study of isoniazid from a two drugs FDC compared to a separate formulation was carried out on a group of 12 healthy volunteers. When evaluated by normal or log transformed confidence interval, Two Way ANOVA and Hauschke analysis, the bioequivalence limits for AUC_{0-8} and AUC_{0-24} were within 0.8–1.25. For C_{max} and T_{max}, these limits were within 0.7–1.43. Hence, isoniazid from a FDC formulation was found to be bioequivalent to a separate formulation at same dose levels.

1. Introduction

In 1993, the World Health Organization (WHO) declared TB as a 'global emergency' because more than 1900 million people are infected with this organism [1].

As *Mycobacterium tuberculosis* is a slowly growing organism, treatment requires at least six months therapy comprising of multiple drugs. To prevent disease transmission and retard the emergence of drug resistance, WHO recommends a standard six months treatment program consisting of rifampicin (RIF), isoniazid (INH), pyrazinamide (PYZ) and ethambutol (ETB) for initial two months followed by RIF and INH for additional four months [2]. However, in practice, because of poor patient compliance, patients tend to take only one drug (monotherapy) thereby promoting drug resistance.

A simple approach to reduce the incidence of monotherapy is the use of Fixed Dose Combinations (FDC) containing two or more first line anti-TB drugs in a single dosage form. Some of the advantages of FDC are a reduced emergence of drug resistant organisms, less risk of medication errors, better patient compliance, reduced cost of treatment, simplify drug supply management, shipping and distribution. Thus, WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) recommend the use of FDCs; also, FDCs form a part of WHO model list of essential drugs [3].

But the major challenge in using FDC formulation is to ensure that only FDCs of good quality are used [4]. It has been shown that the bioavailability of RIF in FDCs is at risk if manufacturing procedures are not followed strictly or poor quality of raw materials is used. WHO and IUATLD advocate the bioequivalence determination of only RIF because of the varied bioavailability reported with this drug [5]. However, for effective therapy, it is necessary that all the components of FDCs are bioavailable at the tissue site. INH, a main component of two drugs FDC exhibits a wide variability as well. Plasma concentrations and clearance are related to hereditary differences in acetylator status and an appreciable 'first pass effect' [6]. The rate of primary and acquired resistance of INH in smear positive patients is much higher compared to other components of anti-TB therapy [7]. Hence, it is necessary to look into the bioavailability of INH in FDC in order to justify the use of FDCs as a routine therapeutic regimen for TB.

2. Investigations, results and discussion

The stability of stock solutions of INH and PYZ in water was studied both by UV spectrophotometry and HPLC. It was found that there was no change in UV spectrum during four weeks, which was further substantiated by the absence of any additional peaks and no change in the retention times in HPLC. Therefore the stock solutions were used within four weeks when stored under refrigeration.

Because of the highly polar nature of INH, the resolution from plasma artifacts is difficult. To overcome this problem, a method described by Gupta [8] was modified which involved the derivatization of INH with p-hydroxy benzaldehyde. This resulted in the formation of a hydrazone, which is more hydrophobic from plasma artifacts. PYZ at a concentration of 20 μg/ml was used as an internal standard. As shown in Fig. 1, the INH hydrazone and PYZ were eluted within a maximum run time of 20 min with retention times of 10.2 and 6.1, respectively. No interference was found from the metabolites or ascorbic acid added to prevent oxidation of the drugs. The mean recoveries of INH and PYZ from plasma were found to be 79% and 85%, respectively.

Both INH hydrazone and PYZ are well separated from plasma artifacts. All the system suitability parameters as K prime, resolution, USP tailing and USP tangent were found to be acceptable. Based on the expected concentration levels in plasma, the standard curve was prepared in the range of 0.5–8 μg/ml. The correlation coefficient obtained was 0.9997.

The mean concentrations of INH as a function of time are shown in Fig. 2 for both combined and separate formulations. The concentration time profiles are very similar.

The pharmacokinetic parameters such as AUC_{0-8}, AUC_{0-24}, C_{max} and T_{max}, are listed in Table 1.

