Pharmacokinetics Evaluation of Acamprosate Tablets in Healthy Human Volunteers

Kiran S. Chaudhari¹, Milind Bagul² and Ketan Shah³

¹RK University, Rajkot, India.
²Head Analytical Services, Raptim Research Ltd., New Mumbai, India.
³Parul Institute of Pharmacy and Research, Parul University, Limda, Vadodara, India.

Authors’ contributions

This work was carried out in collaboration among all authors. Author KSC carried out work and performed the statistical analysis, author MB designed the study, wrote the protocol and author KS managed the analyses of the study, wrote the first draft of manuscript and managed the literature searches. All authors read and approved the final manuscript.

ABSTRACT

Pharmacokinetic data of acamprosate tablets was not accessible on large number of human. Rationale to examine the pharmacokinetic properties of acamprosate calcium in healthy male subject, on single or multiple dosage administration, to evaluate the bioequivalence of two formulations of acamprosate calcium tablets in fast or fed environment. This work engross the study of pharmacokinetic property of Acamprosate calcium tablets in single dosing under fasting condition.

Methods: Bioequivalence study of delayed release acamprosate tablets 333 mg for a randomized, single dose, open label, two treatment, two periods, two sequences and crossover design in 12 healthy, adult human subjects under fasting condition was conducted. The wash out period within each treatment and each stage was 1 week. The quantification of acamprosate was done by LCMS/MS method. Accessibility was evaluated by monitoring adverse events, physical examinations and ECG and laboratory tests.

Results: The entire study was conducted by using 12 male subjects to fulfill all stages in the study. The pharmacokinetic calculations for test and reference formulations are as follows: single dosing,
1. INTRODUCTION

The chemical balance in the brain which would be disrupted due to alcoholism could be stabilizing by using acamprosate calcium. This is working by blocking glutaminergic N-methyl-D-aspartate receptors, while gamma-amino butyric acid (GABA) type A receptors are activated [1]. This is an antipsychotic agent approved by the US Food and Drug Administration (FDA) in 2004 use to decrease alcohol hankering after excess alcohol detoxification [2].

A paragraph on acamprosate calcium was introduced by Lipha, a potentiometric titration method for the assay of the drug and HPLC method for the determination of Homotaurine, which are the precursors of the synthesis and potential degradation product of acamprosate [3]. Acamprosate tablet 333 mg was available in the market since 1989 [4].

Drugs that are easily absorbed from the gastrointestinal tract and having a short half life are eliminated quickly from the blood circulation. However, the intestinal epithelium may constitute a permeability barrier for the absorption of orally administered drugs. This problem stimulated a search for new strategies to overcome mucosal barriers. (Samir Shah, 2018), All values were expressed as mean ± S. D. unless otherwise stated. Statistical differences were assumed to be reproducible when p <0.05 (two-tailed t-test). Swapnil J. Dengle, [5].

The data are presented as Mean ± SEM. The significance of the observed difference in the pharmacokinetic parameter after treatment was assessed by students unpaired t-test. A value of P<0.05 was considered to be statistically significant. TE Gopala Krishna Murthy [6].

The sample preparation involves either liquid-liquid extraction with ethyl acetate, methyl-tert-butyl ether and protein precipitation or solid-phase extraction using C8 and cyclohexyl, cartridges. SR Shinde [7].

Pharmacokinetic profile is linear with respect to dosage, its bioavailability is close to 100%, it undergoes only insignificant hepatic metabolism to inactive metabolites Y.K. Naidu [8].

Worldwide alcohol dependence is a severe problem. According to the World Health Organization, approximately 4% of all deaths in the world, are caused by alcohol abuse (World Health Organization [9]).

Estimates of alcohol dependence is reported to be 12.5% and the price of the health care resulting from alcohol abuse is estimated at more than US$ 26 billion per year (Wright and Myrick, 2006; Saivin et al. [2]. However, these methods suffer from several drawbacks like lack of assessment of matrix effect [10]. Acamprosate is the newly permitted drug in the United States for treatment of alcohol dependence. It is structurally similar to gamma-amino butyric acid (GABA) and the inhibition of neuronal hyper excitability mediated by antagonism or modulation of activity at the NMDA receptor may be one explanation of mechanism of action (Wright and Myrick, 2006; Saivin et al. [2] Scott et al., 2005). Acamprosate tablets have been in clinical use for more than 10 years for the indication of maintaining abstinence in alcohol-dependent.

