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Research Article

MIXED SCALING APPROACH TO ESTABLISH BIOEQUIVALENCE OF LANSOPRAZOLE DELAYED RELEASE CAPSULE IN FASTING SPRINKLE WITH APPLE SAUCE STUDY IN HEALTHY SUBJECTS

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ABSTRACT

Objective: The aim of the present study was to establish bioequivalence of highly variable generic lansoprazole (LSP) delayed release (DR) capsule, exploring minimal number of healthy volunteers by mixed scaling approach as oppose to average bioequivalence approach.

Methods: This was an open-labeled, three-treatment, three-periods, three-sequences, single-dose, partial replicate crossover trial conducted in 36 + 4 (stand by) healthy adult human subject in Indian origin.

Results: Non-parametric Wilcoxon sign rank test at 95% confidence interval failed to conclude significance difference in T_{max} and t1/2 between the formulations. The intra subject standard deviation of the reference formulation was 0.340 for C_{max}, 0.249 for area under curve up to last measurable time point (AUCT) and 0.244 for area under curve up to infinity time (AUCI) parameters. The reference scaling as proposed by Haider *et al*., 2008, was applied for $C_{max,}$ and constant scaling was applied for AUCT and AUCI metrics. No significance difference between two formulations were observed when data were analyzed by Analysis of Variance ($p<0.05$). Westlake 90% confidence limit, as well as two one-sided t-test as proposed by Schuriman and the Anderson-Hauck power analysis all fell under the predefine bioequivalence criteria for mixed scaling.

Conclusion: The generic LSP DR capsules were found to bioequivalent with reference drug under fasting study with apple sauce with respect to rate and extent of absorption. The mixed scaling statistical analysis approach used to establish bioequivalence with a minimum number of subjects was found reliable and utilize 40 subjects as opposed to 110 subjects need to establish bioequivalence in traditional average bioequivalence approach.

Keywords: Mixed scaling, Techniques, Non-parametric, Bioequivalence, Delayed release.

INTRODUCTION

Lansoprazole (LSP) a BCS Class-II drug, chemically 2-([3-methyl-4-[2,2,2-trifluoroethoxy]- 2-pyridyl]methyl) sulfinylbenzimidazole, is a benzimidazole derivative that selectively inhibits the H+/K+-ATPase of the parietal cell of the stomach [1,21]. LSP is widely used in the treatment of active duodenal ulcer, active benign gastric ulcer, gastroesophageal reflux disease, erosive esophagitis, and other pathological hyper secretory conditions [2,3]. The biological mean (n=20) half-life of the drug is approximately 2 hrs, but its acid suppression action persist about 24 hrs, this is due to irreversible inhibition of proton pump by the LSP which in turn prevent the dehydration of pump to make it free for ion transport [3].

A substantial number of studies had confirmed that orally 30 mg/day of LSP provided effective symptoms relief and healing of duodenal ulcer in 75-100% of patients after 4 weeks of therapy in non-comparative and comparative trials [4]. So far, it has been widely used for the *Helicobacter pylori* eradication therapy in combination with clarithromycin and amoxicillin clinically and acid related gastric disorders [5].

LSP is extremely acid labile and degrades rapidly in aqueous solutions with a low pH; at a pH of <4 the degradation half-life is 10 minutes, compared to a half-life of 18 hrs at pH of 6.5. Clear and predictable relationship exists between the degree and duration of intragastric acid suppression and acid-related disease healing and symptom relief [6,7]. To prevent destruction by the stomach acid, all proton pump inhibitors (PPI) are supplied as delayed-release (DR) capsule or tablets, or granules, or buffered suspensions. Enteric coating using acid stable polymers like eudragit, cellulose acetate phthalate, shellac, etc., is the technique to achieve intestinal release at pH near about 6.5 suitable for drug dissolution and absorption.

Even with the enteric coated formulation, pharmacokinetics is extremely variable between individuals. Peak concentrations and bioavailability of all PPI can vary 5-fold. Bioavailability consistently increases after repeated dosing due to alkalinization of the gastric contents, but still remains highly variable and unpredictable [8]. Such a high variability is contributed from the metabolic route. The hydroxylation of LSP (OH-LSP formation) by a polymorphic S-mephenytoin 4'-hydroxylase (cytochrome P450 2C19 [CYP2C19]) is the main metabolic route [9]. The genetic mutation of CYP2C19 [10] divided the population in two distinct metabolizers (extensive and poor). According to the genotyping analysis of *CYP2C19*, poor metabolizers consist of three genotypes (i.e., m1/m1, m2/m2, or m1/m2), while extensive metabolizers (EMs) include homozygous EMs (i.e. wt/wt), and heterozygous EMs (i.e., wt/m1 or wt/m2) [11]. The ratio of poor and rapid metabolizer is approximately 0.03, and found more in Japanese [12], Chinese [13] and Korean [13] compare to Caucasian [14]. An inter-individual difference in the activity of *CYP2C19* has been reported in relation to the metabolic disposition of LSP. The acid-inhibitory effect of LSP has also recently been reported to be affected by *CYP2C19* genotype status [15]. The genetic polymorphism of *CYP2C19* should be of a clinical concern in the treatment of acid-related diseases with LSP. The pharmacokinetics study in healthy volunteers exhibited high variability in C_{max}, area under the curve up to last measurable time point (AUCT) and area under the curve up to infinity time (AUCI) parameters [16,17]. Gastric pH reaches a median of 5.3 after a 1-week course of omeprazole, which is still sufficiently acidic to mandate the need for a buffered suspension or intact enteric formulation [18]. Based on the above finding LSP can be considered as highly variable drug with respect to peak plasma concentration.

