Original Article

In-vivo Pharmacokinetic Study, *in-vitro* Cytotoxic, Cell Cycle Arresting and Proapoptotic Characteristics of Multiple Emulsions for the Co-delivery of Simvastatin and Alendronate Sodium

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ABSTRACT

Purpose: Development of nanocarriers that can provide efficient co-delivery of immiscible hydrophilic/ hydrophobic drugs with established technology for industrial production is crucial. Due to this reason, multiple emulsions (MEs) were selected as the desired carriers to achieve the co-delivery ability of many drugs and the improvement of cancer therapeutic effect. MEs could entrap the drug in the inner oil phase and hence avoid the drug leaking and co-deliver the drugs into the tumor sites. Therefore, in the present study, an attempt is made to develop w/o/w multiple emulsion for co-delivery of lipophilic Simvastatin (SVS) and hydrophilic Alendronate Sodium (ADS) with improved oral pharmacokinetics. **Methods:** The MEs were formulated by the use of Poloxamer-407, TPGS and Soyabean Oil. Tween 80 and Span 80 were used as surfactant and co-surfactant respectively. The MEs was prepared by the process of primary and secondary emulsification and evaluated in terms of visual assessment, turbidity, viscosity, particle size and zeta potential. The optimized batch was evaluated in terms of TEM analysis, X-Ray diffraction, FTIR study, *in-vitro* release and screened for cytotoxicity study, cell cycle arresting, apoptosis study and quantification of SVS and ADS in Rat Plasma. **Results:** The MEs treatment inhibited the cell growth with low IC_{50} value against all cells (A549: 0.030 ± 0.014 μg/mL, MDAMB-231: 0.088 ± 0.013 μg/mL, PC-3: 0.019 ± 0.002 μg/mL).The AUC in case of ADS and SVS was found to be 710.01 ng/mL and 14.413 ng/mL respectively by oral administration and 42.308 ng/mL and 28.902 ng/mL in 12 and 1 hr respectively by IV administration. **Conclusion:** This strategy has improved simultaneous oral bioavailability of very poorly bio-available both ADS and SVS and thus improved the oral therapeutic efficacy of this combination therapy.

Key words: Simvastatin, Alendronate Sodium, *In-vivo* Pharmacokinetic Study, Cytotoxicity Study, Cell cycle arresting, Apoptosis Study.

INTRODUCTION

Statins clinically used to reduce blood cholesterol levels, are the second-most prescribed drugs after analgesics and are considered to be the safest drugs.[1] In cell-based experiments, the hydrophobic statins displayed inhibitory effects on many cancers.[1,2]

Alendronate Sodium is the sodium salt of alendronate, a second generation bisphosphonate and synthetic analogue of pyrophosphate with bone anti-resorption activity. Alendronate sodium binds to and inhibits the activity of geranyl transtransferase, an enzyme involved in terpenoid biosynthesis. Nitrogen containing Bisphosphonates have been proved to reduce and delay bone complications from bone metastasis, and have been used worldwide for the treatment of bone metastasis from solid tumors, bone complications and pain from multiple myeloma.

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In the clinic, NBPs have been demonstrated additional direct anticancer effects.[3]

SVS and ADS are known to affect cholesterol metabolism and biosynthesis by inhibiting the mevalonate pathway via potentially inhibiting the critical enzymes of the mevalonate pathway (HMG CoA reductase and farnesyl pyrophosphate synthase); thus having the negative effects at various levels on cancer cells. The simultaneous inhibition of these enzymes, using a combination of these two drugs, may result in an amplified anticancer effect and allow for use of significantly lower doses of the drugs involved. Further, because of the bone-anabolic properties of SVS and antiresorptive/bone-targeting characteristics of ADS, this combination would be more effective to treat bone cancers, bone metastasis and associated symptoms like bone loss, pain, etc.[4-7]

The main objective of the work is to develop Multiple/Double Emulsion (w/o/w) in the form of self-emulsifying system as a strategy to improve simultaneous oral bioavailability of very poorly bio-available both hydrophilic ADS and lipophilic SVS and thus to improve the oral therapeutic efficacy of this combination therapy.

MATERIALS AND METHODS

Simvastatin was gifted by Tocris Bio-Techne Mumbai, India. Poloxamer 407 was gifted by BASF, India. Alendronate sodium, D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) were purchased from Sigma-Aldrich Mumbai India. Peanut Oil, Soyabean Oil, Olive Oil, Sesame Oil and Cottonseed Oil were purchased from Research Lab Fine Chem Industries. Labrafil M 2125CS, Capryol PGMC, Labrafac PG, Labrasol and Labrafil M 1944CS was gifted by Gattefosse India. All other reagents used were of analytical reagent grade and were used without further purification.

Cell culture

Human triple-negative breast adenocarcinoma (MDA MB-231: Derived from the metastatic site, Pleural effusion), human prostate adenocarcinoma (PC-3: Derived from the metastatic site, Bone) and human lung adenocarcinoma (A549) cell lines were used for *in-vitro* Cytotoxicity Study, Apoptosis Study and Cell Cycle Analysis.

