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FORMULATION AND EVALUATION OF CLARITHROMYCIN – LOADED SELF - MICRO EMULSIFIED DRUG DELIVERY SYSTEM

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ABSTRACT: Clarithromycin is a lipophilic and poorly water-soluble macrolide antibiotic which belongs to class II of the biopharmaceutics classification system. Its oral absorption and related bioavailability are dissolution rate-limited and relatively low. Subsequently, the formulation of oral clarithromycin delivery systems poses a huge challenge to the formulation scientist. Utilization of self - micro emulsifying drug delivery system technology, with the intent of enhancing oral bioavailability of clarithromycin was designed as this study's principal objective. Following successful screening studies involving solubility, compatibility and phase evaluations, series of clarithromycin – loaded self - micro emulsifying drug delivery system formulations were developed. Formulations were subjected to optimization and characterization analyses, from which formulation C2A with compositions (% w/w) of olive oil 5%, Tween 80: ethanol (4:1) 94.42%, clarithromycin 0.5% and aspartame 0.08% was optimized. The optimized/ test formulation was subjected to stability, *in-vitro* drug release, and pharmacokinetic analyses. Estimated parameters included a droplet size of 16.30 ± 3.31 nm, polydispersity index of 0.203 ± 0.11, and zeta potential of -2.01 ± 1.56 millivolts. Test and reference clarithromycin formulations exhibited comparable drug release profiles, inferring pharmaceutical equivalence. The test formulation was stable over a six – month period. No significant difference, at a probability level of 0.05, was observed between the two formulations with respect to pharmacokinetic parameters investigated. Forest plot constructed for test and reference formulations showed compliance with FDA standards, indicating bioequivalence character. The study indicates test formulation's potential of being used as a possible alternative to reference clarithromycin.

INTRODUCTION: Drug discovery techniques which are currently being pursued, have led to a significant increase in the number of poorly water-solublele drug candidates.

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More than 40% of newly discovered pharmacologically active chemical entities are found to be lipophilic and only sparingly soluble or entirely insoluble in water but may possess adequate membrane permeability.

Majority of these molecules belong to class II of the biopharmaceutics classification system (BCS) [1, 2]. Typical examples include clarithromycin, tamoxifen, griseofulvin, fenofibrate, dexamethasone, saquinavir, and cyclosporine A. They have low oral bioavailability and further exhibit high inter- and

intrasubject variabilities as well as a deficiency in the conventional direct dose-response proportionality relationship [3, 4]. However, some of them are established to exhibit enhanced bioavailability when co-administered with fatty- or oily- rich meals or food substances [5]. Examples of molecules in this category are halofantrine, danazole, and griseofulvin. One of the potential challenges facing the formulation scientist is the design and development of these molecules into credible, scientifically, and pharmaceutically acceptable oral delivery systems that possess adequate levels of oral bioavailability. However, judging from the enormous clinical and/or pharmacological potentials of these bioactive molecules, as depicted by those of clarithromycin, there is the urgent and critical need to develop appropriate strategies and/or measures to resolve this oral formulation challenge. A couple of pharmaceutical technique has been explored and applied in a variety of attempts in overcoming some of these solubility, dissolution, and bioavailability challenges. These techniques include micronization, solid dispersions, and use of permeation enhancers [6, 7]. Others are complex formation with cyclodextrins as well as increased solubilization with surfactants and co-surfactants [8, 9]. These techniques have been subjectively applied with a variable level of successes and related limitations. Indeed, some of these approaches have yielded credible results in a couple of selected cases. In recent times, the utilization of lipid-based drug delivery systems has emerged as one of the most popular approaches to improving the oral bioavailability of some of these molecules [10, 11]. This approach has been successfully applied to improve the oral bioavailability profiles of cyclosporine A (Neoral), ritonavir (Novir), saquinavir (Fortovase), efavirenz (Sustiva) and clofazamine (Lamprene). It is partly on the bases of these observations that this study was designed to investigate whether the lipid-based self - micro emulsifying drug delivery system (SMEDDS) technology can be utilized as an effective tool in resolving the oral formulation challenges of clarithromycin, a poorly water-soluble and hydrophobic molecule.

MATERIALS AND METHODS:

Materials: Clarithromycin reference standard C – 9742 (> 95% w/w purity) and clarithromycin industrial standard PH – 1038 were procured from Sigma – Aldrich Co. (St. Louis, MO, USA). Industrial standard erythromycin (66.13% w/w purity) was purchased from Unimax Chemicals Private (PVT) Ltd. India. Acetonitrile, ultra-high purity acetic acid, and diethyl ether solvent systems of liquid chromatography-mass spectrophotometric grade were purchased from Merck Millipore (Billerica, MA, USA). Reagent grade sodium carbonate anhydrous was a bought of amazon.com. Deionized water was produced as and when required at the Pesticide Residue Laboratories of the Ghana Standards Authority, Accra. Reagent grade formulation excipients, including olive oil, castor oil, vitamin E, and soybean oil were purchased from Merck Chemical Company, Germany. Ethanol, Tween 80, Tween 20, Span 80, Span 20, PEG 200, PEG 400 and propylene glycol of reagent grade were also purchased from Merck Chemical Company, Germany. A reference clarithromycin granule/suspension 25 mg/ml (Fromilid brand, manufactured by KRKA d.d. novo mesto, with batch number NA 8977 and expiry date 04/2019), as well as phosphate buffer saline of pH 6.8, were also utilized.

In this current study, animals were handled most appropriately in accordance with the Animal Welfare regulations (Public law 99 – 198, Food Security Act of 1985, subsection F – Animal welfare). Furthermore, due recognition and respect were accorded the Public Health Services Policy on Humane Care and Use of Laboratory Animals before its subsequent implementation in the handling of the rabbit. Serum was harvested from whole blood samples of rabbit (New Zealand White rabbit), while a pharmacokinetic study was conducted based on the same animal model.