In bioequivalence studies a new pharmaceutical formulation so-called generic (test drug, abbreviated as T), will be considered bioequivalence

with respect to the reference listed drug so-called reference (further abbreviated as R) having same pharmaceutical active moiety, If a 90% confidence interval (CI) for the median T/R ratio of a parameter of interest lies fully within the predetermined bioequivalence range (usually 80-125%) [19]. However, such phenomenon does not hold promising for the highly variable drug like LSP and need huge sample size to establish bioequivalence [20,21]. So, the aim of the present reference replicate 3-period crossover study was designed to address the highly variable C_{max} which is otherwise difficult to prove bioequivalence in traditional bioequivalence design.

METHODS

Chemicals

LSP and esomeprazole were obtained from Hetero Drugs Limited, Hyderabad, Andhra Pradesh, India. Acetonitrile, n-hexane and methanol were procured from Sigma-Aldrich, Bengaluru, India. Analytical grade formic acid, amyl alcohol, and sodium hydroxide were purchased from the local chemical store. Water was purified using in-house Milli-Q-System. All chemicals used for method development and validation were of analytical grade.

Equipment

The liquid chromatography (LC) [22] system used was API4000 AB SCIEX (California, USA) LG system equipped with triple quadrupole mass analyzers and Zorbax Eclipse XDB C^{18} (50 × 4.6 mm, 5 µm). A phenomenex security guard column (Bester, 4.0 × 2.0 mm) was used to protect the original column.

Study products

Test product LSP 30 mg DR capsules of Hetero Drugs Limited, Hyderabad, India, administered orally as per randomization code list. Batch no: E100168A.

Reference product

Prevacid® (LSP) DR capsules 30 mg Takeda Pharmaceuticals America, Inc. Deerfield, IL 60015, administered orally as per randomization code list Lot no: 867652E21.

Sample size estimation

Sample size estimation based on intra subject variability has limited influence in reference scaling BE approach [23]. The sample size in reference scaling dose not depends on ISCV (Haider *et al.*, 2008) rather it depends upon the intra subject variability of the test product (ederni and tooth fallaci). However, three-period references replicate pilot BE study has no scope to reliably estimate ISCV of the test product, but a four-period pilot study it dose. Therefore, a new technique of bootstrapping simulation was applied to estimate pivotal sample size which hold promising to the result of present BE study [24-26].

From the pilot study of 12 healthy Indian subject (unpublished data), it was observed that the C_{max} had the highest intra-subject variability which is 35% with T/R ratio 111%, whereas the observed intra-subject variability for the AUC_{0-t} and $AUC_{0-\infty}$ was least intra subject variability which was 24 and 19 respectively. Considering C_{max} variability and guideline of the Food and Drug Administration (FDA) and European medical agency, reference scale bioequivalence study was conducted in 36 healthy Indian volunteer, 4 subjects additionally was included for substitution of drop outs. Pivotal sample size was estimated by resampling of the data obtained from a pilot study to boot sample size of 24, 28, 32, 36, 40, 44, and 48. The individual boot samples were simulated to 1000 times to calculate the power of each boot samples and subjected to pharmacokinetics and statistical analysis to establish bioequivalence similarly as reference scaling techniques. Within-subject reference variability, T/R ratio and 95% CI of the upper limit for C_{max} and AUC were calculated (Table 1). Since 24 subjects were sufficient to show the bioequivalence for the molecule whose intra subject variability is at least 30, hence 24 subjects were chosen as first boot sample and so on. Finally, least boot sample which showed 80% power and ratio fall between 80 and 125 in log scale was selected for pivotal sample size [27-29]. Hence, 36 subjects were chosen to establish bioequivalence at 80% power and 90% CI the log-transformed ratio of test and reference between 80 and 125 at a Type I error rate is 0.05.

Power of boot samples = number of simulation reject null hypothesis (BE PASS)/total number of simulation × 100 1

Bioequivalence study

Study design

The study was an open-labeled, randomized, three-sequence, three-periods, single-dose, reference replicated three-way crossover design with at least 7 days washout period between the doses. The clinical phase of the study was conducted in the Huclin Research Limited, Chennai, Tamil Nadu, India. The subjects who participated in this study, were screened prior, their mean age was 28±8.45 with a range of 18-55 and mean body weight was 68±12.24 beside demographic data, medical history, physical examination vitals including respiratory rate, electrocardiogram (ECG), chest X-ray, hematology, biochemistry, serology includes HIV 1 and 2 antigen and hepatitis B. and urine analysis was performed at baseline screening of the subjects or prior to initiation of the study [30]. In addition, urine pregnancy tests were done for female subjects.

Upon completion of the study, the physical examination and clinical laboratory measurements were repeated. The subjects were instructed to abstain from taking any medication for 1-week prior to and during the study period. Informed consent was obtained from the subjects after explaining the nature and purpose of the study. The study protocols were approved by the independent Ethics Committee called Madras Ethic Committee, Chennai, Tamil Nadu, India.

Drug administration and sample collections

A total of (36+4 stand by) healthy human adult male subjects were enrolled for the study. They were housed at least 11 hrs prior to drug administration until 24 hrs post dose blood sample was drawn in each period of the study. After overnight fasting for at least 10 hrs, one 30 mg DR capsule of LSP (test product [A] or reference product [B]) was administered orally to each subject as per the randomization code list under yellow monochromatic light with the aid of 240 ml apple sauce. The blood samples were collected within 1-hr before dosing and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 7.00, 8.00, 10.00, 12.00, 14.00, 16.00, 20.00, and 24.00 hrs post-dosing in all three-periods under yellow monochromatic light. There was a washout period of 10 days between the first and second dosing and 7 days between the second and third dosing of the study. Following collection of the blood sample 1 ml of sodium citrate buffer pH 9 was added to keep the plasma alkaline and to prevent the degradation of the LSP at alkaline pH. Finally, plasma was separated from whole blood by centrifugation for 15 minutes at 3200 RPM at −10°C and separated plasma was kept in K3 ethylenediaminetetraacetic acid (EDTA) and stored for bioanalysis at −20°C.