The lower and upper limits for the bioequivalence assessment of INH obtained by statistical evaluation of pharmacokinetic parameters at 90% confidence intervals are shown in Table 2.

With reference to AUC_{0-8} and AUC_{0-24}, the primary parameters for bioequivalence, formulations were found bioequivalent (within 80–125% of the separate formulation) for INH by all the statistical tests. In the case of C_{max} and T_{max}, the bioequivalence parameters also were within the acceptable limits of 0.7–1.43.

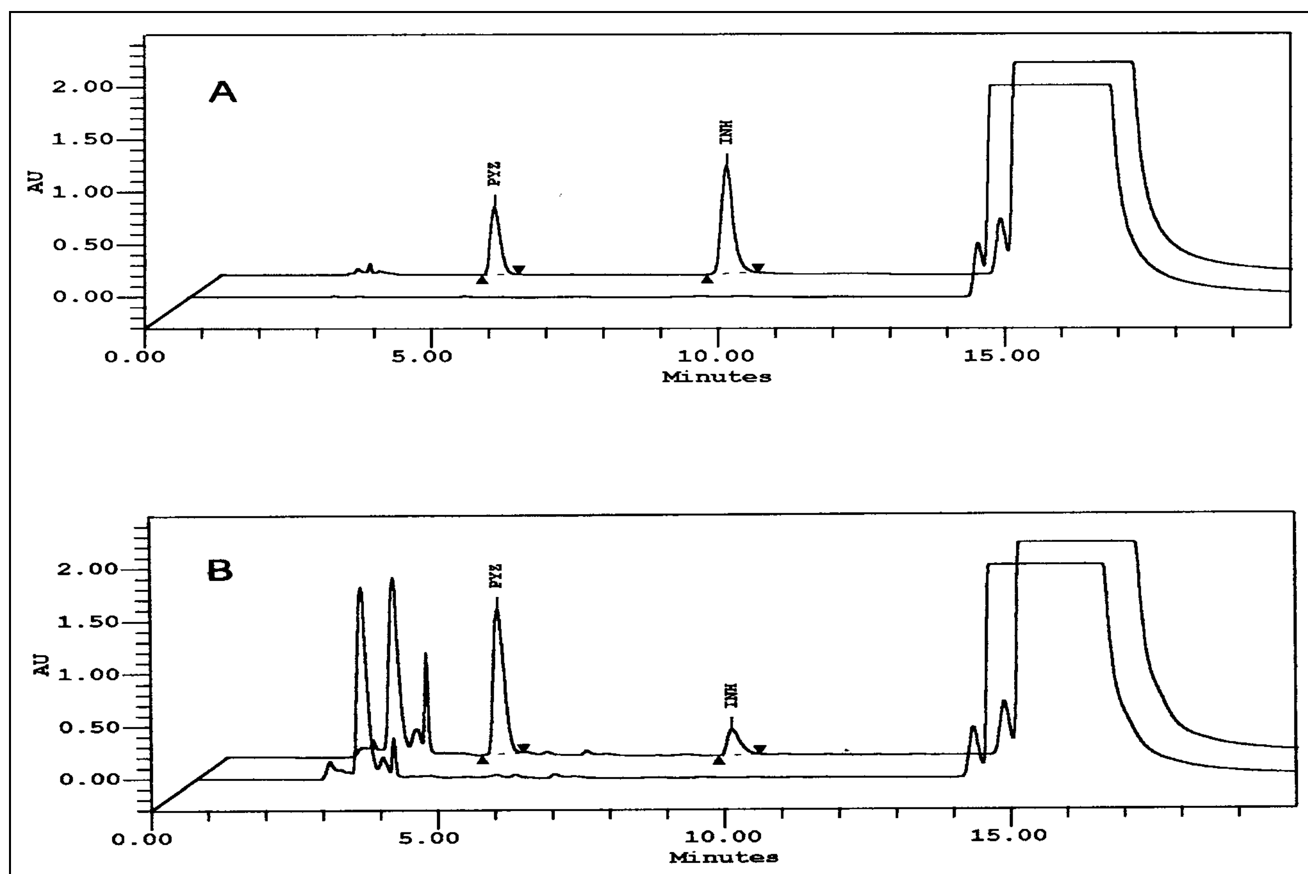

Fig. 1: A: Chromatograms representing reagent blank and standard drugs processed B: Chromatograms representing plasma blank and drugs in plasma processed