A Bioanalytical method for the determination of acamprosate from human plasma, dog plasma and urine was reported by ample of authors. [11-14].

Acamprosate calcium is a white and odorless powder. It is freely soluble in water and insoluble in absolute ethanol and dichloromethane. The chemical structure is C$_{10}$H$_{20}$N$_2$O$_4$S$_2$Ca and the molecular weight is 400.48.

A detailed literature survey reveals capillary zone electrophoresis methods [11-13], the bioanalysis of Acamprosate calcium was done by using LCMS [11-22]. We here report a totally new, precise, accurate and linear isocratic LCMS method for the quantitative estimation of

| Parameter | Value |
|-----------|-------|
| $T_{\text{max}}$ | 8.54 ± 5.24 and 10.71 ± 5.41 h |
| $C_{\text{max}}$ | 146.06 ± 99.73 and 115.01 ± 26.26 ng · mL$^{-1}$ |
| AUC$_{0-t}$ | 1391.95 ± 731.24 and 1557.03 ± 962.84 ng·mL$^{-1}$·h |
| AUC$_{0-\infty}$ | 1987.40 ± 962.84 and 2720.21 ± 1931.79 ng·mL$^{-1}$·h |

Conclusions: As per regulatory guidelines, pharmacokinetics parameters for acamprosate calcium were found to be within the acceptance criteria.

Keywords: Bioequivalence; Pharmacokinetics.
Acamprosate calcium in ACAMPROL Human Plasma analysis.

Compliance with good laboratory practices (GLP) for conducting sample analysis of nonclinical (also known as preclinical) laboratory studies and clinical studies are intended to ensure the quality and integrity of the safety data filed in support of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation [1-7].

In varieties of matrices like human plasma, and dog urine, dog plasma. Among all reported methods, LC-MS method gives the best results. Ghosh C. et al explained more about the matrix effect of acamprosate in biological matrices and they developed the method by using precipitation extraction method and reported for quantitation with linearity range 7.04 to 702.20 ng/mL using LCMS in Human Plasma. As of our knowledge the reported method does not provide the stable, easily reproducible method for a matrix with high sensitivity.

The present study aimed to explore the pharmacokinetics parameters of acamprosate calcium in healthy male subjects after the administration of single and multiple doses to evaluate the bioequivalence of two formulations beneath fasting conditions of acamprosate calcium.

2. METHODS

2.1 Study Design and Drug Administration

A randomized, open label, single dose, two-treatment, two-period, two-sequence, crossover bioequivalence study of Acamprosate Calcium Delayed Release Tablets 333 mg in 12 healthy, adult, human subjects under fasting condition was conducted. A total of 12 subjects were enrolled in the study. A total of 12 subjects completed both the periods of the study successfully. Plasma samples of 12 subjects were analyzed and data was considered for statistical analysis. The washout period between each treatment in a stage and between each stage was 1 week. Fig. 1 shows the flowchart of the whole study. In the first stage, each subject received “test” or “reference” formulation of 333 mg acamprosate calcium tablets randomly under fasting conditions (overnight fast for 12 h). The study drug was administered with 200 mL water. Additional water intake was permitted 2 h after dosing.

The study was carried out with all stipulations in accordance with the Declaration of Helsinki, in compliance with the Study Protocol, SOPs, and requirements of ICH-GCP guideline, CDER guideline, the principles enunciated in the Declaration of Helsinki and relevant National Laws and Regulations.

The bioequivalence of the “test” Product A was compared to “reference” Listed Drug B, determined by analyzing Acamprosate from human plasma, which was obtained from 29 blood samples, with 96.00 hours as the last sampling point. Analysis of Acamprosate was carried out using a LC-MS/MS analytical method. The pharmacokinetic parameters Cmax, AUC0-t, AUC0-inf, Tmax, t½ and Kel were estimated for each subject and treatment based on the data generated in this study, the 90% confidence Interval for geometric means ratio (“test” Product A / “reference” Listed Drug B) on log transformed Cmax, AUC0-t and AUC0-inf didnot fall within acceptable bioequivalence limits of 80.00% to 125.00% for Acamprosate calcium. Hence, “test” Product A was not bioequivalent with the “reference” Listed Drug B.