Equipment and instrumentation requirements for this study included Agilent 1290 HPLC system, Agilent 6460 triple – quadrupole LC/MS detector, and Agilent Zorbax Eclipse - Plus C_{18} (100 mm x 2.1 mm, 1.8 µm) column (Agilent Technologies, Santa Clara, CA). Others were BD vacutainer tubes of 2.0 ml capacity with clot activator and gel separator (BD Berliner industrial estate, Plymouth, UK), Oven (Bench oven UF 30, Memmert GmbH & Co.KG), Refrigerator (LSFC324UK, Upright Lab Fridge Freezer 324L Lec Medical UK), Centrifuge (Rotofix 32A, Medical expo, Andreas

Hettich GmbH & Co.KG), Flask shaker (Stuart Tube and Flask shaker SF1, Camlab UK) and thermo water bath system (ThermoFisher Scientific Precision™ General Purpose Bath). Other relevant equipment included the following: ultraviolet-visible spectrophotometer (UV-1800 double beam Spectrophotometer, Perkin Elmer, Germany), DT 6; USP II dissolution apparatus (Erweka GmbH, Germany), Malvern Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, UK), SpectraPor® Floate-A-Lyzer® G2 dialysis device (volume 5 ml, MWCO 0.1 – 0.5 kD, Spectrum Laboratories, USA), Binder stability chamber (model APT. LineKBF – ICH 240, USA), Sonicator (Bandelin sonorex® RK 510, Bandelin electronics, Germany) and Rotary evaporator (RC 900: KNF Neuberger, Trenton, NJ, USA). GraphPad Prism software (version 7.04, developed by GraphPad software Inc.), ProSim ternary diagram software (version 1.0, developed by ProSim Inc.) and PK Solution software (version 2.0, developed by Summit Research Services) were also utilized.

Methods:

HPLC - MS/MS Method Development and Validation: A reverse-phase high-performance liquid chromatography-mass spectrophotometric analytical method for determinations of clarithromycin in both formulations and biological systems was successfully developed. In this method development, the chromatographic process was investigated on an Agilent 1290 L/C system that was equipped, among others, with a binary pump, binary solvent manager, autosampler, degasser, refrigeration unit, and a column oven. The separation was performed on an Agilent Zorbax Eclipse - Plus C_{18} (100 mm × 2.1 mm, 1.8 μm) column at an oven temperature of 40° C. The mobile phase consisted of acetonitrile and 0.1% acetic acid in water and the gradient chromatographic flow program was selected.

Following a couple of screening analyses, a flow rate of 0.5 ml/min, an injection volume of 10 μl, and a chromatographic run time of 13 min were optimized for this method development. Validation of the method was assessed under these optimized conditions. A triple - quadrupole mass spectrophotometer that was coupled with electrospray ionization source was the detector system utilized. Detection was conducted in the multiple reaction monitoring (MRM) mode under optimized conditions of ion spray voltage 4000 V, fragment or voltage 110 V and collision energy 25 eV or 15 eV. The MRM pairs were *m/z* 748.5→*m/z* 158.2 for clarithromycin and *m/z* 734.5→*m/z* 158.0 for erythromycin. Stock standards, working standards, and standard calibration solutions of clarithromycin and erythromycin the internal standard were prepared and utilized under the above-mentioned conditions in regular runs to develop the method. Optimized and acceptable analyte concentration range, correlation coefficient, and cumulative linear regression equation were among other relevant parameters that were estimated from this analysis. Validation of the method was conducted by utilizing the guidelines provided by the international conference on harmonization [12]. Nominal and quality control solutions of clarithromycin in both solvent and drug – free serum were prepared and engaged in the validation process. By utilizing a liquid-liquid extraction technique involving sodium carbonate and diethyl ether systems, quality control samples of clarithromycin and erythromycin were prepared. These were also used in the estimation of the response factor, which was the principal tool that was employed in this current study for the estimation of clarithromycin concentrations in serum. Validation parameters that were assessed included recovery, accuracy, and precision, as well as inter-day and intraday variability. For the estimation of the limit of detection (LD) and limit of quantitation (LQ), the signal to noise ratio technique was utilized.

Solubility Study: Solubility of clarithromycin was investigated in different types of lipid-based formulation excipients, which included olive oil, castor oil, vitamin E, and soybean oil. Series of surfactants that were investigated for the solubility analysis included Tween 20, Tween 80, Span 20, and Span 80. Co – surfactant systems employed were propylene glycol, ethanol, PEG 200, and PEG 400. These were selected from the 'generally recognized as safe – GRAS' category of lipid-based excipients list and the Inactive Ingredients Database [13]. The flask shake technique was the solubility method employed in this study. An excess amount of clarithromycin was added to 5 ml of each formulation excipient in respective vials and isothermally shaken for 48 hours at 25 ± 1 °C

after which it was observed for equilibrium. To those vials' indicating non – attainment of equilibrium, further clarithromycin crystals were added. All the mixture vials were subjected to a further 24 h isothermal shaking at 25±1.00 °C, making a total of approximately 72 h shaking, to ensure that a state of equilibrium was attained. The samples were centrifuged for 15 min at 3000 rpm. The concentration of clarithromycin in the supernatant was determined by the developed and validated HPLC – MS/MS method. These analyses were repeated thrice per each formulation excipient (n = 3).

Compatibility Study: Both physical and chemical compatibility analyses of clarithromycin with various surfactants and co-surfactants were employed in the surfactant/co-surfactant pair selection procedure. A fixed amount of each of the surfactant: co-surfactant (1:1) mixes was placed in a glass vial with a known amount of clarithromycin. The samples were stored at 25 °C for one month, after which they were visually observed for physical changes such as precipitation, phase separation, and color change. For chemical changes, the systems were subjected to quantitative analyses by the developed and validated HPLC – MS/MS method after the storage period.

Construction of Pseudo Ternary Phase Diagrams: To obtain an optimum composition for the clarithromycin - loaded SMEDDS formulation, the phase titration technique was utilized to generate related pseudo ternary phase diagrams. From the results of preliminary studies, olive was selected as the oil phase, Tween 80 as the surfactant, and both propylene glycol and ethanol were used as co-surfactant, respectively, in two modules of formulations that were investigated. A variety of surfactant: co-surfactant (Smix) systems in fixed ratios of 1:1, 2:1, 3:1, and 4:1 by weight were prepared. These mixtures (Smix) were dispersed in calculated amounts of oil to give a series of oil: Smix systems of weight ratios 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. Water was added dropwise to the above systems, at room temperature, and stirred continuously until the solution became cloudy or turbid. After each addition, the sample was examined with the naked eye for the appearance of turbidity, which was an indication of a phase boundary and the endpoint of the titration. Visual observations of transitions in the sample appearance from cloudy to transparent; and transparent to cloudy *etc*. was the tool employed in the phase boundaries identification.