Table 1: Pharmacokinetic parameters of INH determined from concentration time profile

Vol. No.	Sequence	T_{max} (h) Combined	T_{max} (h) Separate	C_{max} (μg/ml) Combined	C_{max} (μg/ml) Separate	AUC_{0-8} (μg/ml).h Combined	AUC_{0-8} (μg/ml).h Separate	AUC_{0-24} (μg/ml).h Combined	AUC_{0-24} (μg/ml).h Separate
1	CS	2	1	4.5	6.2	20.97	31.33	25.73	34.95
2	SC	1	1	8.72	7.5	31.69	32.82	38.71	39.2
3	SC	2	1	4.38	4.83	14.815	14.75	15.815	16.36
4	CS	1	2	4.93	5.9	22.56	26.48	26.08	34.36
5	SC	1	1	4.73	4.55	20.26	18.83	24.54	21.33
6	CS	1	1	4.14	5.1	11.18	16.895	11.96	30.695
7	SC	1	1	5.94	6.38	27.65	21.625	33.47	23.357
8	SC	1	1	6.03	5.07	23.815	25.145	25.895	27.585
9	CS	1	2	4.24	5.13	21.56	22.37	23.9	28.71
10	CS	1	1	6.15	7.07	22.71	28.54	25.57	30.6
11	CS	1	1	3.57	4.15	12.91	12.605	13.65	12.925
12	SC	1	1	5.68	5.09	22.395	22.93	28.535	29.03
Mean		1.17	1.17	5.25	5.58	21.04	22.86	24.49	26.59
SD		0.39	0.39	1.38	1.03	5.84	6.36	7.73	7.87
CV		33.36	33.36	26.24	18.43	27.74	27.84	31.55	29.59

Table 2: Pharmacokinetic parameters of INH for bioequivalence estimation of combined and separate formulations at 90% confidence interval based on ratios of combined/separate

Pharmacokinetic Parameter	Normal LL	Normal UL	Log Transformed LL	Log Transformed UL	Parametric LL	Parametric UL	Non Parametric LL	Non Parametric UL
AUC(0–8)	0.8665	1.0020	0.8570	0.9950	0.8510	0.9760	0.9998	1.2210
AUC(0–24)	0.8520	1.0240	0.8350	1.0000	0.8480	0.9800	0.9815	1.1847
C_{max}	0.8807	0.9993	0.8750	1.0070	0.8950	0.9790	1.0150	1.3999
T_{max}	0.8880	1.2780	0.8480	1.1800	0.7968	1.2031	0.7071	1.414

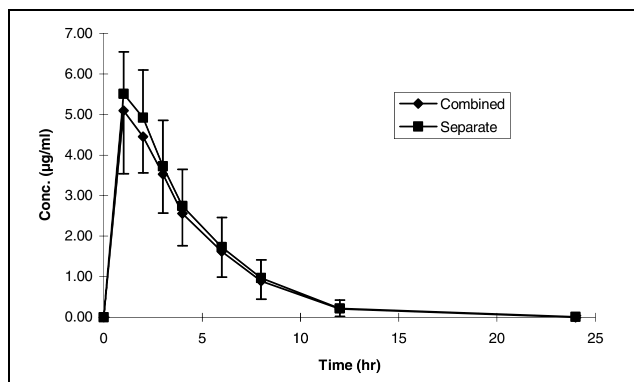

Fig. 2: Concentration-time profiles of INH in plasma, obtained from the bioequivalence study of combined and separate formulations, conducted in 12 healthy volunteers
(Mean +/− SD, n = 12)

An earlier study [10] showed that RIF was bioequivalent in this fixed dose formulation. Now the bioequivalency of INH has also been proven. This confirms the fact that there is no negative pharmacokinetic interaction between the two drugs when administered separately or in fixed dose combinations.