Fig. 1. Structure of acamprosate calcium
2.2 Study Population

Healthy male volunteers aged from 18 to 40 and with a body mass index between 19 and 24 kg/m² were eligible for recruitment. Additional inclusion criteria included a healthy status confirmed by medical history, physical examination, 12-lead ECG, and laboratory tests (hematology, blood biochemistry, hepatic function, urinanalysis, hepatitis B surface antigen, tests for alcohol and other drugs of abuse) and nonsmoking status. Those with any allergic history or history of cardiac, pulmonary, renal, hepatic, gastrointestinal, or hematologic abnormality or any other acute or chronic disease were excluded.

Subjects were screened for demographic data, medical history, physical examination, 12 lead ECG, hematology, biochemistry, serology and urine analysis. Urine screen for drugs of abuse was done before check-in for each study period. Breath alcohol test was done before check-in for each study period and at each ambulatory sample visit.

2.3 Formulation

Acamprosate calcium enteric-coated tablets (CAMPRAL®, 333 mg; lot no. A294744) purchased from Merck Santé s.a.s. and acamprosate calcium enteric-coated tablets (333 mg) were used as the “reference” and “test” formulations, respectively.

2.4 Sampling and Medical Supervision

Total number of blood samples: Twenty nine (29), Sampling hours: Pre-dose sample (collected within 1 hour prior to drug administration), 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 9.00, 9.50, 10.00, 10.50, 11.00, 12.00, 16.00, 20.00, 24.00, 48.00, 72.00 and 96.00 hours (post-dose) within 2 minutes of scheduled sampling time (except for ambulatory sample which was collected up to 4.00 hours from the scheduled time), Blood samples were collected in pre-labeled K3EDTA vacutainers. The subjects were under continuous medical supervision throughout the study. Tolerability was evaluated by monitoring adverse events, physical examinations, 12-lead ECG, and laboratory tests. All laboratory tests were performed at the in house laboratory, which was authenticated by external authority.

2.5 Method for Sample Analysis

Plasma acamprosate calcium was quantified by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed and validated before the clinical study. API 4000 LC-MS/MS system and ZIC®-HILIC, 4.6×150 mm, 5μ was used. Nimodipine used as internal standard. The solid phase extraction used to extract Acamprosate and Nimodipine (Internal standard) from plasma samples. The calibration curve was relied on the concentration of 10.01 ng/mL to 709.36 ng/mL for acamprosate in human plasma.

Mass Spectrometry API 4000 triple quadrupole instrument from (AB Sciex) frontend was used as Ultra Performance Liquid Chromatography from Shimadzu. Data processing was done by using Analyst software 1.6.3.

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization were in the negative ion mode. Sources dependent parameters were as follows. Ion Spray Voltages 4500 and temperature was 400°C. The compound dependent parameters such as declustering potential (DP), Focusing potential (FP), Entrance potential (EP), Collision Energy (CE) and cell exit potential (CXP) were optimized during tuning as 60, 400, 12, 30 and 15 eV for acamprosate and 40, 400, 8, 30 and 5 eV for Nimodipine respectively. The collision activated dissociation (CAD) gas was set at 3 psi by using nitrogen gas. Dwell time was set at 200 msec for acamprosate and Nimodipine. The mass transition was selected as 179.9 – 80.0 for acamprosate and 417.0-122.0 for Nimodipine.

A hybrid method is used for separation of drug from plasma. Acetonitrile used for precipitation and solid phase extraction is used for separation of drug from plasma. The 300 μL of plasma was taken into the RIA vial, then 50 mL of internal standard was added and vortex approximately, followed by addition of 0.5 mL of 0.1% formic acid in acetonitrile and vortex for 2 minutes. These samples were loaded to SPE cartridges Orochem 1CC phospholipids cartridges which were pre-conditioning with 1 mL methanol followed by 1 mL water. The supernatant liquid of samples was loaded and eluted liquid directly transferred to the auto sampler vial and 5µL samples injected from the auto sampler vial in optimized chromatographic condition.
The limit of quantification (LOQ) in plasma was 10.01 ng·mL$^{-1}$. Recovery was determined on three different levels. (LQC, MQC, and HQC) Recovery was found 77.69, 82.89 and 79.11 respectively; the intraday RSD less than 5% and inter-day RSD less than 9%. Matrixes effect of acamprosate calcium was below 12%. The results of all stability studies were found within the acceptance criteria.