Quantitative analysis

Plasma LSP concentration at each time point was quantitated by validated LC-mass spectrometry (MS)-MS method using esomeprazole as an internal standard at Huclin Research Limited, Chennai, India. Subject sample was prepared for the determination of LSP by using liquid - liquid extraction technique. For extraction of the LSP from plasma, 200 µl of subject sample and 50 µl of IS (1 µg/ml in 0.1 M sodium hydroxide solution) was mixed and vortexed with 100 µL of 0.1 M di-sodium hydrogen ortho-phosphate in milli-Q water and 2.5 ml extraction solution (tertiary butyl methyl ether and dichloromethane in 7:3 ratio) for 10 minutes at 2500 RPM. The resultant mixture is further centrifuge at 4000 RPM for 5 minutes at 4°C to separate organic layer as a supernatant. 2 ml of the supernatant liquid is then transferred to the labeled Ria vial to evaporate to dryness at 40°C. the residue was reconstituted with 250 µL of the mobile phase containing acetonitrile and 5 mM of ammonium phosphate (PH 9.0) in 40:60 ratio. The final solution was then transferred to the auto sampler vial at 10°C. All the

Table 1: LC-MS/MS method validation results for quantitative estimation of lansoprazole in human plasma in K_2 EDTA matrix

Validation parameters for QC	List of QC samples (ng/mL), analyte: LSP IS: Esomeprazole (%)				
	QCLLQ 10.536	QCL 27.726	QCM1 277.260	QCM 792.172	QCH 1800.392
Intra-day precision	6.20-8.45	1.56-5.42	1.73	1.56-5.42	1.56-5.42
Intra-day accuracy	97.44-102.54	96.01-102.16	100.33	96.01-102.16	96.01-102.16
Inter-day precision	7.30	2.39-4.46	1.73	2.39-4.46	2.39-4.46
Inter-day accuracy	100.38	97.57-101.38	100.33	97.57-101.38	97.57-101.38
Bench-top stability (hrs)	5 hrs 55 minutes at room temperature				
Stock solution stability (days)	31 days (2-8°C)				
Processed stability (hrs)	Wet extract: 2 hrs 4 minutes @ room temperature				
Freeze-thaw stability (cycles)	4 freeze-thaw cycles				
Long-term storage stability	245 days				
Dilution integrity	Concentration diluted two-fold and six-fold				
Selectivity	No interfering peaks noted in blank plasma samples				

LC-MS/MS: Liquid chromatography-mass spectrometry, LSP: Lansoprazole, EDTA: Ethylenediaminetetraacetic acid, QCLLQ: Quality control limit of quantification, QCL: Quality control level, QCM: Quality control medium, QCH: Quality control high

process was carried out under monochromatic light in order to prevent degradation of the light sensitive LSP. 2 µL of the reconstituted solution was injected into the high-performance LC (HPLC) system equipped with a Zorbax Eclipse XDB C^{18} (50 × 4.6 mm, 5 µm) column, turbo ion spray and API 3200 LCMS/MS detector.

The data was collected and calculated on a millennium chromatography manager software system version 4.00. Linear regression, with 1/x weighting factor to obtained the best fit of the data for the calibration curves. The lower limit of quantitation (LLOQ) for LSP was 10.242 ng/mL; concentrations below the LLOQ were reported as 0.0 ng/mL. Inter-assay coefficients of variation of quality control samples spiked with 10.536, 792.172, and 1800.392 ng/mL of LSP were 8.21%, 7.71%, and 6.56%, respectively.

Pharmacokinetics analysis

Individual subject's plasma LSP concentration at each time point was used to obtain the plasma profile of the drug. C_{max} and T_{max} were obtained directly from the plasma profile by visual inspection. The mean plasma concentration with standard deviation versus time graph was computed for both the treatment after calculating mean plasma concentration at each time point as given in the Fig. 1. Rest of the PK metric was calculated based on the non-compartmental modeling of the concentration versus time data. AUC from 0 to last measurable concentration (Ct) was calculated by the linear trapezoidal method. Area under the concentration curve from time zero to infinite was computed from the formula of $AUC_{0-t} + C_t/K_{el}$. Terminal phase elimination rate constant (K_{el}) was calculated from the linear regression of at least 4 terminal nonzero concentrations. Elimination half-life of LSP was determined by using the formula $\ln(2)/K_{el}$. All pharmacokinetics analysis was done using WinNonlin V 5.3.2.

Statistical analysis method

Traditional two one sided test procedures were applied for the comparison of two formulations in a two treatment two-period cross-over design (Schuirmann) [30] for the assessment of average bioequivalence. Computational methods for log-transformed data were illustrated (Midha *et al.*) [21] in a comparison of two formulations of LSP. Traditional average bioequivalence method was used for those PK metric having S_{WR} within-subject standard deviation (SD) of the reference product is <0.294 and scaling was done for those PK metric having s_{WR} more than 0.294. Equation-1 was used for the computation of S_{WR} for all individual PK metrics.