As both the components of two drugs FDC were found to be bioequivalent, this FDC can be used as a routine therapeutic regimen to increase patient compliance and to reduce the emergence of drug resistant strains.

However, further studies are required with PYZ and ETB.

3. Experimental

3.1. Conduction of bioequivalence study

The clinical trial for the assessment and comparison of the bioavailability of INH and RIF in separate or combined formulation was conducted at NIPER Bioavailability Center after approval of the NIPER Ethical Committee.

3.1.1. Design of bioequivalence study

The trial was designed as an open, randomized, crossover clinical study.

3.1.2. Recruitment of volunteers

Twelve healthy male volunteers [11] were included in the study after screening on the basis of following inclusion parameters.
i) Weight not less than 50 kg, ii) age between 18–50 years, iii) physical examination, iv) medical history, and v) laboratory tests (hematology, blood biochemistry and urine analysis) vi) refrain from drug abuse, alcohol or cigarettes.

3.1.3. Dosing schedule

The test (combined) and reference (separate) formulations at the same dose levels were given to the volunteers on two occasions separated by a washout period of one week. On one occasion standard separate daily drug formulations of INH and RIF were given, while on the other occasion the corresponding combined formulation of the two drugs were given to the volunteers. The formulations contained 450 mg of RIF and 300 mg of INH either in a combined formulation or as separate formulations. Out of 12 volunteers, 6 were randomly allocated to ingest the combined formulation first, while other 6 were initially given separate formulations of the individual drugs. On each occasion the test dose was swallowed on an empty stomach after an overnight fast with a glass of water (200 ml). A light breakfast was provided only after 2 h in order to reduce the effect of food on drug absorption.

3.1.4. Collection of blood samples

Venous blood samples of 3 ml were collected immediately before the ingestion of each test drug dose, and exactly 1, 2, 3, 4, 6, 8, 12, and 24 h thereafter. The samples were collected in heparinized (10 IU/ml) tubes and centrifuged immediately to separate plasma. To prevent the oxidation of drugs, ascorbic acid (0.5 mg/ml) was added to plasma and stored at −20 °C until further analysis.

3.2. Determination of pharmacokinetic parameters of INH

3.2.1. Bioanalysis of INH

A HPLC method described by Gupta [9] was modified for the determination of INH in the presence of other anti-tubercular drugs.

3.2.1.1. Materials used

INH, RIF and PYZ were gift samples from Lupin Laboratories, Mumbai. All other reagents were either of HPLC or AR grade procured from Loba Chemie, Mumbai, E. Merck (India) Ltd., Mumbai, Sigma, Aldrich or Ranbaxy Laboratories Ltd., New Delhi. Triple distilled water, filtered through a 0.45 µm membrane filter, was used in all the experiments.

3.2.1.2. Instrumentation

Waters HPLC system (Milford, MA) consisting of two 510 pumps, 717 plus autosampler, and 486 tunable UV detector was used in the study. Millennium software (version 2.1, Waters) was used for data acquisition and processing. Other instruments used included Mettler electronic balance AG 245 (Greifensee, Switzerland), Branson 3210 sonicator (The Hague, The Netherlands), Heraeus Centrivac and Biofuge-13 (Hanau, Germany), Nichipet from Nichiryo (Tokyo, Japan) and microlitre syringes from Hamilton (Bonaduz, Switzerland).

3.2.1.3. Column

A Spherisorb C-8 reversed phase column (250 × 4mm i.d., 4 µm), supplied by Waters Associates, linked to C-8 precolumn, was used.

3.2.1.4. Mobile phase

The mobile phase was comprised of methanol, water, 70% perchloric acid and 40% tetrabutyl ammonium hydroxide (2 : 8 : 0.005 : 0.0025, v/v). The mobile phase was filtered through a Millipore filter (pore size 0.45 µm) and sonicated for 30 min before use.

