Pharmacokinetic evaluation of Inhalable microparticles in *Rhesus* Monkeys

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Introduction:

Several bacteria of the genus *Mycobacterium* have evolved to survive in lung macrophages of the mammalian host, leading to granulomatous or latent tuberculosis (TB) (Russell et al, 2007)**.** Chemotherapeutic regimens for TB require prolonged administration of multiple drugs through the oral route, but are not yet established to provide a lasting cure(Cox et al ,.2008) Isoniazid (INH), a first line drug, prodrug which needs to be activated by catalase-peroxidase enzyme katG to form isonicotinic acyl anion or radical. These forms react with a NADH radical or anion to form isonicotinic acyl-NADH complex and subsequently bind tightly to ketoenoylreductase known as InhA and prevents access of the natural enoyl-AcpM substrate and inhibits the synthesis of mycolic acid in the mycobacterial cell wall (Agrawal et al ,2004). Rifabutin (RFB), an analog of rifampicin, inhibits DNA-dependent RNA polymerase activity but does not inhibit the mammalian enzyme. Because of rapid emergence of resistant bacteria, its use is mainly restricted to treatment of mycobacterium infections and a few other indications (Arevalo et al, 2001).Current therapeutic regimens for treatment of tuberculosis have recognized drawbacks like the long duration of therapy and emergence of multidrug resistance due to non-compliance. The biopharmaceutics of both isoniazid and rifabutin are well-known; both in humans and mice, and good correlates have been observed between the two species in terms of both pharmacokinetic data and therapeutic outcome. This report addresses single-dose pharmacokinetics of isoniazid and rifabutin in *poly* (lactide) microparticles, administered as a dry powder inhalation to *Rhesus* monkeys in comparison to intravenous route.

Material and Methods:

Pure reference standards of INH and RFB (purity > 99%) were generously provided by Lupin Research Park Pune, India. HPLC grade acetonitrile (ACN), n-hexane and methanol (MeOH) were procured from Thomas Baker, USA. Dichloromethane (DCM) of HPLC grade was procured from Ranbaxy laboratories, SAS Nagar, India. Analytical grade potassium dihydrogen orthophosphate, orthophosphoric acid, triethylamine were procured from Qualigens Fine Chemicals, Mumbai, India. Glacial acetic acid and ammonium acetate GR were obtained form E. Merck Ltd., Mumbai, India. Triple distilled water (TDW) used for buffer preparation was prepared in-house using distillation assembly made up of Pyrex glass. Parafilm (Parafilm "M" laboratory Film, American Can Company, CT, USA) was used for sealing tubes.

Microparticles containing 1 part rifabutin, 1 part isoniazid and two parts *poly* (D, L-lactic acid) of intrinsic viscosity 0.8 as well as free drugs were donated by Lupin Laboratories Research Park, Pune, India. Characterization has been done using particle size analyzer (Malvern 2000) for micro size determination (Fig 1) and dissolution apparatus (DISSO, Lab India) for the in-vitro drug release profile of the microparticles.The animal study procedures were approved by the Institutional Animal Ethics Committee of the Central Drug Research Institute, Lucknow, India. Monkeys were bred, housed and fed ethically in the animal house of the Institute. Monkeys were housed singly in standard cages in light controlled room. The pharmacokinetics studies were performed in group of 4 rhesus monkeys of either sex.

The HPLC system (Shimadzu, Kyoto, Japan) met all the specification for in-house developed and validated method (Kaur et al, 2008). RFB samples were eluted in a mobile phase of 50 mM Potassium dihydrogen orthophosphate (KH_2PO_4)-Acetonitrile (55:45) using an isocratic method at a flow rate of 1 ml/min. INH samples were eluted in a mobile phase of triethyl amine acetate (TEA) buffer (pH – 6.0) and acotenitrile (97:3) at a flow rate of 1 ml/min Column was equilibrated with mobile phase for 30 mins. INH and RFB were detected at λ_{max} of 262 and 275 nm, respectively. Column was equilibrated for approximately 30 min with initial condition before commencement of analysis and chromatography was carried out at ambient temperature. (Fig 2). In-house Inhalation chamber was fabricated and calibrated for the dose dependence and time dependence of the inhalable powder coming out of apparatus as exhaled microparticles. Calibration was done using reweighed cotton swabs (Kaur et al, 2007) and instrument is shown in Fig 3.