$$S^2_{WR} = \frac{\sum_{i=1}^{m}\sum_{j=1}^{n}(Dij - Di)2}{2(n-m)} \quad (1)$$

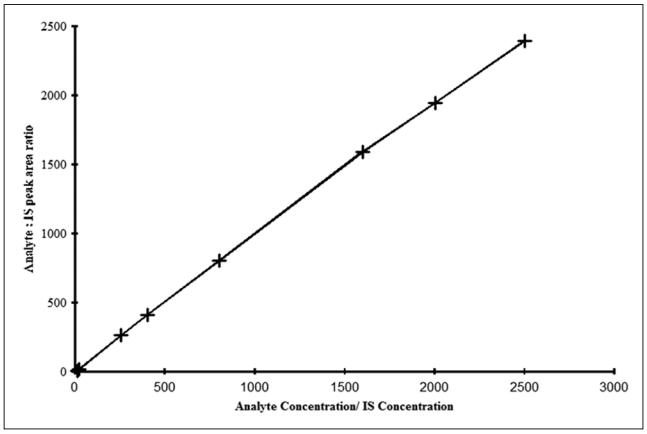

Fig. 1: Standard calibration curve of lansoprazole concentration/ internal standard concentration versus mean analyte to internal standard peak area ratio (n=6), linear curve equation (linear regression (1/(x*x) weighing) is Y=0.000916X - 0.000429 R^2=0.9995, Where Y stands for mean analyte to internal standard peak area ratio, X stands for l lansoprazole concentration/ internal standard concentration (ng/mL)

Where: *i* = number of sequences *m* used in the study (*m*=3 for partially replicated design: TRR, RTR, and RRT), *j* = number of subjects within each sequence, T = Test product, R = Reference product and *Dij = Rij1–Rij2* (where 1 and 2 represent replicate reference treatments). The DI and n of the equation (1) were calculated by using equation 2 and 3 respectively.

$$Di = \frac{\sum_{j=1}^{n} Dij}{n_i} \quad (2)$$

$$n = \sum_{i=1}^{n} n_i \quad (3)$$

For reference scaling of PK metric, 95% upper confidence bond was calculated by using equation 4.

$$(Yt\text{-}Yr) - \theta S^2_{WR} \quad (4)$$

Where: Yt and Yr are the means of the ln-transformed PK endpoint (AUC and/or C_{max}) obtained from the BE study for the test and reference products, respectively.

$$\theta=(\frac{\ln(1.25)}{\sigma_{W_0}})^2 \text{ (scaled average BE limit)} \quad (5)$$

and $\sigma_{W0} = 0.25$ (regulatory limit) (6)

The formula used to expand the BE limit is given below Boddy *et al.* and Tothfalusi and Endrenyi of the scABER criterion are described by Eq. (7):

$$\text{Upper / Lower BE limits} = \exp[\pm\ln(1.25)*\frac{Swr}{Swo}] \quad (7)$$

RESULTS

Clinical study

A total 36 of 40 subjects completed all the three-period of the study. 4 subjects were dropped out, subject 16 and 18 withdrew consent in period two and subject 28 and 36 were excluded from the study due to vomiting within the labeled dosing interval. Four stands by subjects were replaced to get a total of 36 subjects. Finally, 36 subject's data were analyzed to evaluate pharmacokinetics property of the molecules and statistical analysis to conclude bioequivalence. There were no serious adverse events and adverse events reported for the study except, diarrhea, abdominal pain, and vomiting reported for few subjects.

HPLC method validation

The HPLC equipped with mass detector was used to quantify the plasma concentration. The retention time for the drug was 2.33 minutes and an internal standard (esomeprazole) was 1.58 minutes, chromatogram is shown in the Fig. 1. Under described analytical conditions, the relationship between the concentration and peak area ratio was linear from 10.242 ng/mL to 2500 ng/mL (LSP, Y=0.000916X -0.000429 R^2=0.9995, LLOQ was 10.242 ng/mL). The linear calibration curve of peak area ratio (analyte to internal standard) versus analyte concentration to internal standard concentration is depicted in the Fig. 1. The method was validated as per the regulatory requirements. The absolute recovery, precession and accuracy of the method was found to (mean ± SD) 82.77±4.51, 91.423±7.34 and 3.97±2.52 (Table 1).

Assay validation

The assay procedure was validated by analyzing six single standard curve sets per day for a total of 3 days. The standard curve concentrations range from 10.242 to 2500.589 ng/mL. On each day of validation, five sets of QC samples at 10.536 ng/mL (low), 792.172 ng/mL (medium) and 1800.392 ng/mL (high) (Table 1) were assayed for a total of 12 sets of QC samples for all 6 days. The QC concentration levels were selected to represent the full calibration range. An integrator was used to determine the chromatographic peak heights of LSP and internal standard. The peak height ratios of LSP to internal standard were used for the calculation of unknown concentrations.

Linearity

Linearity was defined as the correlation coefficient (r) obtained from weighted (l/concentration) linear regression analysis of the standard curve. Acceptable standard curves were required to have "R^2" values of 0.99 or greater. During validation, the assay was linear over the range of 10.242-2500.589, ng/mL all "R^2" values obtained were 0.999 or better. The mean value for the *y*-intercept was −0.000429 and for the slope was 0.000916 (Fig. 1).

Matrix effects

Matrix factors ranged from 0.96% to 1.01% (% CV 1.02) for LSP and from 0.98% to 1.02% (% CV 1.00) for IS (esomeprazole), respectively. Matrix effects were within acceptance criteria (Table 1) indicating absence of significant matrix effect.

Signal-to-noise (S/N) ratio

S/N ratios were >5 for the matrix lots evaluated, demonstrating acceptable signal intensity data are presented in Table 1.

Carryover

LSP carryover was 0.00% of the extracted analyte LLOQ sample response and IS carryover was 0.00% of the IS area (Table 1). Both values were within acceptance limits.

Calibration curve precision and accuracy

Inter-batch calibration standard accuracy for LSP ranged from 0.50% to 3.11% with inter-batch precision values of 95.93-103.69% during the course of validation, demonstrating acceptable assay linearity. Correlation coefficient (r) was consistently >0.99. A representative calibration curve for LSP in K_2 EDTA Human plasma is shown in Fig. 1.