3.2.1.5. Sample preparation

Calibration stock solutions of INH and PYZ were prepared in water. Calibration stock of INH (1 mg/ml) was suitably diluted to give a working stock solution of 100 µg/ml and from this working stock solution; calibration standards were prepared in plasma (0.5–8 µg/ml of INH). The calibration concentration range for the drug was selected on the basis of its expected steady state concentration levels in plasma. PYZ was added as an internal standard to give a concentration of 20 µg/ml in plasma samples. Quality control samples were prepared by adding different amounts of INH to plasma to give concentrations within the calibration range.

3.2.1.6. Extraction procedure

A plasma sample of 0.5 ml was taken in 1.5 ml Eppendorf tubes. PYZ was added as an internal standard to give a concentration of 20 µg/ml of plasma and was vortexed for 30 s. 200 µl of 1.5% methanolic solution of *p*-hydroxy benzaldehyde and 400 µl of a10% aqueous solution of trifluoroacetic acid were added, vortexed for 2 min and centrifuged at 13000 rpm for 30 min. The supernatant (800 µl) was separated into another Eppendorf tube and vacuum dried. The residue obtained was reconstituted in 250 µl of mobile phase and centrifuged at 13000 rpm for 15 min. After centrifugation 200 µl of the supernatant was injected onto the HPLC column. Both the analytes were detected at 267 nm.

3.2.1.7. Analytical method validation

The developed analytical method was validated in terms of accuracy, precision, specificity, limit of detection, limit of quantification, linearity and range. The various system suitability parameters such as plate count, tailing factor, resolution, capacity factor and reproducibility were also determined.

3.2.2. Pharmacokinetic analysis of INH

AUC_{0-24} was calculated by the trapezoidal method; in 24 h almost all the plasma INH is excreted. In 8 h more than 85% of the INH is excreted hence AUC_{0-8} was also calculated. The C_{max} (the highest drug level measured) and T_{max} (the time to reach the highest concentration) were directly read from the concentration time plots.

3.2.3. Statistical analysis for the bioequivalence of INH

All the bioequivalence parameters (AUC_{0-8}, AUC_{0-24}, C_{max} and T_{max}) were evaluated by normal confidence interval, log transformed confidence interval, parametric (Two Way ANOVA) and nonparametric test (Hauschke analysis) at a 90% confidence interval.

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References

1 Sinder, D. E. Jr.; Kochi, A.: J. Am. Med. Assoc. **273**, 220 (1995)
2 Mather, D.; Chaulet, P.; Spinaci, S.; Harries, A.: Treatment of Tuberculosis: Guidelines for National Programmes, 2. Ed. World Health Organization. WHO/TB/97.220, Geneva, 1997
3 Fixed Dose Combination tablets for the treatment of tuberculosis. World Health Organization. WHO/CDS/CPC/TB/99.267, 1999
4 International Union Against Tuberculosis and Lung Disease/ World Health Organization: Tubercle Lung Dis. **75**, 180 (1994)
5 Fourie, B.; Pillai, G.; Mclleron, H.; Smith, P.; Panchagnula, R.; Ellard, G.: Establishing the bioequivalence of RIF in fixed dose formulations containing INH with or without PYZ and/or ETB compared to single drug reference preparations administered in loose combination (Model protocol), World health Organization, WHO/CDS/TB/99.274, 1999
6 Holdiness, M. R.: Clin. Pharmacokin. **9**, 511 (1984)
7 Bretzel, G.; Aziz, M.; Wendl-Richter, U.; Adatu, F.; Aisu, T.; van Wijnen, A. Sticht-Groh, V.: Int. J. Tuberc. Lung Dis. **3**, 810 (1999)
8 Gupta, R. N.: J. Chromatogr. Biomed. Appln. **425**, 441 (1988)
9 Accocella, G.; Nonis, A.; Gialdroni-Grassi, G.; Grassi, C.: Am. Rev. Respir. Dis. **138**, 882 (1988)
10 Panchagnula, R.; Kaur, K. J.; Singh, I.; Kaul, C. L.: Meth. Find. Exp. Clin. Pharmacol. **21**, 625 (1999)
11 Govt of India: Schedule Y: Requirement and guidelines on clinical trials for import and manufacture of new drug under Drugs and Cosmetic Rules, 1945 amended up to 1999

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