evaluated pulmonary and systemic bioavailability of the two formulations of fluticasone propionate with and without volumatic spacer device under fasting conditions.

Materials and Methods

Volunteers

Healthy male subjects aged 18-45 years (body mass index ≥18.5 kg/m^2 and ≤25.00 kg/m^2), in good health participated in both the studies. All subjects had a Forced Expiratory Volume in One Second (FEV_1) ≥80% of predicted normal. The volunteers were assessed to be healthy based on physical examination, medical history, ECG, pulse oximetry, chest X-ray, and clinical laboratory test results prior to inclusion in the study.

Volunteers were excluded if they took prescription medications or over-the-counter products including herbal products within the 14 days prior to the study drug dosing and also during the study. Exclusion criteria included a history of drugs of abuse, heavy alcohol consumption, active smoking, and inability to use metered dose inhaler satisfactorily.

Informed consent and ethics approval

The studies were conducted in accordance with the Declaration of Helsinki, current ICH GCP guidelines, European Guidelines on bioequivalence, and the relevant national laws and regulations [14-18].

The study protocols were approved by an independent ethics committee before the start of the study procedures. All volunteers gave written informed consent voluntarily for participating in both the studies after they were explained about the purpose, procedures and anticipated risks of the study. Study 1 (without spacer) was conducted from November, 2011 to January, 2012 and study 2 (with spacer) from July, 2012 to September, 2012.

Study design

Both studies were similar in design (open-label, randomized, two-treatment, two-sequence, single dose, studies in healthy fasting volunteers), except that the study 1 (without spacer) was a replicate study (4-way crossover) and the study 2 (without spacer) was a two way-crossover study.

The treatment periods were separated by a washout period of at least 14 days in both the studies. The study treatments consisted of the test product fluticasone propionate HFA pMDI 250 mcg/actuation manufactured by Cipla ltd., India, and the reference product Flixotide® 250 Evohaler® (containing fluticasone propionate 250 mcg per actuation) manufactured by Glaxo Wellcome Production, France.

On check in day, at least 12 h prior to each dosing, all subjects were screened for drugs of abuse (cocaine, cannabinoids, benzodiazepines, Opioids, Amphetamines, and barbiturates) by urine test and for alcohol consumption by breath alcohol test. Additionally the, volunteers' belongings were thoroughly checked for any restricted items.

Study drug administration

The investigational products were primed within 10 min prior to dosing by releasing 2 test sprays into a cardboard box, away from the subjects. This activity was performed within 10 min prior to individual subject's dosing. After an overnight fast of at least 10 h, subjects self-administered a single dose of 1000 µg (250 µg per actuation × 4 puffs) of test product or reference product as per the randomized sequence in a standing position. In study 2, the subjects inhaled the dose with the aid of the volumatic spacer.

Dosing was performed under the supervision of a trained and qualified pharmacist, sponsor's monitor and quality assurance personnel. Both the test and reference formulations of fluticasone propionate HFA pMDI 250 mcg/actuation were stored in the pharmacy where the temperature and humidity conditions were monitored continuously till the completion of the study temperature conditions (22 ± 3°C) and relative humidity (50 to 60%).

Time of first puff was considered zero for all post-dose activities. Volunteers were vigorously trained on the standardized inhalation technique with the help of training aids and practice placebo inhalers before the dosing during each of the treatment period.

Volunteers were checked-in to the clinical unit at least 12 h prior to dosing on the previous day during each study treatment period, and they continued to stay at the clinical unit for a minimum of 24 h after the dosing. They stayed overnight for two consecutive days and two nights, and were provided standard meals at appropriate intervals during their stay. The volunteers refrained from consuming any food and beverages containing xanthine or alcohol 48 h before dosing and for 24 h after each dose, grapefruit 7 days before dosing and throughout the study, or vitamins throughout the confinement period.

The volunteers were instructed to be seated for at least the first 2 h after dosing and were provided standard meals post dose in all the treatment periods of the study. They were not permitted to drink water 1 h prior to dosing till 2 h after dosing. Safety evaluations included assessment of vital signs, clinical laboratory parameters and monitoring of adverse events.

Blood sampling

In both the studies, the pre-dose blood sample (5 mL) was followed by serial blood sampling (5 mL each) at 0.08, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 18.00, 24.00 and 36.00 h post dose. At each time point, blood samples were collected via an indwelling catheter (intra-venous) in vacutainers containing (dipotassium ethylene diamine tetraacetic acid) K_2EDTA anticoagulant. Plasma was harvested by centrifuging the blood samples at 3000 rpm for 10 min and was then divided in two portions (main and reserve). Then plasma samples were stored at -30°C or below until sample analysis.