2.6 Selection of Study Population

The selection of the study population is based on the screening reports (demographic data, medical history, physical examination, vital signs, 12 lead ECG, clinical laboratory tests), inclusion and exclusion criteria as per study protocol. The subjects are screened and enrolled from the local population.

2.7 Screening and Enrollment

Prior to screening of potential subjects, the written Consent for Screening is obtained. All subjects are provided information about the study through the Subject Information Sheet and Informed Consent Form. Subject selection is done on the basis of the inclusion and exclusion criteria, specified in the Study Protocol. Subjects are deemed healthy on the basis of their demographic data, medical history, physical examination, 12 lead ECG and clinical laboratory results including hematology, biochemistry tests, serology and urine analysis. Subject Information Sheet is explained and the Informed Consent obtained from the participating subjects, on the IEC approves Informed Consent Forms, prior to their enrolment. A total of 12 healthy, adult, human subjects are enrolling in this study. Subjects are admit to the clinical facility for the study.

Approximately, 12.00 hours prior to dose administration i.e., on the day of check-in of both periods. Urine screen for drugs of abuse was done before check-in for each study period. Breath alcohol test was done before check-in for each study period and at each ambulatory sample visit.

2.8 Demographic Data

The mean age, weight, height and BMI of all enrolled subjects were 31.2 years, 61.5 kg, 167.7 cm and 21.8 kg/m$^2$ respectively. All subjects participating in this study were males of Asian origin. The demographic data of subjects was found as per the requirement of guidelines.

2.9 Dose Administration, Blood Sample Collection and Processing

Subjects were administered a single oral dose of one tablet of "test" Product A or "reference" Listed Drug B orally, in a crossover fashion according to the Formulation Randomization Schedule with 240 mL of water in sitting posture. It was ensured that the Tablet was swallowed by the subject by performing a thorough check of the oral cavity, by using a tongue depressor and flash light immediately after drug administration.

The drug was administered in a staggered manner to maintain subsequent blood collection schedule, the time for dosing of each subject in both study period.

Subjects did not remain in a reclining position for the first two (02) hours, after drug administration, in each study period. Drinking water was not allowed from 1.00 hour before drug administration until 1.00 hour after dose except during administration of the dose. Record of drug administration for individual subject was maintained in Case Report Form. The pre-dose blood sample was collected within 1 hour prior to drug administration. Total number of blood samples per period: Twenty nine (29) Sampling hours: Pre-dose sample (collected within 1 hour prior to drug administration), 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 9.00, 9.50, 10.00, 10.50, 11.00, 12.00, 16.00, 20.00, 24.00, 48.00, 72.00 and 96.00 hours (post-dose) within 2 minutes of scheduled sampling time (except for ambulatory sample which was collected up to 4.00 hours from the scheduled time). Blood samples at 48.00, 72.00 and 96.00 hours post-dose was collected as ambulatory blood sample. All the blood samples were collected in pre-labeled K3EDTA vacutainers. Blood sample of 5 mL was collected at each sampling point in both the study periods.

2.10 Pharmacokinetics and Bioequivalence

Pharmacokinetic parameters of acamprosate calcium were calculated with Win Nonlin Version 5.3 (Pharsight Corporation, Mountain View, California) by non compartmental analysis method. $C_{\text{max}}$ and $T_{\text{max}}$ were obtained directly...
from the concentration – time data. \( \text{AUC}_{0-t} \) was calculated using the linear trapezoidal rule. \( \text{AUC}_{0-\infty} \) was calculated as the sum of \( \text{AUC}_{0-t} \) and \( C_t/\lambda \). \( C_t \) was the last measured concentration and \( \lambda \) was the slope of linear regression of the log-transformed concentration – time curve, and \( t_{1/2} \) was calculated as 0.693/\( \lambda \).