The quantities of aqueous phase required to effect these respective changes were used to determine the boundaries of the homogenous and stabilized microemulsion zone corresponding to the selected optimum fixed weight ratios of oil and surfactant: co-surfactant mix system. ProSim ternary diagram software was employed in the generation of pseudo ternary phase diagrams.

Effect of Incorporation of Clarithromycin on Selected Systems: Investigations into the effects of incorporation of clarithromycin into the micro-emulsions were conducted. A therapeutic or dose amount of clarithromycin was dissolved in the above-selected systems, and the aqueous titration method, as described above, was utilized in the construction of the corresponding ternary phase diagrams.

Preparation of CLA – SMEDDS Formulations: A series of clarithromycin - loaded self - micro emulsifying drug delivery system (CLA – SMEDDS) formulations were prepared with varying ratios by weight of oil and optimized surfactant: co-surfactant system (Smix). The first module of formulations coded C1A, C1B and C1C were prepared by using olive as oil, Tween 80 as surfactant and propylene glycol as co-surfactant. The second module of formulations coded C2A, C2B and C2C were also prepared by using olive oil, Tween 80 as surfactant and ethanol as co-surfactants. In each module, three varieties of formulations were prepared by varying the ratio of oil at three different levels of 5%, 7.5% and 10% w/w with the optimized surfactant: co-surfactant (Smix) corresponding ratio of 95%, 92.5% and 90% w/w respectively.

For Tween 80: propylene glycol (Smix) systems, the largest one phase region indicating most stabilized micro emulsified zone was found in the 1:1 ratio system. This system was thereby selected for further development and preparation. For Tween 80: ethanol (Smix) systems, the largest one phase-stabilized micro emulsified region was found

in the 4:1 ratio system and hence selected for further development. The detailed compositions of oil, surfactant: co-surfactant (Smix) required for each CLA - SMEDDS formulation are as shown in **Table 3a** and **3b**.

In this present study, each formulation batch containing fixed proportions of the therapeutic dose of clarithromycin and the calculated amount of requisite excipient(s) - sweetener dissolved in varying ratios of oil and optimized surfactant: co-surfactant (Smix) were prepared via the self – micro emulsification technique. The oil and optimized surfactant: co-surfactant (Smix) were accurately weighed (or measured) into a 'dry' vial or bottle and thoroughly mixed by using a magnetic stirrer. Formulation amounts of clarithromycin and aspartame were accurately weighed and dispersed in this oil and surfactant: co-surfactants (Smix) system and were mixed by gentle stirring. This was followed by vortex at 37 °C until the drug was completely dissolved. The resultant formulation of clarithromycin - loaded self - micro emulsified drug delivery system (CLA – SMEDDS) were sealed in appropriate glass vials or bottles. These were suitably labeled and stored at room temperature until used and/or analyzed.

Optimization and Characterization of CLA – SMEDDS Formulations:

Physical Evaluation - Appearance: The CLA – SMEDDS formulations were gradually diluted with dropwise addition of distilled water up to a hundred times, and the transitional appearance of the resulting systems were examined by the naked eye under normal atmospheric conditions. Observations were made for changes in the formulation appearance following the dilution in terms of clarity through transparency to cloudy and *vice versa*.

Clarity: One milliliter of each CLA - SMEDDS formulation batch was diluted with water to a hundred milliliters. Aliquots of these diluted formulations were subjected to ultraviolet-visible spectrophotometric analysis at a detecting wavelength of 210 nm for clarithromycin. The resultant absorbance (A) value obtained was converted to the percentage transmittance (%T) parameter, which was estimated as the clarity parameter of the related formulation.

Thermodynamic Stability Study: These analyses, which involve heating - cooling stress cycles, centrifugation, and freeze-thawaw stress cycles, were employed to evaluate the thermodynamic stability of the formulations. Each of the pre-concentrated SMEDDS formulations was subjected to three cycles of between refrigerator temperature 5 °C and oven temperature 45 °C. A minimum of 48 h was provided as the storage period at each temperature limit. These were then visually observed for precipitation, phase separation, and color changes. The CLA - SMEDDS formulations were further subjected to centrifugation for 30 min at 3500 rpm. These formulations under this stress test were also visually observed for any signs of phase separation, precipitation, or color change.

The final stress test involved exposure of the formulations to three cycles of freeze-thawaw processes. The temperature limits for this test were – 21 °C and +25 °C, respectively, with a minimum of 48 h as a storage period at each temperature. These were also visually observed for stress test indices of precipitation, phase separation, and coloration changes.

Dispersity and Emulsification Speed Studies: Self – emulsification efficiency and emulsification speed of formulations were assessed by using a modified version of the Erweka GmbH DT 6, USP II dissolution apparatus technology [14]. One milliliter of each formulation was dispersed in five hundred milliliters of distilled water, and the entire system maintained at a temperature of 37 ± 0.5 °C. By using a rotating standard stainless steel dissolution paddle, these dispersions were agitated at a speed of 20 rpm. By visual observations, the in vitro performance of the formulations and related emulsification time were assessed via the grading scheme shown in **Table 1** [15].

Grade A and Grade B classified formulations shall produce and remain as self - micro emulsified systems when dispersed in the gastrointestinal tract. Formulations that are classified in Grade C may serve as possible candidates for SMEDDS development and may thereby be proposed. Formulations that are categorized in Grade D and Grade E are not potential candidates requiring development as SMEDDS [15].

TABLE 1: GRADES OF DISPERSITY AND EMULSIFICATION TIME STUDIES

S. no.	Observations	Emulsification time	Grade
1	Rapidly forming emulsion having a clear or slight yellowish color	Less than 1 min	A
2	Rapidly forming emulsion having slightly less clear or yellowish color	Within 1 min	B
3	Fine milky or creamy and viscous emulsion	Within 2 min	C
4	Dull, greyish white emulsion having slightly oil appearance that is slow to emulsify	More than 2 min	D
5	Formulation exhibiting poor emulsification with large oil globules present on the surface	More than 2 min	E

Droplet Size - Z, Polydispersity Index - PDI and Zeta Potential - ZP Analyses: Droplet size, polydispersity index, and zeta potential of CLA – SMEDDS formulations were determined by using the Malvern Zetasizer equipment technology [16]. One milliliter of each formulation was dispersed in twelve milliliters distilled water at a temperature of 37 ± 0.5 °C. A rotating magnetic stirrer that was placed in these systems was utilized as a source of agitation to produce related micro-emulsions that were used in these analyses. Droplet size and polydispersity index were assessed by using a clear disposable zeta cell or cuvette. Zeta-potential was determined by using the folded capillary zeta cell or cuvette.