Weighted scheme

Linear regression with 1/x2 weighting was selected as the weighting scheme. Weighting scheme analysis data are provided in Table 1.

LLOQ

A LLOQ of 10.242 ng/ml was determined for this method, where mean LSP accuracy and % CV were 99.86% and 1.50%, respectively (Table 1).

Sensitivity

The mean LSP accuracy and % CV were 101.71% and 6.83% respectively (Table 1).

Intra-batch

Intra-batch precision (% CV) for LSP in quality control limit of quantification (QCLLQ) samples ranged from 2.57% to 9.11% across the six precision and accuracy batches. Intra-batch precision (% CV) for LSP in quality control level (QCL), quality control medium (QCM) and quality control high (QCH) samples ranged from 0.88% to 5.82% (Table 1). Intra-batch precision (% CV) for LSP was 1.73% in QCM1 samples.

Intra-batch accuracy (% nominal) for LSP in QCLLQ samples ranged from 92.01% to 106.46% across the six precision and accuracy batches. Intra-batch accuracy (% nominal) for LSP in QCL, QCM and QCH samples ranged from 95.25% to 103.49%. Intra-batch accuracy (% nominal) for LSP was 100.33% in QCM1 samples (Table 1).

Intra-batch precision and accuracy values were within established acceptance limits.

Inter-batch

Inter-batch precision (% CV) for LSP was 7.30% in QCLLQ samples and ranged from 2.39% to 4.46% in QCL, QCM and QCH samples. Inter-batch precision (% CV) for LSP was 1.73% in QCM1 samples (Table 1).

Inter-batch accuracy (% nominal) for LSP was 100.38% in QCLLQ samples and ranged from 97.57% to 101.38% in QCL, QCM and QCH samples. Inter-batch accuracy (% nominal) for LSP was 100.33% in QCM1 samples (Table 1).

Inter-batch precision and accuracy values were within established acceptance limits.

Intra-day

Intra-day precision (% CV) for LSP in QCLLQ samples ranged from 6.20% to 8.45% across the six precision and accuracy batches. Intra-day precision (% CV) for LSP in QCL, QCM and QCH samples ranged from 1.56% to 5.42%. Intra-day precision (% CV) for LSP was 1.73% in QCM1 samples (Table 1).

Intra-day accuracy (% nominal) for LSP in QCLLQ samples ranged from 97.44% to 102.54% across the six precision and accuracy batches. Intra-day accuracy (% nominal) for LSP in QCL, QCM and QCH samples ranged from 96.01% to 102.16%. Intra-day accuracy (% nominal) for LSP was 100.33% in QCM1 samples (Table 1).

Intra-day precision and accuracy values were within established acceptance limits.

Recovery
Mean LSP recovery was 74.89% and IS (esomeprazole) recovery was 68.15%.

Reinjection reproducibility
Mean calculated LSP concentrations in stored QCL and QCH samples were 1.19% and 0.36% (% change) and 5.43% and 3.03% (% CV), respectively (Table 1).

Dilution integrity
Mean calculated LSP concentrations at 2 times and 6 times dilution levels were 100.57% and 103.51% (% nominal) and 2.97% and 1.07% (% CV) of the expected concentration, respectively demonstrating acceptable sample dilution integrity (Table 1).

Stability

Long term

LSP and IS (esomeprazole) stock solutions were stable for at least 31 days when stored in the refrigerator temperatures (2-8°C). The % nominal concentration was 100.22% and 99.82% for LSP and IS (esomeprazole) (Table 1).

Bench top
Mean calculated LSP concentrations in stability samples were 105.37% and 98.11% (% nominal) and 4.05% and 1.59% (% CV) at QCL and QCH concentrations, respectively. In addition, LSP concentrations in stability samples were 103.97% and 106.23% relative to bulk spiked samples at QCL and QCH concentrations, respectively (Table 1). Acceptable bench-top stability was demonstrated for at least 5 hrs 55 minutes.

Freeze thaw
Mean calculated LSP concentrations in stability samples were 104.89% and 97.55% (% nominal) and 2.44% and 1.09% (% CV) at QCL and QCH concentrations, respectively. In addition, LSP concentrations in stability samples were 103.49% and 105.62% relative to bulk spiked samples at QCL and QCH concentrations, respectively (Table 1). LSP was stable in K_2 EDTA Human plasma for at least four freeze-thaw cycles.

Wet extract
Mean calculated LSP concentrations in stability samples were 105.64% and 92.42% (% nominal) and 3.2% and 1.64% (% CV) at QCL and QCH concentrations, respectively. In addition, LSP concentrations in stability samples were 104.23% and 100.07% relative to bulk spiked samples at QCL and QCH concentrations, respectively. LSP samples were stable as wet extracts when stored for at least 2 hrs 4 minutes at room temperature (Table 1).

In-injector
Mean calculated LSP concentrations in stability samples were 99.82% and 89.44% (% nominal) and 2.51% and 1.83% (% CV) at QCL and QCH concentrations, respectively. In addition, LSP concentrations in stability samples were 98.49% and 96.83% relative to bulk spiked samples at QCL and QCH concentrations, respectively. Mean IS (esomeprazole) concentration in QCM samples were 102.59% relative to bulk spiked samples and the % CV was 2.45%. LSP and IS (esomeprazole) were stable in mobile phase stored at 10°C in the auto sampler for at least 50 hrs 8 minutes.

The method demonstrated acceptable performance and was, therefore, suitable for the determination of LSP in Human K_3 EDTA plasma over the range of 0.01-25 µg/mL in the bioequivalence study. Under the described analytical conditions, the relationship between the concentration and peak area ratio was linear form 0.01 to 25 µg/ml (LLOQ, 0.05) (LSP, Y=0.000916x - 0.000429 R^2=0.9995). The linear calibration curve of peak area ratio (analyte to internal standard) versus concentration is shown in Fig. 1.