Randomization and blinding

The volunteers were randomized to either of the treatment groups as per the randomization sequence generated using SAS® Version 9.2 software. Since, the study was open-label where investigators had knowledge of the formulation being administered to the volunteer, however, the analyst was blinded.

Analytical methods

Bio-analysis was performed using liquid chromatography-mass spectrometry (LC-MS)/MS based method. The bio-analytical method for estimation of fluticasone propionate from human plasma was developed and validated as per the international guidelines [19,20].

An aliquot of 500 µL of human plasma containing the analyte and the internal standard was extracted using solid phase extraction technique. The internal standard for fluticasone propionate assay was fluticasone D3. About 12.5 µL of the internal standard working solution were added to 500 µL of plasma sample. After vortexing the tubes,

500 µL of 30% Methanol solution was added and the tubes were again vortexed. This sample was transferred to a pre-conditioned Agilent Bond Elute C18 SPE cartridge. After loading the entire sample, low vacuum was applied to the cartridge till the entire sample has flowed out of the cartridge. Subsequently the cartridge was washed with 1 mL of 30% acetonitrile followed by 1 mL of water. After washing was complete, the analytes were eluted with 0.3 mL of 100% Acetonitrile under low vacuum. About 0.2 mL of this final extract is transferred to glass vial and to it 0.2 mL of ammonium trifluoroacetate buffer is added and vortexed. This extracted sample is then taken for analysis using LC-MS/MS.

The extracts were analysed on the LC-MS/MS system comprising of Schimadzu UFLC XR series HPLC and AB SCIEX Q TRAP 5500/ AB SCIEX TRIPLE QUAD5500 LC-MS/MS System using turbo ion spray source. Positive ions were monitored in the Multiple Reaction-Monitoring (MRM) mode. Following ion transitions using analyst 1.5.1 were monitored 501.20/293.20 and 504.20/313.20 for fluticasone propionate and internal standard respectively. Linearity for fluticasone propionate was assessed by plotting area ratios versus standard concentrations and using a linear regression weighted 1/concentration^2. The calibration standard ranges for fluticasone propionate for both the studies were 3.0-1000 pg/mL and 3.0-750 pg/mL respectively. The column used for the analysis is ACE 3 C18 3 µ 3.0 × 100 mm and the mobile phase composition was a mixture of acetonitrile and trifluro ammonium acetate buffer (65:35). The retention time of fluticasone propionate was 2.34 min and the acquisition time was 5.0 min.

Method validation was performed according to the current international approach and the applicable regulations regarding bio-analytical method validation. The intra-batch and inter-batch accuracy and precision was evaluated at five different concentrations of control samples. The inter-batch accuracy ranged from 99.77 to 106.67% and the inter-batch precision ranged from 1.15 to 6.10%. The selectivity of the method was assessed by analysing plasma samples from six sources. Matrix effect was evaluated by performing post-extraction addition and post-column infusion experiments. Stabilities such as stock solution stability, short-term stability of analyte in plasma, freeze-thaw stability, post-preparative stability and long-term stability in plasma were assessed. Fluticasone propionate is stable in plasma for 179 days when stored at -70 ± 10°C.

Incurred sample reanalysis was performed for both studies, and the percentage of samples meeting the acceptance criteria (±20%) was 100 and 96.50% respectively.

Pharmacokinetic analysis

The primary PK variables were maximum plasma concentration (C_{max}), and area under the plasma concentration curve from administration to last observed concentration at time t (AUC_{0-t}). The maximum plasma concentration (C_{max}) and the time until C_{max} (T_{max}) were taken directly from the plasma concentration time profiles of individual subjects. The area under the plasma concentration time curve (AUC_{0-t}) was calculated by the linear trapezoidal rule from measured data points from the time of administration until the time of the last quantifiable concentration. The other PK variables were area under the plasma concentration curve extrapolated to infinite time ($AUC_{0-\infty}$), residual area, half-life ($t_{1/2}$), and terminal elimination rate constant (Kel). The above PK parameters were calculated using validated PK software (WinNonlin version 5.3 for study-1; and WinNonlin version 6.3 for study-2).

These parameters were derived individually for each subject from the respective plasma concentrations analysed for fluticasone. The actual blood sampling time was used for the PK and statistical calculations. Concentrations below the lower limit of quantification were set to zero for all PK and statistical evaluation. A non-compartmental method was used to calculate the PK parameters using drug concentrations versus time profile.