The statistical method for testing bioequivalence was based upon the 90% Confidence Interval for the Ratio of the geometric means ("test" Product A/ "reference" Listed Drug B) for \( \text{C}_{\text{max}} \), \( \text{AUC}_{0-t} \) and \( \text{AUC}_{0-\infty} \) of log-transformed data. The geometric mean value was reported for the log-transformed parameters. Ratios of means were expressed as a percentage of the LSM for the "reference" treatment.

The relative bioavailability of the "test" formulation was calculated as \( F = \text{AUC}_{0-t} \) (test)/\( \text{AUC}_{0-t} \) (reference) \times 100%. 90% CIs for the test/reference ratio of log-transformed \( \text{C}_{\text{max}} \) and \( \text{AUC} \) were evaluated by analysis of variance (ANOVA) using Win Nonlin Version 6.1. \( T_{\text{max}} \) was tested by paired Wilcoxon test for significant differences. Test Product A was considered as Bioequivalent with the Reference Listed Drug B, if 90 % Confidence Interval for ratio (Test Product A/ Reference Listed Drug B) of geometric means based on log transformed \( \text{C}_{\text{max}} \), \( \text{AUC}_{0-t} \) and \( \text{AUC}_{0-\infty} \) falls within acceptable Bioequivalence limits of 80.00% to 125.00% for *Acamprosate calcium.

**3. RESULTS AND DISCUSSION**

**3.1 Pharmacokinetics of Acamprosate**

Six different screen lots of human plasma along with haemolyzed and lipemic plasma collected from the different donors for selectivity. This screened plasma used for validation experiments to test for interference at the retention time of analyte and internal standard.

There was no adverse event during the study. No significant abnormalities were reported in the post-study physical examination, vital signs and 12 lead ECGs and post study Clinical Investigation reports for all subjects.

**3.2 Pharmacokinetic Study**

This validated method was used for the determination of Acamprosate in plasma samples to study the bioequivalence of delayed release acamprosate calcium tablets 333 mg in the healthy adult human subject under fasting conditions. There was no adverse event reported during the study. No significant abnormalities were reported in the post-study physical examinations, vital sign, ECG and post clinical investigation report for all subjects.

The mean plasma concentration of analyte versus time profile was mentioned in the Fig. 2.

Plasma concentrations of the acamprosate were within the calibration curve range. The pharmacokinetic parameters for the "test" and "reference" are mentioned in Tables 1-2. The LSM mean ratio of \( \text{AUC}_{0-t} \), \( \text{AUC}_{0-\infty} \) was higher than 90% as per the bioequivalence guidelines. The LSM ratio (Test/Reference) and 90% confidence interval for the bioequivalence study was concluded between 80-125%. Therefore, it can be concluded that the two acamprosate formulations (Test and Reference) analyzed are bioequivalent in the pharmacokinetics of fasting conditions.

![Concentration Mean Plot against Time](image_url)

**Fig. 2. Concentration mean plot against time**
Table 1. Mean pharmacokinetics parameters

| Pharmacokinetics Parameters | Reference | Test       |
|----------------------------|-----------|------------|
| AUC$_{0-t}$                | 1557.03   | 1591.95    |
| AUC$_{0-\infty}$           | 2720.21   | 2987.4     |
| C$_{\text{max}}$           | 115.01    | 122.06     |
| K$_{\text{el}}$            | 0.06      | 0.07       |
| T$_{\text{max}}$           | 7.5       | 7.6        |

Table 2. Pharmacokinetics Parameters

| Pharmacokinetics Parameters | AUC$_{0-t}$ | AUC$_{0-\infty}$ | C$_{\text{max}}$ |
|-----------------------------|-------------|------------------|------------------|
| Test / Reference Ratio      | 87.57       | 91.24            | 95.87            |

3.3 For Log-transformed Data

Ratio and 90% Confidence Interval for C$_{\text{max}}$, AUC$_{0-t}$ and AUC$_{0-\infty}$ C$_{\text{max}}$