Pharmacokinetics and statistical analysis
The linear mean plasma concentration versus time curves of 2 LSP formulations to the 36 subjects under fasting conditions are given in Fig. 1. The primary and secondary PK parameters for both the formulation under non-fasting conditions are shown in Table 2. The mean (±SD) C_{max} (ng/ml) of test and reference formulation were 1476.6638 (±468.91) and 1458.8766±594.50 respectively. The mean (±SD) AUCT (ng.hr/ml) of test was 5112.3210±3915.64 and reference was 5191.6770 (±3942.79) while mean (±SD) AUCI (ng.hr/ml) of test and two reference formulations were 5250.1054 (±4199.89) and 5320.9689±4165.06. The mean (±SD) of T_{max} (hr), Kel (hr^{-1}) and half (hr) of test and two reference formulations of LSP were 1.61 hrs (±0.85), 1.77 hrs (±0.68); 0.4533 rs^{-1} (±0.19), 0.4624 hrs^{-1} (±0.21) and 2.03 hrs (±1.47), and 2.04 hrs (±1.43), respectively. The results of descriptive statistics of primary pharmacokinetics parameters of the test and two reference products are presented in Table 3.

The reference variability of the primary pharmacokinetics parameters of C_{max}, AUCT (AUClast) and AUCI (AUC total) were 35%, 25.33%, and 24.77%, respectively details of which is presented in Table 2.

The least square mean of the primary PK parameters calculated from the ANOVA. the ratio and 95% CI upper limit of primary PK parameters C_{max}, and 90 % CI for AUCT (AUC last) and AUCI (AUC total) assuming equal variance between the group were 1.04, −0.042694, 1.0167, 91.81-112.58 and 1.0161941, and 91.92-112.32, respectively for details refer Table 4 for 95% CI and 66 for 90% CI.

DISCUSSION

A total of 36+6 subjects were enrolled for apple sauce study under fasting conditions. The demographic characteristics of all participants were, mean (±SD) age, body mass index, height and weight of the all participants were 35.38±11.75, 24.19±3.62, 165.69±12.16 and 66.12±10.16 respectively. There were 6 subject's drops out, one is dropped out due to adverse events of vomiting, and 5 subjects were not reported, self-withdrawn from the study. Some of the adverse events like mild headache, abdominal pain and diarrhea had occurred for few of the subjects which had no significant impact on the study result.

The HPLC equipped with mass detector was used to quantify the plasma concentration. The retention time for the drug was 25 minutes, and an internal standard (esomeprazole) was 10 minutes, a chromatogram is represented in Fig. 2.

A standard calibration curve (Fig. 1) was constructed at LLOQ of 10.536 ng/mL and ULOQ was 1800.392 ng/mL. The method was validated as per the regulatory requirements. The absolute recovery of LSP and internal standard for the method was found 74.89% and 68.15%, 91.423±7.34 and 3.97±2.52, the intraday precision and accuracy range of the substance was 6.20-45% (QCLLQ) and

Table 2: Reference scale bioequivalence of C_{max}, and within reference standard deviation, intra subject CV and 95% upper confidence bond for all PK parameters (C_{max}, AUCT ans AUCI) of lansoprazole DR capsules 30 mg

Statistical parameters	C_{max}	AUCT	AUCI
δ (μ_T-μ_R)	0.0439	0.0141	0.135
σ WR within reference standard deviation	0.340	0.249	0.244
Intra subject CV of reference	35	25.33	24.77
σ^2_{WR} within reference variability	0.1156	0.0622	0.0595
$(\mu_T-\mu_R)^2/\sigma^2_{WR}$	0.01667	0.0032	
95% upper confidence bond for $(\mu_T-\mu_R)^2/\sigma^2_{WR}$	−0.0482	0.0914	0.0876
Ratio (%)	104.49	101.67	101.61
Power	0.8422	97.42	97.77
Observed BE limit	90.93-120.06	91.81-112.58	91.92-112.32
Permitted BE limit	73.82-135.46	80.07-124.89	80.43-124.33

Table 3: Descriptive statistics of primary and secondary PK parameters for test and reference product under fasting (sprinkle with apple sauce) conditions

Test statistics	Kel (Hr)	Thalf (Hr)	C_{max} (ng/ml)	T_{max} (Hr)	AUCT (hr)*(ng/ml)	AUCI (hr)*(ng/ml)
Test drug						
N	36	36	36	36	36	36
Mean	0.4553	2.03	1476.6638	1.61	5112.3210	5250.1054
SD	0.19	1.47	468.91	0.85	3915.64	4199.89
Min	0.0954	0.85	176.8200	0.50	662.2508	699.7754
Median	0.4555	1.52	1520.7525	1.50	4049.0019	4085.9046
Max	0.8164	7.26	2133.4510	4.00	18730.7317	20714.5117
CV%	42.64	72.41	31.75	52.52	76.59	80.00
Geo-mean	0.4047	1.71	1367.4866	1.42	4066.5496	4136.9180
Har-mean	0.3415	1.52	1158.7614	1.25	3246.0520	3305.7867
Reference drug						
N	72	72	72	72	72	72
Mean	0.4624	2.04	1458.8766	1.77	5191.6770	5320.9689
SD	0.21	1.43	594.50	0.68	3942.79	4165.06
Min	0.1150	0.79	258.6960	1.00	787.6087	799.8131
Median	0.4534	1.53	1420.7430	1.50	3978.4062	4023.2180
Max	0.8786	6.03	2878.2470	4.00	18341.0367	19810.6662
CV%	45.10	70.02	40.75	38.29	75.94	78.28
Geo-mean	0.4056	1.71	1316.2030	1.66	4014.6036	4084.8215
Har-mean	0.3394	1.50	1134.2636	1.56	3114.2220	3166.4514