Safety analysis

The safety assessment was based on recording Adverse Events (AEs) throughout the study durations. Assessment of vital signs through physical examination, laboratory parameters, and ECGs were performed at the time of screening and at the end of the study. Safety follow up visit was scheduled for individual subjects after 7 days but not later than 15 days of the last treatment period (or earlier if subject dropped out or discontinued early from study or as decided by investigator).

Statistical analysis

Statistical analysis in both the studies were performed using SAS® version 9.2 and above. In both the studies, Analysis of Variance (ANOVA) was performed for the PK endpoints, C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$. The analysis of variance model included sequence, subjects nested within sequence, period, formulation, and group as factors. Least square means (LSMs) for each parameter, derived from ANOVA, were then calculated for ratio analysis. Ratios of LSMs were calculated for both untransformed and log-transformed parameters; and 90% CI for the difference between formulations were calculated for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$. Wilcoxon signed rank test was used for T_{max} comparisons. Concentration values below the LOQ of the assay for fluticasone propionate (3.0 pg/mL for study-1 and 2) were set to zero. The 90% confidence interval of the ratio of C_{max}, and AUC_{0-t} is required to fall between 80.00-125.00% (transformed values) to conclude bioequivalence. For safety endpoints, descriptive statistics (number and percentage) was used.

The study without spacer was conducted in 28 healthy subjects while the study with spacer was conducted in 32 healthy subjects. The sample size calculation was based on the expected variances in each study. Considering an intra-subject variability of 36.9% for C_{max} of fluticasone propionate (data provided by Cipla Ltd.) for the study without spacer and assuming a test to reference ratio of 0.95, a minimum of 56 volunteers were required for two period cross-over study design to get 90% confidence interval (CI) within 80% to 125% range, with 90% power at significance level of 5% when calculated using SAS® Version 9.2. Therefore, 32 volunteers were recruited for the four period crossover replicate study design to account for any dropout or discontinued subjects if any during the study.

Considering an intra-subject variability of 22.61% for AUC_{0-t} of fluticasone propionate (data provided by Cipla Ltd.) for the study with spacer and a test to reference ratio of 1.0556, a minimum of 23 volunteers were required for two period cross-over study design to get 90% confidence interval (CI) within 80% to 125% range, with 90% power at significance level of 5% when calculated using SAS® Version 9.2. Therefore, 28 volunteers were recruited for two period crossover study design to account for any dropout or discontinued subjects if any during the study.

Results

Study population

The details of demographic characteristics of the study population included in the individual PK studies are mentioned in Table 1.

In study 1, 32 volunteers were randomised of which 28 volunteers completed the study. 3 volunteers discontinued from the study due to personal reasons and 1 volunteer was discontinued due to unacceptable inhaler technique. In study 2, 28 volunteers were randomised of which 25 volunteers completed the study. There were 3 volunteers who discontinued from study 2 due to personal reasons.

Safety

In study-1, 4 volunteers (12.50%) experienced an adverse event. In study-2, 8 volunteers (28.57%) experienced an adverse event. Summary of the adverse events is presented in Table 2.

All AEs were of mild to moderate severity and resolved completely without any sequalae. No serious adverse events were reported in both the studies. During vital signs examination, ECG and post study clinical laboratory data assessment, there were no clinically significant changes observed from baseline. All volunteers were medically fit in post-study safety assessment.

Pharmacokinetics and statistics

The mean plasma concentration profiles of fluticasone propionate over 36-h for study 1 and 2 are presented in Figures 1 and 2 respectively. In both the studies, the plasma profile curves between the test and reference product were comparable.

The statistical results of the primary pharmacokinetic parameters of fluticasone propionate for both the studies are presented in Table 3. In both the studies, the 90% CI for the ratio of the LSMs of test and reference products of ln-transformed data for both the primary parameters C_{max} and AUC_{0-t} were within the predefined bioequivalence range of 80% to 125% (Table 4). Hence, the bioequivalence criteria has been met for fluticasone with and without spacer. The mean extrapolated AUC values were found to be less than 20% for both the formulations in both the studies.

The median T_{max} for both test and reference was 1.00 h. in study-1 while the median T_{max} for test was 2.00 hr. and for reference was 1.50 h. in study-2. Overall, the T_{max} was comparable.

Discussion

In both the studies, bioequivalence was assessed between the test and reference formulations of fluticasone propionate HFA pMDI 250 mcg/actuation. It is challenging to demonstrate bioequivalence of inhaled drugs due to extremely low levels of drug concentration in the plasma which are not easily detectable. In both the studies conducted, sensitive bio-analytical method with an LLOQ of 3.0 pg/mL was used to ensure adequate PK profiling which was done until 36 h post dose. Standardized inhalation technique training across both the PK studies also minimized variability in the inhalation of the drug across the treatment periods.