Determination of Drug Content: Clarithromycin from selected CLA – SMEDDS formulation batches C1A, C2A, and C2B were extracted in acetonitrile by using a liquid-liquid extraction technique. The extracts were centrifuged at 3500 rpm for 15 min, and the supernatant filtered through a 0.22 µm membrane filter to obtain refined extracts of the analyte, clarithromycin. The developed and validated HPLC – MS/MS method was used to quantitatively determine the clarithromycin content in the formulations.

***In-vitro* Drug Release Study:** Drug release studies for the optimized CLA – SMEDDS formulation batch C2A, and a reference clarithromycin suspension were performed by using a modified version of the Erweka GmbH DT 6, USP II dissolution apparatus technology [14]. The release vessel was filled with 900 ml dissolution medium and operated at a temperature of 37 ± 0.5 °C at a rotating paddle speed of 50 rpm. Five milliliters of each formulation was introduced into a cylindrical cellulose ester dialysis membrane device; SpectraPor® Floate-A-Lyzer® G2 of 5 ml capacity and a molecular weight cutoff (0.1 – 0.5 Kd). This was conducted in six replicates per each formulation. The filled membrane tubes were floated or suspended vertically in the dissolution medium consisting of 900 ml phosphate buffer saline (PBS) at pH 6.8 and subjected to release analyzes *via* agitation under the above-stated conditions. Aliquots of samples (5 ml) were initially withdrawn just before agitation and further serially withdrawn from the receiver medium at predetermined time intervals of 20, 40, 60, 80, 100, and 120 minutes following the agitation process. In order to maintain sink conditions, an equivalent volume (5 ml) of fresh dissolution medium that was preheated at 37 °C was added after each sampling. The developed and validated HPLC - MS/MS method was used to quantitatively analyze the withdrawn samples for released clarithromycin content. The cumulative amounts of clarithromycin at each sampling time point were calculated. A comparison of drug release profiles was performed by estimation of difference factor (f1) and similarity factor (f2) by using equations (1) and (2), respectively.

Difference factor (f1) = $\{[\Sigma_{t=1}^{n}|Rt - Tt|] / [\Sigma_{t=1}^{n}Rt]\} \times 100$……………………Equation (1)

Similarity factor (f2) = $50 \times \log \{[1 + Wt\,(1/n)\,\Sigma_{t=1}^{n}(Rt - Tt)^2]^{-0.5} \times 100$..……..Equation (2)

Where Rt and Tt are the percentage mean cumulative drug dissolved/released at each of the selected n^{th} time point of the reference and test product respectively. Wt is optional weight which is assigned a value of unity (1) in conventional approach of (f2) estimation.

Stability Study: Stability study of the optimized/test CLA - SMEDDS formulation was conducted in accordance with the International Conference on Harmonization guidelines [17]. A modified version of the real-time, long - term stability approach was the technique employed in this study. Subsequent to this, freshly prepared, optimized CLA - SMEDDS formulation was appropriately packaged in sealed glass bottles and stored at 25 °C/60% RH in a binder stability chamber for a six - month period. At predetermined

time intervals of 0, 15, 30, 60, 90, 120, 150, and 180 days after storage, aliquots of samples were withdrawn and analyzed. Withdrawn samples were characterized for appearance, percentage transmittance, globule size, zeta potential, poly-dispersity index, and drug content. These parameters were compared with those prior to storage by using the quantitative statistics tool.

Pharmacokinetic Study: The pharmacokinetic profiles of test CLA – SMEDDS formulation and reference clarithromycin suspension were investigated in an animal model. A randomized, open-labeled, oral single - dose, and two - way crossover design was employed. Twenty (20) healthy white New Zealand rabbits, weighing 1.8 – 2.4 kg, with an approximate average weight of 2.0 kg were utilized. The animals were kept and maintained under normal atmospheric conditions of 12h day-night cycle at 25 °C with access to food and water. Animals were subjected to fasting for 12h with access to only water, prior to the beginning of the study. Based on random sampling, animals were divided into two groups, coded group A and group B. Each group of animals received to test and reference formulations alternatively after a washout period of fourteen days. Blood samples from animals were serially collected via the marginal ear vein at predetermined time intervals of 0, 0.5, 1.5, 2.5, 3, 3.5, 5, 9, 17, and 24 h post-dosingng. Serum was harvested from whole blood samples and stored at -20° C until further analysis. The developed and validated HPLC – MS/MS analytical method was used to determine clarithromycin concentrations in the serum samples and the resulting data utilized for pharmacokinetic analyses. In this current study, pharmacokinetic parameters were estimated from serum concentration-time data via a computer model by the Pharmacokinetic Solutions® software version 2.0: [18]. This utilizes the linear trapezoidal and curve – stripping techniques coupled with the non - compartmental analytical model.

RESULTS AND DISCUSSION:

HPLC – MS/MS Method Development and Validation: The HPLC – MS/MS analytical method that was developed in the current study revealed reproducible results within a validated calibration curve range of 1.00 – 1000.00 ɳg/ml. The correlation coefficient (r^2) following a cumulative regression analysis of the calibration curves was 0.9995. Intra-day precision and accuracy of the method were within the range 0.53 - 2.00% and 99.03 – 102.31% respectively, while the corresponding inter-day estimates were 0.04 – 0.67% and 100.05 – 100.41% respectively. The limit of detection (L_D) was estimated to be 0.0018 ɳg/ml, while the limit of quantification (L_Q) was 0.01 ɳg/ml. Accuracy and precision of quality control samples were 99.20 ± 0.67% and 1.52 – 2.00%, respectively. With respect to quantitative determinations of clarithromycin in serum, the results indicate the developed analytical method to be suitably validated, simple, sensitive, and reproducible. Subsequently, the method was successfully applied in pharmacokinetic investigations of clarithromycin formulations in an animal model.