Four monkeys of group-I were used as control while, group-II animals received inhalation dose of microparticles as 10mg and 100mg using in-house apparatus. For group-II, 80 mg and 500 mg of drug loaded microparticles were charged respectively into in-house apparatus and aerosolized by pumping 30 actuations for 30 seconds, in order t get 10 mg and 100mg of microparticles inhaled. While dosing, monkeys were restrained and mask of in-house apparatus was kept on the nares, keeping mouth closed, and inhaled the aerosolized dry powder microparticles in a controlled manner under optimum pressure.Group-II monkeys (washing period 10 days) initially received inhalation were administered intravenous injection of combination of 2.5 mg comprising each of soluble rifabutin and isoniazid; Blood sampling was done at specified time interval. After wash out period of 10 days, animals received 25 mg each of rifabutin and isoniazid and blood sampling was done.

Sample processing and bio-analysis was done using the in-house developed and validated HPLC method separately for both the drugs using liquid-liquid extraction.(Kaur et al ,2007) Obtained amounts were calculated and PK data was calculated by WinNonLin ^{R} as shown in Table1 and 2. Non-compartmental mode is applied for comparison as inhalation data dose not show any type of compartmental model fitting in comparison to intravenous route which clearly shows two compartmental models in case of rhesus monkeys.

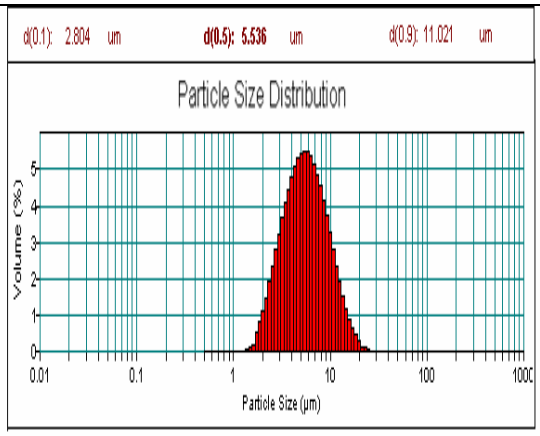

Fig. 1 Particle size analysis of formulated microparticles

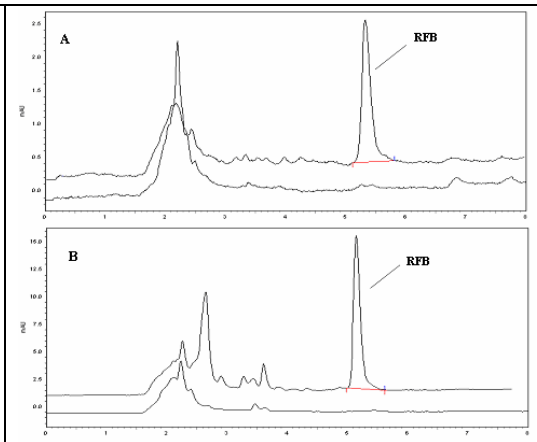

Fig 2. HPLC chromatogram for rifabutin in serum as biomatrics

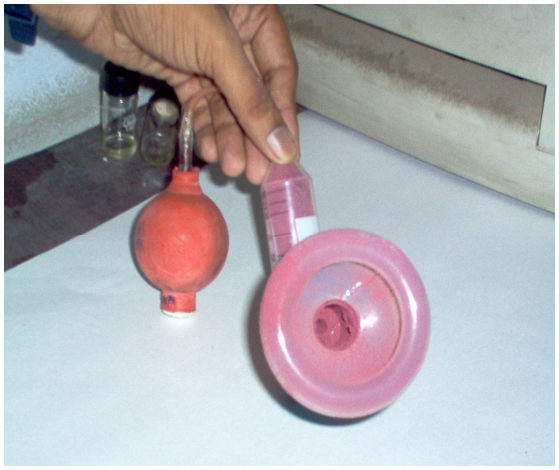

Fig 3 In-house fabricated apparatus used for inhalation dosing to monkeys

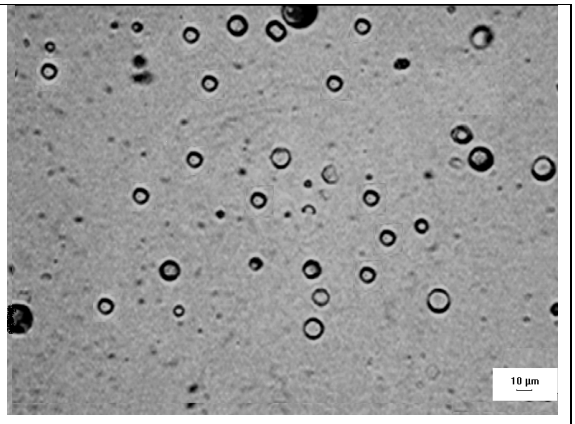

Fig 4 Microparticles under light microscope, 10X magnification. The bar indicates 10 µm.