The Orally Inhaled Product (OIP) guidelines acknowledge that pharmacokinetic studies are valid for demonstrating equivalent safety and efficacy of two OIPs. Equivalence in terms of efficacy is established via a PK systemic exposure equivalence study where charcoal is administered to block gastro-intestinal absorption so that only the exposure of the active pharmaceutical ingredient absorbed via the lung is compared. Equivalence in terms of safety is assessed via a PK systemic exposure equivalence study but where charcoal is not administered,

	Study-1				Study-2			
	Age (years)	Weight (kg)	Height (m)	BMI (Kg/m²)	Age (years)	Weight (kg)	Height (m)	BMI (k g/m$_2$)
Number of observations	32	32	32	32	28	28	28	28
Mean	27.56	63.5	1.69	22.3	29	60.7	1.66	22.1
Standard Deviation	4.86	7.3	0.05	2	6	6.2	0.04	1.8
Median	26.5	63	1.68	22.9	27	61	1.65	22.4
Minimum	21	52.9	1.58	18.7	21	51.2	1.56	18.8
Maximum	39	82.8	1.83	24.8	41	72.6	1.76	24.8

Table 1: The demographics of all recruited volunteers in Study-1 (fluticasone propionate HFA pMDI 250 mcg per actuation without a spacer device) and Study-2 (fluticasone propionate HFA pMDI 250 mcg per actuation with a spacer device) are summarized.

Adverse Event (Preferred Term)	Frequency (Percentage)	Relationship	Number of Adverse Events	
			Test product (T)	Reference product (R)
Study-1				
Injection site thrombosis	3.13%	Not Related	0	1
Abdominal pain	3.13%	Not Related	1	0
Hordeolum	3.13%	Not Related	0	1
Eyelid oedema	3.13%	Not Related	1	0
Study-2				
Nasopharyngitis	7.14%	Not Related	1	1
Pyrexia	3.57%	Not Related	1	0
Dizziness	3.57%	Not Related	0	1
Oropharyngeal pain	3.57%	Related	1	0
Cough	3.57%	Not Related	0	1
Abdominal pain	3.57%	Not Related	1	0
Mouth ulceration	3.57%	Not Related	0	1

Table 2: Adverse events of Study-1 (fluticasone propionate HFA pMDI 250 mcg per actuation without a spacer device) and Study-2 (fluticasone propionate HFA pMDI 250 mcg per actuation with a spacer device) are summarized.

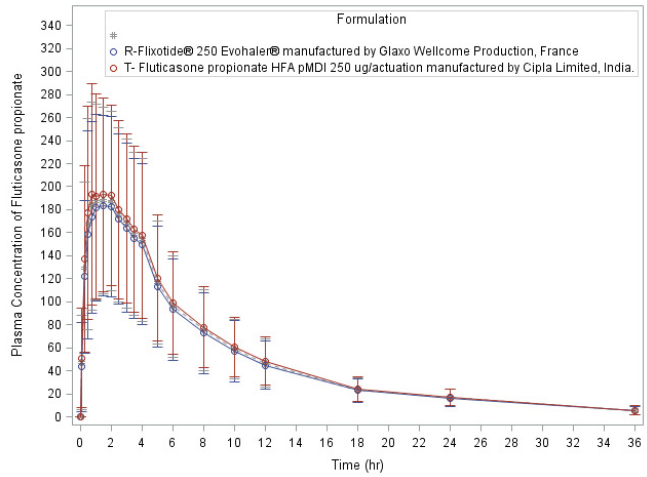

Figure 1: Mean graph (linear) for plasma concentration vs. time profile of Fluticasone propionate after inhalational dose of Fluticasone propionate HFA pMDI 1000 mcg in Study-1 (Fluticasone propionate HFA pMDI 250 mcg per actuation without a spacer device).