SD: Standard deviation

Table 4: Average bioequivalence limits for AUCT and AUCI of lansoprazole

PK parameters	Geometric mean of test treatment	Geometric mean of reference treatment	Ratio	90% lower confidence limit	90% upper confidence limit	Power
AUCT	4026.5200	3960.4276	101.67	91.81	112.58	97.42
AUCI	4094.4548	4029.6991	101.61	91.92	112.32	97.77

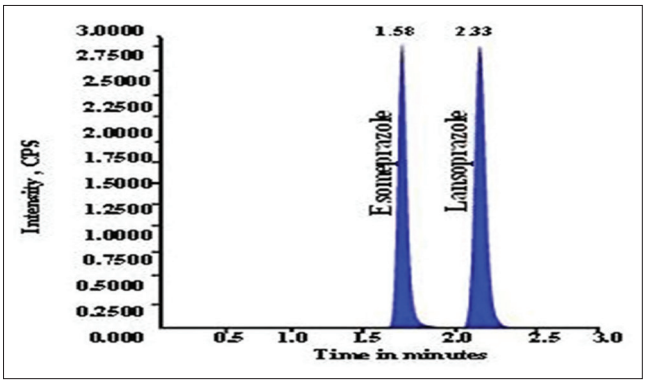

Fig. 2: Chromatogram of analyte (extracted Blank K_2 ethylenediaminetetraacetic acid human plasma sample) and internal standard (esomeprazole) showing peak area and retention time in human plasma

97.44-102.54% (QCLLQ) respectively (Table 1) which indicated the reproducibility of the method. The LLOQ of the method was found to be 20 ng/mL indicating the sensitivity of the method.

The calibration curves (from at least 6 batches of rug with 8 concentration points) for LSP were constructed by plotting concentration ratio versus peak area ratio with internal standard and showed good linearity in the 10.242-2500.589 ng/mL range. Weighing with 1/x2 was used to remove heterogeneity of the data by assuming equal variance. The representative linear equation was Y = 0.000916X - 0.000429 with a correlation coefficient (0.9995) highly significant for the method. As shown in Fig. 1, excipients used as additives in pharmaceutical formulations did not interfere in the proposed procedures.

The robustness of the LC-MSMS method was determined by analysis of samples under a variety of conditions such as matrix effects, reinjection reproducibility, S/N ratio, dilution integrity, carryover effects and various stability.

Signal-to-noise ratios for the method were >5 for the matrix lots evaluated, demonstrating acceptable signal intensity. Data indicated ability of the instrument to separate the two peaks within 5 unit distance. LSP carryover was 0.00% of the extracted analyte LLOQ sample response and IS carryover was 0.00% of the IS area (Table 1). Both values were within acceptance limits. Reinjection reproducibility and dilution integrity of the method was assessed from the mean calculated LSP concentrations in stored QCL and QCH samples were 1.19% and 0.36% (% change) and 5.43% and 3.03% (% CV), respectively and Mean calculated LSP concentrations at 2 times and 6 times dilution levels were 100.57% and 103.51% (% nominal) and 2.97% and 1.07% (% CV) of the expected concentration, respectively demonstrating acceptable sample dilution integrity.

Finally, the stability of the method was carried out with plasma quality control samples (10. 536, 792.172 and 1800.392 ng/ml) in bench top, short term and long term as mentioned in the results samples showed no significant degradation under the prescribe conditions (Table 1). The inter- and intra-day precision and accuracy was also in the accepted limit (2). Hence, LC-MSMS method used for the estimation of the LSP was adequate and given a reproducible result. The calibration curve was linear throughout the CC range of 10.242-2500.589 ng/ml. The method showed acceptable stability in the room temperature, refrigerator and bench top. The EDTA matrix used for the sample collection was not affected in the analysis of the plasma sample, the detail of the method validation and intraday accuracy and precession is given in Table 1.

The mean graph in turn helps to establish equivalence between two formulations through the naked eye without doing statistical analysis. The graph (Fig. 3) also tells about the mean C_{max} and T_{max} for test and reference formulation. Higher the difference of two C_{max} increases the ratio which tend to be failed in the BE study.

The mean concentration-time profiles for the two brands of LSP 30-mg capsules are shown in Fig. 3. All calculated pharmacokinetic parameter values were in good agreement with the previously reported values [1,17]. The pharmacokinetic parameters for both formulations are shown in Table 3. For bioequivalence evaluation various statistical modules were applied to AUCT, AUCI and C_{max} as per current FDA guidelines for reference scaling [19]. The justification of reference scaling requirement for the C_{max} parameter are given in the statistical analysis table of within reference variability (S_{WR}) which was 35 (Table 3). Tables 3 and 4 show the results of the statistical analysis for AUCT, AUCI, and C_{max}. According to the mean plasma levels of the 36 subjects completing the study, the relative bioavailability was found to be 101.68%, 101.9 and 101.61% on the basis of mean AUC02t, AUC02/ and C_{max}, respectively.

Area under the curve (AUCT)

The geometric mean AUCT was 4026.5200 ng/mL*hr and 3960.4276 ng/mL*hr for test and reference products, respectively; these values were in good agreement with reported ones (Landes *et al.*). On the basis of these values, it was concluded that the two products did not show any unusual pharmacokinetics values for LSP.

ANOVA did not show any significant differences for periods effects and treatment (formulations). 90% CI also fell within the bioequivalence acceptance criteria. Two one-sided t-tests [31] and (Schuirmann) were also performed on the ratio (r) of mean AUCT of test to mean AUCT of reference. The probability for the ratio (T/R) to lie within 0.8 and 1.25 was 1.02 (Table 4).