Results and Discussion:

Pharmacokinetic parameters are well established in humans and mice for both the drugs. Inhalation route was evaluated by comparing pharmacokinetic parameters in *Rhesus* monkeys after single dose at two dose-levels, viz. 10 and 100 mg/monkey with intravenous (IV) administration. There is significant increase in area under the curve (AUC) for isoniazid and rifabutin, in both routes with increase in dose of drug. AUC value is more in case of intravenous route as comparison to inhalation route showing the sustained drug delivery system up to fixed time interval Maximum Concentration (C_{max}) achieved $_{for}$ rifabutin shows higher change with dose in case of intravenous route in comparison to inhalation route. Half life ($T_{1/2}$) for isoniazid and rifabutin shows major

ISONIAZID					
Inhalation Route					
Dose	**mg**	**10 mg dose**		**100 mg dose**	
		Mean	**SD**	**Mean**	**SD**
$T_{1/2}$	hr	4.869	0.104	10.443	0.518
C_{max}	ug/mL	0.926	0.151	3.116	0.098
AUC	hr*ug/mL	8.55	0.617	54.873	8.244
Vd	mL	8231.357	419.11	27900.73	4162.005
Cl	mL/hr	1172.634	84.589	1851.654	261.339
Intravenous Route					
T1/2	hr	3.502	0.816	8.919	0.853
Cmax	ug/mL	6.193	0.358	17.152	0.37
AUC	hr*ug/mL	17.164	0.0751	80.251	8.357
Vd	mL	2935.109	592.286	16211.951	2806.555
Cl	mL/hr	587.195	60.31	1256.856	138.192

Table1SelectedPharmacokinetic parameters of isoniazid through the intravenous and inhalation routes in the serum of monkeys

RIFABUTIN					
Inhalation Route					
Dose	**mg**	**10 mg dose**		**100 mg dose**	
		Mean	**SD**	**Mean**	**SD**
$T_{1/2}$	hr	9.206	0.253	13.811	0.833
C_{max}	ug/mL	0.726	0.056	3.164	0.269
AUC	hr*ug/mL	10.276	2.563	70.979	13.182
Vd	mL	12924.980	25.369	29092.581	4083.696
Cl	mL/hr	973.182	2.639	1472.041	283.037
Intravenous Route					
T1/2	hr	9.206	0.235	7.687	3.04
Cmax	ug/mL	0.726	0.056	17.152	5.869
AUC	hr*ug/mL	10.276	0.253	65.258	30.289
Vd	mL	12924.98	0.253	18.026	3.547
Cl	mL/hr	973.182	0.235	896.023	256.356

Table 2 Selected pharmacokinetic parameters of Rifabutin through the intravenous and inhalation routes in the serum of monkeys

difference in case of 10 mg dose and not for 100 mg dose .Half life change can be explained by auto-induction of enzymes responsible for metabolism of drugs. With increase in dose, induction will be more; results in more metabolisms of drugs and half life will be less in higher doses. Basic aim of study comprising two different doses of inhalation shows there is no major change in PK parameters responsible for the bio-analytical profile of drugs. Intravenous route is selected for comparison of various serum concentrations obtained by inhalation drug delivery systems as microparticles can not be administrated through IV route, so soluble drugs are selected for comparison purpose.PK parameters are shown in Table 1 (Isoniazid) and Table 2 (Rifabutin).

Conclusions: Various pharmacokinetics parameters obtained by inhalation and intravenous route shows a correlation with increase in level of various PK parameters with change of dose and also with change of route of administration also. Selection of animal is based on the pharmacokinetics data evaluation (by WinNonLin) of mice and rat as animal model (Muttil et al, 2007). Primate as animal model is selected to identify any type of toxicity and various metabolites of drugs at a specified time interval. In comparison to literature values of PK parameters using different animal models shows major difference in $T_{1/2}$ values and C_{max} values .Various PK parameters obtained by administering microparticles and soluble drugs reflects sustained drug delivery of both the drugs by inhalation route. Lesser values obtained in case of inhalation route in comparison to intravenous route shows targeting of these drugs to lungs which can be proved by analysis biodistribution of these microparticles size <10 µm (Fig 4) in various tissues such as lungs, liver and spleen. (Muttil et al, 2007).This PK study provides us sufficient information comprising difference of distribution of various drugs using non-compartmental mode of analysis.

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