For log - transformed data, the C$_{\text{max}}$ ratio between the Test Product - A and the Reference Listed Drug B was found to be 132.92% and 90% Confidence Interval of it was 80.74% (Lower limit) and 215.55% (Upper limit) AUC$_{0-t}$

For log- transformed data, the AUC$_{0-t}$ ratio between the Test Product A and the Reference Listed Drug B was found to be 100.77% and 90% Confidence Interval of it was 62.01% (lower limit) and 163.76% (upper limit) AUC$_{0-\infty}$

For log transformed data, the AUC$_{0-\infty}$ ratio between the Test Product A and the Reference Listed Drug B was found to be 60.81% and 90% Confidence Interval of it was 42.46% (lower limit) and 87.11 % (upper limit)

3.4 Range, Mean and SD of C$_{\text{max}}$ and T$_{\text{max}}$

The C$_{\text{max}}$ for Test Product A ranged from 47.65 to 341.98 ng/mL, with mean SD of 146.06 ± 99.73 ng/mL 122.06 ± 99.73 ng/mL. The C$_{\text{max}}$ for the Reference Listed Drug B ranged from 32.58 to 341.27 ng/mL with a Mean ± SD of 115.01 ± 86.26 ng/mL.

The T$_{\text{max}}$ for the Test Product A ranged from 4.50 to 24.00 hours with a Mean ± S.D. of 8.54 ± 5.24 hours.

The T$_{\text{max}}$ for the reference Product B ranged from 4.50 to 20.00 hours with a Mean ± S.D. of 10.71 ± 5.11 hours.
3.5 Elimination Rate Constant (Kel) and Elimination Half-Life (t½)

The individual elimination rate constants and elimination half-life for the Test Product A and the Reference Listed Drug B the Mean ± S.D. values were found to be 17.61 ± 17.42 hours and 32.74 ± 47.60 hours respectively for untransformed Data.

Elimination Rate Constant (Kel) the Mean ± S.D was found to be 0.07 ±0.05 hours⁻¹ and 0.06 ± 0.05 hours⁻¹ for Test Product A and for Reference Listed Drug B respectively of untransformed Data.

4. CONCLUSION

A randomized, open label, single dose, two-treatment, two-period, two-sequence, crossover bioequivalence study of delayed release Acamprosate Calcium Tablets 333 mg in 12 healthy, adult, human subjects under fasting condition was conducted, to determine the bioequivalence of the Test Product A and Reference Listed Drug B by evaluation of the Pharmacokinetic parameters $C_{\text{max}}$, $\text{AUC}_{0-t}$, and $\text{AUC}_{0-\text{inf}}$ for Acamprosate.

The bioequivalence was determined, by analyzing Acamprosate from human plasma, obtained from 29 blood samples, with 96.00 hours as the last sampling point for twelve (12) subjects completing the clinical part of this study. Analysis of Acamprosate was carried out using a prevalidated LC-MS/MS method. The pharmacokinetic parameters $C_{\text{max}}$, $\text{AUC}_{0-t}$, $\text{AUC}_{0-\text{inf}}$, $T_{\text{max}}$, Kel and $t\frac{1}{2}$ were calculated for each subject and treatment.

The Ratio and 90% confidence limits of log transformed pharmacokinetic parameters $C_{\text{max}}$, $\text{AUC}_{0-t}$ and $\text{AUC}_{0-\text{inf}}$ for the Test Product A, Reference Listed Drug B were calculated and compared. Based on the data generated in this study, the 90% Confidence Interval for geometric means ratio (Test Product A/Reference Listed Drug B) on log transformed Cmax, $\text{AUC}_{0-t}$ and $\text{AUC}_{0-\text{inf}}$ fall within acceptable bioequivalence limits of 80.00 % to 125.00 % for Acamprosate calcium.

In this pilot study, the Test Product A was not found to be bioequivalent with the Reference Listed Drug B. However, the intra-subject coefficient of variation is observed in this study which may be due to variability in the data. Hence if a study would be conducted with more number of subjects, there would be a good chance that the two products may prove to be bioequivalent.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by producing company rather it was funded by personal efforts of the authors.

CONSENT AND ETHICAL APPROVAL

The study protocol was approved by the Independent Ethics Committee. All subjects provided written informed consent.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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