Area under the curve (AUCI)

The mean AUCI was 4094.4548 ng/mL*hr and 4029.6991 ng/mL*hr for test and reference products, respectively; these values were in good agreement with reported ones (Baradell *et al.* and Landes *et al.*). These values again confirmed the conclusion that the two products did not show any unusual pharmacokinetics for LSP.

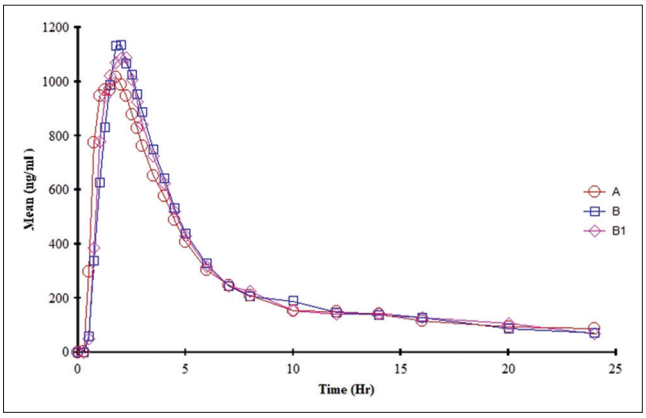

Fig. 3: Plasma lansoprazole concentration (mean [ng/mL], n=36), at each time point. A stands for "test drug," B and B1 stands for "reference drug"

ANOVA did not show any significant differences for periods effects and treatment (formulations). 90% CI ranges also fell within the bioequivalence acceptance criteria. Two one-sided t-tests [32] and (Anderson) were also performed on the ratio of mean AUCI of test to mean AUCI of reference. It was accepted that the probability for the ratio (T/R) to lie within 0.8 and 1.25 was 1.02 (Table 4).

Peak plasma concentration (C_{max})

The mean C_{max} was 1476.6638 and 1458.8766 ng/mL for test and reference products respectively; these values were in good agreement with reported ones (Baradell *et al.* and Landes *et al.*), assuring further the lack of any unusual pharmacokinetics for LSP.

ANOVA did not show any significant difference; for periods effects the observed p=0.092. In terms of treatment (formulations), no significant difference was observed; observed p=1.06 while table F value at corresponding degree of freedom was 4.26. 95% CI ranges among the reference and test products also fell within the bioequivalence acceptance criteria for C_{max}. Two one-sided t-tests [33-36], and (Anderson) were also performed on the ratio of mean C_{max} of test to mean C_{max} of reference. The probability for this ratio to lie within 0.8 and 1.2 was 0.81. For T_{max} the parametric point estimate of difference (test to reference) was 20.39 h, (mean T_{max} of test was 1.61 hrs and reference was 1.71) which showed an improved rate of bioavailability, though it was very close to acceptance limits (20% of reference mean).

The PK profile of the two drugs was comparable (Table 5). There were no significance difference observed among sequence, formulation, and period based on the two-way analysis of variance. The reference SD, ratio and 95% CI of LSP was C_{max} 0.34, 1.0448, and −0.042694 for AUCT 0.2533, 1.0167, and 90% CI (91.81-112.58) and for AUCI was 0.2477, 101.61, and 90% CI (91.92-112.32) (Tables 3 and 4).

The statistical analysis result of mixed scale design showed good agreement with bootstrapping prediction of C_{max} and AUCT ratio (Table 5).

Safety and tolerability

The most common drug-related adverse event was abdominal pain and diarrhea, which occurred in association with the reference capsule formulations in 10, 15, and 11 volunteers, respectively. There were no serious adverse events, subject 2 discontinued due to adverse events, or clinically important abnormalities in laboratory test results, vital signs, or ECG.

CONCLUSION

The sample size calculation for SABE from the pilot data using bootstrapping resampling techniques was adequate to estimate T/R

Table 5: Bootstrapping sample size prediction for pivotal study (data taken from pilot study of 12 subjects in 3 period 3 sequence reference replicated study design)

Boot sample	No of simulation	Predicted T/R ratio		Power %		95% CI upper limit	
		C_{max}	AUC_{0-t}	C_{max}	AUC_{0-t}	C_{max}	AUC_{0-t}
12 (pilot study)		1.31	1.21	68	64	0.0243	0.0113
24	1000	1.23	1.14	72	82	−0.0276	0.0167
28	1000	1.19	1.06	76	85	−0.0321	0.0201
32	1000	1.10	1.02	79	86	−0.0368	0.0268
36	1000	1.06	1.01	82	86	−0.0389	0.0289
40	1000	1.04	1.02	84	88	−0.0467	0.0311
44	1000	1.1	1.01	85	93	0.0665	0.0312
48	1000	1.02	1.00	84	92	0.0072	0.0309
52	1000	1.04	1.02	87	94	0.0081	0.0334

CI: Confidence interval

ratio with only 1% deviation from the observed data. The LC-MSMS method used for the analysis of plasma samples was reliable with a good reproducibility of the results. The reference scale semi-replicate 3 periods, 3 sequence, 2 treatment *in vivo* clinical study was adequate to establish the difference of their geometric mean for all the primary PK metric.

There were no serious adverse events of the study and no change in the abnormal laboratory value for the subjects except diarrhea in the subjects 10, 11, and 15 in period two which were mild in nature and resolved without sequale.

The assumption of mixed scaling for the bioequivalence study is proved since the within-subject reference variability (S_{WR}) for C_{max}, AUCT and AUCI are 0.34, 0.249, and 0.244, respectively under fasting conditions, therefore mixed scaling approaches was used to establish bioequivalence and test product found bioequivalence with respect to the reference formulation.

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