Solubility Study: Results of solubility studies of clarithromycin in various vehicles are as represented in **Table 2**. Olive oil, Tween 80, and ethanol showed the highest solubilization capacity for clarithromycin in their respective group analysis. The excipients used in SMEDDS formulations should be able to solubilize the drug to a reasonably high degree in the resultant dispersion. Thereby for this present study, selected primary components were olive as oil, Tween 80 as a surfactant, and ethanol as co-surfactant for formulation development. Though propylene glycol did not display the highest clarithromycin solubility, it was further selected as co-surfactants in a parallel formulation development study, owing to its ready availability and accessibility.

TABLE 2: SOLUBILITY OF CLARITHROMYCIN IN VARIOUS OILS, SURFACTANTS AND CO – SURFACTANTS

Oil type	Solubility of CLA (mg/ml)
Olive oil	51.16 ± 0.01
Castor oil	45.75 ± 0.02
Soybean oil	38.86 ± 0.02
Vitamin E	50. 26 ± 0.04
Surfactant type	**Solubility of CLA (mg/ml)**
Tween 80	81.58 ± 0.11
Tween 20	33.22 ± 0.04
Span 80	55.25 ± 0.42
Span 20	35.42 ± 0.13
Co – surfactant type	**Solubility of CLA (mg/ml)**
PEG – 200	108.79 ± 0.03
PEG - 400	89.53 ± 0.24
Propylene glycol	45.16 ± 0.21
Ethanol	117.85 ± 0.31

All concentration values are stated as (mean ± s.d.): n = 3.

Compatibility Study: Results of the analysis of the compatibility of clarithromycin with oil and surfactant/co-surfactants pair selection indicated compliance with specifications. It was observed that none of the selected pairs of surfactant/co-surfactants and clarithromycin mix, as well as the oil and clarithromycin mix, showed any sign of physical changes in the form of color change, precipitation nor phase separation. Furthermore, the drug content in the combined systems after the storage period was estimated to fall within the range of 97% - 98% of the initial amount employed. This is an indication of the high solubilization capacity of the selected excipients for clarithromycin, which constitutes a relevant SMEDDS formulation development requirement.

Pseudo Ternary Phase Diagram Study: Pseudo ternary phase diagrams that were constructed are generally represented in **Fig. 1**. The selection of formulation components' analyses was conducted through two main ternary phase diagram modules. In the first module, olive oil was tested for phase transitions with Tween 80 and propylene glycol as the surfactant/co-surfactant system - Smix in varying ratios. An indirect relationship was observed as the microemulsion area decreases with a corresponding increase in the surfactant/co-surfactant ratio. Subsequently, a surfactant/co-surfactant system in a ratio of 1: 1 was selected in this module of the SMEDDS formulation development. In the second parallel study module, olive oil was tested for phase transitions with Tween 80 and ethanol as the surfactant/co–surfactant system – Smix. Under these conditions, it was observed that the microemulsion area increased as the surfactant/co-surfactant ratio was increased. Hence a surfactant/co-surfactant system in the ratio of 4: 1 was selected for this series of formulation development.

Clarithromycin was incorporated into these selected systems to investigate the possible effects on the zones of the stable micro and self-micro-emulsions. It was expected that the incorporation of clarithromycin would influence the phase behavior and the areas of micro and self - microemulsion formation. However, the study indicated an insignificant influence.

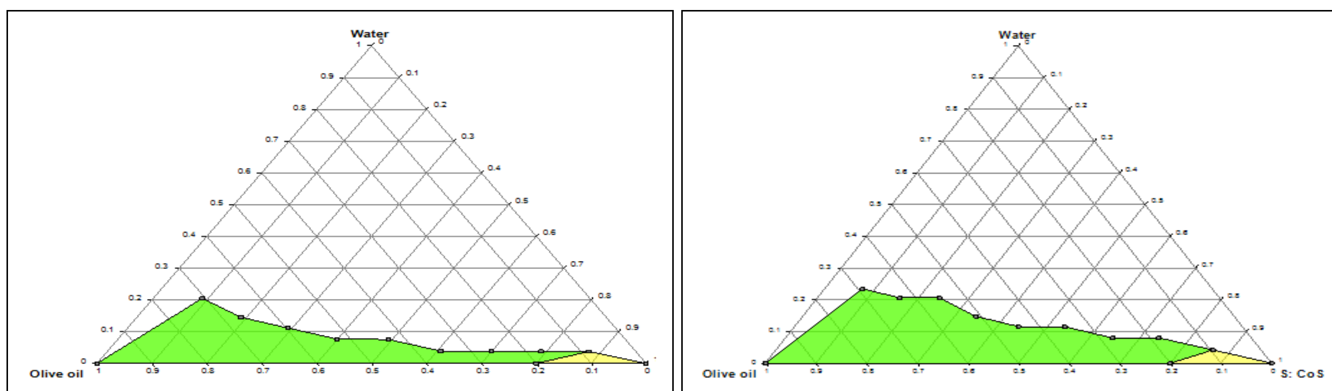

FIG. 1: PSEUDO TERNARY PHASE DIAGRAMS OF OLIVE OIL, TWEEN 80: PROPYLENE GLYCOL (1: 1) AND WATER SYSTEM (LEFT) AND OLIVE OIL, TWEEN 80: ETHANOL (4: 1) AND WATER SYSTEM (RIGHT).
Key: White-colored zone represents metastable macro emulsion; green-colored zone represents microemulsion, and the yellow colored zone represents a stable self – microemulsion system.

Preparation of CLA – SMEDDS Formulations: The detailed compositions of the CLA – SMEDDS formulations are as indicated in **Tables 3a** and **3b**.