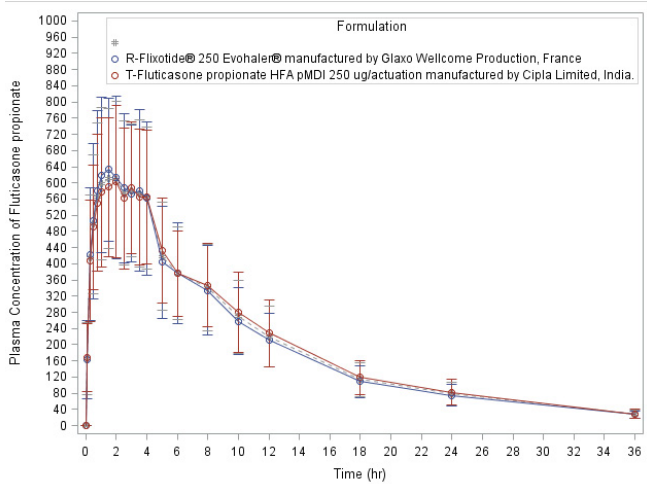

Figure 2: Mean graph (linear) for plasma concentration vs. time profile of Fluticasone propionate after inhalational dose of Fluticasone propionate HFA pMDI 1000 mcg in Study-2 (Fluticasone propionate HFA pMDI 250 mcg per actuation with a spacer device).

Pharmacokinetic Parameters	Study-1		Study-2	
	Test (T) (Mean ± SD)	Reference (R) (Mean ± SD)	Test (T) (Mean ± SD)	Reference (R) (Mean ± SD)
N	56	56	25	25
C_{max} (ng/mL)	222.97 ± 96.55	208.34 ± 86.73	656.50 ± 180.37	687.25 ± 199.70
AUC0-t (h.ng /mL)	1798.83 ± 772.15	1683.67 ± 744.36	7127.75 ± 2139.82	6898.32 ± 2111.43
AUC0-∞ (h.ng /mL)	1890.35 ± 798.98	1769.58 ± 765.67	7529.96 ± 2299.02	7252.67 ± 2194.47
*T_{max} (h)	1.00 (0.50–3.50)	1.00 (0.25–4.00)	2.00 (0.50–4.00)	1.50 (0.75–10.00)
Kel (1/h)	0.087 ± 0.018	0.085 ± 0.018	0.082 ± 0.009	0.083 ± 0.013
T1/2 (h)	8.40 ± 2.06	8.54 ± 1.73	8.58 ± 1.07	8.58 ± 1.35

*Median (range)

Table 3: The statistical results of primary pharmacokinetic parameters for fluticasone propionate of Study-1 (fluticasone propionate HFA pMDI 250 mcg per actuation without a spacer device) and Study-2 (fluticasone propionate HFA pMDI 250 mcg per actuation with a spacer device) are presented.

Pharmacokinetic Parameters	Geometric Mean		*(%)T/R	90% Confidence Interval	Power (%)	Intra subject CV%
	Test	Ref				
Study-1						
N	56	56	-	-	-	-
C_{max} (pg/mL)	199.41	190.57	104.64	97.46-112.34	99.97	22.61**
AUC_{0-t} (h.pg/mL)	1610.36	1525.58	105.56	98.55-113.06	99.98	22.61**
Study-2						
N	25	25	-	-	-	-
C_{max} (pg/mL)	631.5617	656.8985	96.14	88.13 - 104.88	99.33	18.08
AUC_{0-t} (h.pg/mL)	6832.3683	6604.943	103.44	96.21 - 111.22	99.92	15.02

*(%) T/R is ratio of Test Geometric Mean/Ref Geometric Mean
**intra-subject variability for reference product

Table 4: The Geometric mean ratios, 90% CIs, power and intra subject coefficient of variation of test and reference for Ln transformed pharmacokinetic parameters C_{max} and AUC_{0-t} for fluticasone propionate of Study-1 (fluticasone propionate HFA pMDI 250 mcg per actuation without a spacer device) and Study-2 (fluticasone propionate HFA pMDI 250 mcg per actuation with a spacer device) are presented.

so that the total systemic exposure of the generic versus the reference product are compared, not just what is absorbed via the lung [18,21].

Drug concentrations in the blood represent fractions of the aerosolized dose that has reached the blood after absorption from the gastrointestinal tract and the peripheral absorption from the lungs, which determines the safety of the administered drug. The extent to which the swallowed portion is absorbed depends on the oral bioavailability of the drug. Fluticasone propionate has negligible oral bioavailability (<1%) due to a combination of incomplete absorption from the gastro-intestinal tract and extensive first-pass metabolism, therefore systemic exposure arises only from pulmonary absorption i.e., lungs [22-24]. The amount of drug that reaches the blood via absorption from the lungs is same as the total bioavailability of the drug. Therefore, PK study using oral charcoal blockade to block the gastrointestinal absorption of inhaled fluticasone propionate for comparison of the pulmonary bioavailability of the test product and the reference product is not required.

Hence, both the PK studies conducted were useful to compare the