TABLE 3A: FORMULATION INGREDIENTS COMPOSITIONS OF CLA – SMEDDS BATCHES C1A, C1B AND C1C

Formulation Batch	C1A		C1B		C1C	
Ingredients Compositions	Quantity (% w/w)	Quantity (g)	Quantities (% w/w)	Quantity (g)	Quantity (% w/w)	Quantity (g)
Clarithromycin	0.5	0.25	0.5	0.25	0.5	0.25
Olive oil	5.0	2.5	7.5	3.75	10.0	5.0
Tween - 80	47.21	23.605	45.96	22.98	44.71	22.355
Propyl. Glycol	47.21	23.605	45.96	22.98	44.71	22.355
Aspartame	0.08	0.04	0.08	0.04	0.08	0.04

TABLE 3B: FORMULATION INGREDIENTS COMPOSITIONS OF CLA – SMEDDS BATCHES C2A, C2B AND C2C

Formulation Batch	C2A		C2B		C2C	
Ingredients Compositions	Quantity (% w/w)	Quantity (g)	Quantities (% w/w)	Quantity (g)	Quantity (% w/w)	Quantity (g)
Clarithromycin	0.5	0.25	0.5	0.25	0.5	0.25
Olive oil	5.0	2.5	7.5	3.75	10.0	5.0
Tween - 80	75.536	37.768	73.536	36.768	71.536	35.768
Ethanol	18.884	9.442	18.384	9.192	17.884	8.942
Aspartame	0.08	0.04	0.08	0.04	0.08	0.04

Optimization and Characterization of CLA – SMEDDS Formulations:

Physical Evaluation – Appearance and Clarity Analyses: Results of analyses involving physical evaluations of CLA – SMEDDS formulations in terms of appearance and clarity (assessed in percentage transmittance - % T notation) following aqueous dilutions are as presented in **Table 4**. It was observed that formulation batches C1C and C2C, with the highest concentration of oil, initially became turbid upon dilution and subsequently converted to the macro emulsion at the hundred times dilution point.

This observation may be partly due to the high concentrations of oil which could not be solubilized by the given surfactant: co-surfactant system (Smix) and oil ratio. This may also be attributed to the concentration of the surfactant falling below its critical micelle concentration (CMC) under the prevailing circumstances. Percentage transmittance (% T) parameters for these systems were less than the standard 98% and thereby could not meet specifications of the clarity assessment test as per the ICH protocols. However, percentage transmittance parameters for formulation batches C1A, C1B, C2A, and C2B were all greater than the 98% standard limit and hence may be inferred to be clarity compliant.

While formulation batches C1A and C1B produced slightly clear macro emulsions upon a hundred times dilution, those of batches C2A and C2B remained clear under similar conditions, indicating a high clarity of the related microemulsions. Formulations batches C2A and C2B were thereby expected to possess droplet size within the range of microemulsions or SMEDDS and were considered to be potential candidates requiring further development.

TABLE 4: TRANSITIONAL APPEARANCES AND %T ASSESSMENTS FOLLOWING DILUTIONS OF CLA – SMEDDS FORMULATIONS

Formulation Batch	Transitional Appearances following 100 times dilution of formulations	%T at 210nm 100X dilution
C1A	Clear, transparent, slightly clear macro emulsion	99.60 ± 0.17
C1B	Clear, transparent, slightly clear macro emulsion	99.32 ± 0.14
C1C	Clear, turbid, slightly clear macro emulsion	55.04 ± 0.15
C2A	Clear, transparent, clear micro emulsion.	99.95 ± 0.12
C2B	Clear, transparent, clear micro emulsion.	99.98 ± 0.23
C2C	Clear, gel, turbid, slightly clear macro emulsion.	57.43 ± 0.18

%T values are expressed as (mean ± s.d.): n = 3.

Thermodynamic Stability and Dispersity Studies: Results of thermodynamic stability studies in terms of cooling - heating stress cycles; centrifugation and freeze-thawaw stress cycles as well as dispersity tests of the CLA - SMEDDS formulations are as presented in **Table 5**. With the exception of formulation batch C2C that formed fine milky emulsion within approximately two minutes and thereby appeared to have failed the dispersity test, all the remaining CLA - SMEDDS formulations passed. While formulation batches C2A and C2B exhibited instant formation of clear microemulsions in less than a minute, those of C1A, C1B and C1C formed rapid and slightly less clear emulsions within approximately one minute. Although formulation batches in the C1 series passed the dispersity test, they failed the freeze-thawaw cycle stress test. Furthermore, formulation batches C1B and C1C could not pass the heating-cooling cycle stress test either. Formulation batches C2A, C2B, and C2C passed all the thermodynamic stress tests.

TABLE 5: THERMODYNAMIC STABILITY AND DISPERSITY TESTS OF CLA – SMEDDS FORMULATIONS

Formulation Batch	H - C cycle	Centrifugation 3500 rpm	F - T cycle	Dispersity Grade	Inference
C1A	√	√	X	B	Failed
C1B	X	√	X	B	Failed
C1C	X	√	X	B	Failed
C2A	√	√	√	A	Passed
C2B	√	√	√	A	Passed
C2C	√	√	√	C	Failed

Key: A- clear microemulsion, B- slightly whitish, less clear emulsion, and C- whitish or milky emulsion: √- passed and X- failed. H – C: Heating – Cooling cycle. F – T: Freeze-Thawaw cycle.

On the bases of the overall results of the above optimization studies, formulation batches C1B, C1C, and C2C do not appear to be potential candidates requiring further development, whereas formulation systems C2A, C2B, and C1A seem to be potential candidates requiring further development and evaluation. However, all formulation batches were carried forward and subjected to the zeta-sizer based characterization analyses.

Droplet Size, Polydispersity Index and Zeta Potential Assessments: The results of droplet size, polydispersity index, and zeta potential assessments of the CLA – SMEDDS formulations are as presented in **Table 6**. Droplet size is a crucial factor in self-emulsification performance as it influences to a greater extent, both absorption and bioavailability profiles of drugs [19]. Documented reports have it that smaller droplet size facilitates the surfactant's potential of forming a better packed film at the oil-waterer interface, leading to improved stabilization of the oil droplets [20]. Formulation batches C1A, C1B, C2A, and C2B, possess droplet size measurements, which are below the 100 nm standard size requirement. This is an indication of the potential of developing these into stable SMEDDS formulations. Among the C2 formulation series, the least droplet size was observed in formulation batch C2A with mean droplet size 16.30 ± 3.31 nm in water. The analysis revealed the existence of a direct relationship between droplet size measurements and concentration levels of oil in the formulation. Thereby formulations with minimum droplet size were found to possess the least oil concentration while those with the highest oil concentration were found to possess the largest globule size, which was over and above the 100 nm standard. Based on these observations, formulation batches C1A, C2A and C2B were optimized and selected for further development and evaluation.

Results of zeta potential analyses of the CLA - SMEDDS formulations indicate that these parameter values for all the formulation batches were generally low ranging between negative (-) 3.92 to negative (-) 2.01 mV. This represents the existence of relatively weak repulsive forces within the micro-emulsion droplets. It has been established that an increase in the repulsive forces between micro-emulsion droplets prevents their coalescence, and subsequently lead to the formation of more stable deflocculated system [21, 22]. Thus, if the droplets are highly charged (positive if basic and negative if acidic), then a strong electrostatic repulsion will be set up between them which will prevent coalescence and flocculation. If the magnitude of the zeta potential is reduced, the system may become unstable as the electrostatic repulsion can no longer overcome the momentum of colliding particles leading to coalescence and flocculation [23]. Zeta potential values more than +30 mV or less than -30 mV typically offer high levels of stability [24]. The higher the 'absolute' magnitude of the zeta potential the more stable is the system. Zeta potentials of the optimized formulations C2A, C2B and C1A were -2.01, -3.32 and -3.29 mV, respectively. As a result of these relatively low zeta potential values estimated, the related influence on stability of SMEDDS does not appear to be so prevalent in these systems. It appears that the stability profile of these formulations is not zeta potential - limited.

Polydispersity index (PDI) analysis involves assessment of droplet size distribution or homogeneity in a given system. An ideal SMEDDS formulation should thereby be widely distributed with globules with size measurement less than 100 nm. Thus in a given sample, the constituent droplets having size measurement more than 100 nm should be maximum up to 33%. However, in practice, samples with PDI values of approximately

0.5 are generally regarded to be of good quality and thereby acceptable. The ratio of the standard deviation to mean droplet size of microemulsion may be used as an assessment of the related PDI, which is a reflection of the uniformity of droplet size distribution in the related system. An inverse relationship exists between the PDI and the extent of uniformity of droplet size distribution. Higher PDI values are associated with lower uniformity of droplet size distribution and vice versa [21]. The results of polydispersity index analysis for the CLA – SMEDDS formulations indicate that all the formulation batches have PDI values lower than 0.5, specifically within a range of 0.203 ± 0.11 to 0.536 ± 0.16. The lowest PDI value was observed in formulation C2A, which indicates the potential of developing this system into a more stable SMEDDS formulation.

TABLE 6: DROPLET SIZE, POLYDISPERSITY INDEX AND ZETA POTENTIAL OF CLA – SMEDDS FORMULATIONS

Formulation batch	Composition (%w) Oil: (S: CoS)	Droplet size (d nm)	Polydispersity index	Zeta potential (mV)
C1 A	5: 47.5: 47.5	16.48 ± 3.17	0.292 ± 0.01	-3.29 ± 2.93
C1 B	7.5: 46.25: 46.25	46.18 ± 12.49	0.271 ± 0.11	-3.41 ± 2.99
C1 C	10: 45.0: 45.0	236.70 ± 76.55	0.323 ± 0.13	-3.74 ± 2.91
C2 A	5: 76.0: 19.0	16.30 ± 3.31	0.203 ± 0.11	-2.01 ± 1.56
C2 B	7.5: 74.0: 18.5	21.88 ± 8.61	0.416 ± 0.12	-3.32 ± 2.64
C2 C	10: 72.0: 18.0	358.2 ± 150.55	0.536 ± 0.16	-3.31 ± 2.62

All values are expressed as (mean ± s.d.): n = 3

Drug Content Determination: The assay of clarithromycin in selected and optimized formulation batches CIA, C2A, and C2B revealed the content to fall within the range 98% - 99% (w/w) of the added formulation amount. This is an indication of compliance with content uniformity specifications as per ICH protocols of the formulation. Thus, the assay results signify uniform drug dispersion in the formulations. Furthermore, these results indicate the suitability of the formulation system for high entrapment of the drug in the internal phase.

Stability Studies: Results of stability studies of optimized CLA – SMEDDS formulation batch C2A are as presented in **Table 7**. In all the parameters investigated involving appearance, phase separation, transmittance, droplet size, zeta potential, polydispersity index, and drug content, no significant differences, at probability level p = 0.05, were observed after the stability study period between a freshly prepared and the stored formulations. This is an indication that the optimized CLA – SMEDDS formulation is stable over the storage period of six months.

TABLE 7: STABILITY STUDIES OF OPTIMIZED CLA – SMEDDS FORMULATION BATCH C2A

Evaluation	Optimized CLA – SMEDDS formulation C2A				
	0 day	30 days	60 days	120 days	180 days
Appearance	Clear and Transparent	Clear and Transparent	Clear and Transparent	Clear and Transparent	Clear and Transparent
Phase separation	No	No	No	No	No
Transmittance (%T)	99.95 ± 0.12	99.94 ± 0.11	99.93 ± 0.21	99.87 ± 0.12	99.83 ± 0.13
Droplet size (d. nm)	16.30 ± 0.14	16.31 ± 0.12	16.32 ± 0.14	16.34 ± 0.11	16.34 ± 0.14
Zeta potential(mV)	-2.01 ± 0.11	-2.01 ± 0.12	-2.01 ± 0.10	-2.02 ± 0.11	-2.02 ± 0.12
Polydispersity index	0.203 ± 0.11	0.203 ± 0.12	0.204 ± 0.12	0.204 ± 0.11	0.205 ± 0.12
Drug content (%)	99.58 ± 0.12	99.46 ± 0.12	99.57 ± 0.23	99.51 ± 0.35	99.45 ± 0.18

All values are expressed as (mean ± s.d.): n = 3.

***In-vitro* Drug Release Study:** Results of *in-vitro* drug release studies performed on test CLA – SMEDDS formulation and reference clarithromycin suspension is presented in **Table 8**. From this data, it appears that there is no significant difference between the release profiles of the test and reference clarithromycin formulations. The release profiles were characterized by the variabilities associated with drug release at each sampling time point of both the test and reference formulations. Percentage coefficient of variation (% CV) values of approximately less than or equal to 20% for both test and reference formulations at early sampling time points of 20, 40, and 60 min were observed. The only exception was the reference formulation that exhibited a (% CV)

value of 0.23 at the time point 60 min. At the later sampling time points of 80, 100, and 120 min, the percentage coefficient of variation (%CV) values of approximately less than or equal to 10% were estimated for the formulations. On the bases of these observations, the test CLA - SMEDDS formulation and reference clarithromycin suspension were subjected to the similarity factor (f2) and difference factor (f1) comparative drug release profile analyses [25]. A similarity factor value of 51.5 was estimated, and this was observed to meet the acceptance criteria of the standard range 50 – 100 set up by the FDA. This is an indication of significant similarity or sameness of the release profiles of the two formulations. A difference factor value of 12.93 was estimated. This difference factor value is less than the 15 standard marks set up by the FDA; indicating that there is no significant difference between the release profiles of the two clarithromycin formulations [26]. Based on these findings, it appears that the drug release profiles of the test CLA - SMEDDS formulation and reference clarithromycin suspension compare favorably and may be inferred to be pharmaceutically equivalent.

TABLE 8: *IN-VITRO* DRUG RELEASE ANALYSES OF TEST AND REFERENCE CLARITHROMYCIN FORMULATIONS

Time	% Cumulative drug release in phosphate buffer saline (PBS) - pH 6.8			
min	Test CLA (mean ± s. d)	%CV	Reference CLA (mean ± s. d)	%CV
0	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00
20	22.44 ± 0.041	0.18	8.72 ± 0.016	0.18
40	29.62 ± 0.044	0.15	13.87 ± 0.022	0.16
60	47.62 ± 0.095	0.20	51.71 ± 0.118	0.23
80	66.12 ± 0.073	0.11	70.21 ± 0.096	0.14
100	89.84 ± 0.106	0.12	96.05 ± 0.091	0.09
120	99.86 ± 0.103	0.10	99.89 ± 0.088	0.09

All values are expressed as (mean ± s.d.): n = 6.

Pharmacokinetic Study: Results of pharmacokinetic studies involving test CLA – SMEDDS formulation and reference clarithromycin suspension in an animal model are as presented in **Table 9**. Upon a paired – t statistical analysis at a significance level of ($p = 0.05$), no significant difference was observed between the two clarithromycin formulations with respect to the main pharmacokinetic parameters that were assessed. The significant similarity exhibited by the AUC's is an indication that the total drug exposure by both formulations is similar, and thereby, the test formulation may produce similar therapeutic responses as the reference formulation. It thereby appears that the two formulations possess similar bioavailability profiles.

In a bioequivalence analysis, the geometric mean ratios of the parameters Ln AUC $_{(0-24)}$, Ln AUC $_{(0-\infty)}$, and Ln Cmax of test to reference clarithromycin formulations were estimated as 99.34%, 97.86% and 102.07% respectively. These were observed to fall within the FDA's bioequivalence criterion range of 80% - 125% limits. Furthermore, the 90% confidence intervals around the geometric mean ratios of Ln AUC $_{(0-24)}$, Ln AUC $_{(0-\infty)}$, and Ln Cmax were observed to fall within the standard boundaries of the FDA bioequivalence criterion range. These findings are as depicted in a forest plot in **Fig. 2**.

Accordingly, it appears that the two clarithromycin formulations comply with the FDA bioequivalence criteria, and thereby, the reference clarithromycin suspension can adequately be interchanged with the test CLA – SMEDDS formulation.

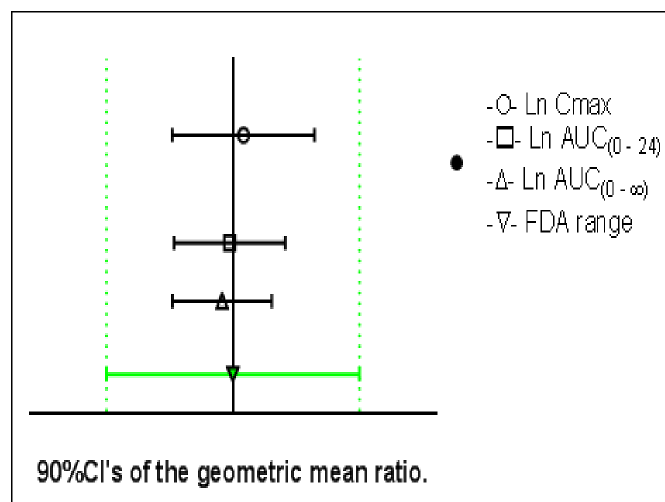

FIG. 2: FOREST PLOT SHOWING 90% CI's AROUND THE GEOMETRIC MEAN RATIO PLOTTED OVER FDA'S STANDARD BOUNDARIES (0.8 – 1.25) WHICH IS PRESENTED IN GREEN COLORED LINES

TABLE 9: MAIN PK PARAMETERS OF TEST AND REFERENCE CLARITHROMYCIN FORMULATIONS

PK - Parameter	Units	Test CLA - SMEDDS	Reference CLA suspension
Cmax	ŋg.ml^{-1}	1612.08 ± 27.19	1378.55 ± 24.17
Ln Cmax	ŋg.ml^{-1}	7.385 ± 0.017	7.237 ± 0.021
E – rate (Kel)	h^{-1}	0.1192 ± 0.011	0.1117 ± 0.016
E – Half-life (t ½)	h	5.8151 ± 0.579	6.059 ± 0.399
AUC $_{(0-24)}$	ŋg-h.ml^{-1}	13465.17 ± 596.06	13403.18 ± 360.69
AUC $_{(0-\infty)}$	ŋg-h.ml^{-1}	14438.67 ± 601.1	14496.77 ± 381.85
AUMC $_{(0-\infty)}$	ŋg-h*h.ml^{-1}	107725.28 ± 12905.99	142134.52 ± 17285.81
MRT	h	7.59 ± 0.62	9.67 ± 0.94
Ln(AUC$_{(0-24)}$)	ŋg-h.ml^{-1}	140.57 ± 2.6519	141.5 ± 1.159
Ln(AUC$_{(0-\infty)}$)	ŋg-h.ml^{-1}	327.05 ± 20.147	334.20 ± 22.05

All values are expressed as (mean ± s.d.): n = 20.

CONCLUSION: The study indicates the existence of both pharmaceutical equivalence and bio-equivalence characteristics of the two formulations and that the test CLA – SMEDDS formulation can be used as a possible alternative to conventional oral formulations of clarithromycin. Furthermore, there are indications that the formulation technique employed has been able to enhance to a respectable and comparable degree, the oral bioavailability of clarithromycin, a drug molecule which is characterized by poor water – solubility and lipophilic properties.

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