Solubility study

Solubility of Simvastatin in various oils and surfactants was checked. Excess amount of drug was added in 2 ml of each individual vehicle contained in stopper vial separately and after sealing; it was heated at 40°C and sonicated for solubilization. Vials were then shaken at 37°C±1°C and then allowed them for equilibrium. Then samples were centrifuged (5000 rpm) for 5-10 min to separate the undissolved drug and the supernatants were filtered by membrane filter (0.45µm, 13mm, Whatman, India) and after appropriate dilution with methanol, the absorbance was measured against respective blank by UV spectroscopy at λ_{max}. The concentration of Simvastatin was calculated by using the calibration curve.[8]

Screening of surfactants for emulsifying ability with Soyabean Oil were checked

The % transmittance values and number of inversions required for uniform emulsion of various dispersions.

Preparation of Multiple/Double Emulsion (MEs/DEs) by suitable method

STEP-I: Briefly, weighed quantity of Simvastatin, TPGS and Poloxamer-407 and were dissolved in beakers containing 5mL of methanol. The solvent was then evaporated at room temperature and the resultant film at the bottom of beaker was redispersed with 5 mL of distilled water using bath sonicator for 5 min. The resultant solution was then centrifuged at 5000rpm for 10 min and the supernatant solution was prepared. In that solution Alendronate Sodium equivalent to weight of simvastatin was added with continuous stirring.

STEP-II: Two step emulsification techniques were used to prepare MEs/DEs:

Primary Emulsification (w/o emulsion)

Briefly, accurately weighted the quantities of soyabean oil and in that oil, lipophilic surfactant span 80 was added drop wise and mixed with continuous stirring on magnetic stirrer at 100rpm for 30 min. The prepared W1 phase was then added into above mixture with continuous stirring at1000rpm for 1hr. The prepared primary emulsion was then subjected to Ultra-Turrax for some cycles at 8000rpm for 15 min.

Secondary Emulsification (Self Emulsifying Composition)

The prepared primary emulsion was then mixed with Hydrophilic Surfactant Tween 80 with continuous stirring on magnetic stirrer at 150 rpm for 30 min. The prepared Multiple/Double Emulsion was then mixed with distilled water upto 100ml with continuous stirring on magnetic stirrer at 100-200rpm for 30 min and subjected to High Pressure Homogenization (HPH) to convert into nanoemulsion and characterized in terms of various parameters.[8,9] Various Formulation

Table 1: Formulation Batches of Self Emulsifying composition (Multiple Emulsions).

Ingredients	F1	F2	F3
Simvastatin	20mg	20mg	20mg
Alendronate Sodium	20mg	20mg	20mg
TPGS	150mg	200mg	250mg
Poloxamer 407	150mg	200mg	250mg
Soyabean Oil	04ml	05ml	06ml
Span 80	04ml	05ml	06ml
Tween 80	04ml	05ml	06ml
Distilled Water	1.5 ml	02 ml	2.5 ml

batches of Multiple/Double Emulsion (MEs/DEs) were given in Table 1.

Characterization of Prepared Multiple Emulsions[10-13]

Visual Assessment

The prepared MEs formulations were observed visually for any turbidity or phase separation.

Turbidity Measurement

Turbidity of the prepared MEs formulations was measured using a turbidimeter (TurbiDirect, Lovibond, U.K). Turbidity measurements were performed by storing the MEs in screw capped sample vials. A quantity about 0.2 ml of MEs was introduced into 100 ml of 0.1 N HCl under gentle magnetic stirring rotates under a constant speed at room temperature. The measurement was carried out to monitor the growth of droplet after emulsification.

Viscosity Determination

Brookfield LVDV Ultra III Rheometer using spindle S60 was used to determine the viscosity of various formulations at 25±1.0°C rpm at room temperature.

Particle Size and Zeta Potential

The mean particle size and zeta potential of prepared MEs formulations were determined using Horiba particle size and zeta potential analyzer (HORIBA SZ-100) (HORIBA Scientific Ltd. Japan). The measurements were performed in triplicate at 25°C.

Transmission Electron Microscopy (TEM) Analysis

Transmission Electron Microscopy Analysis was carried out for samples before High Pressure Homogenization and after High Pressure Homogenization (HPH). An extremely small amount of material is suspended in water/ethanol. The solution was homogenized using Ultrasonicator to disperse the particles. A drop of the solution was then pipette out and cast the drop on carbon-coated grids of 200 mesh the grid is dried and fixed in the specimen holder.

Crystallinity study by X-Ray Diffraction

X-ray powder diffraction study of prepared MEs formulations were analysed by Miniflox 600 x-ray Diffractometer. Samples were irradiated with mono-chromatized Cu Kα-radiation (1.542A0). The voltage and current used were 30k V and 30Ma respectively. The range was 5 ×103 cycle/s and chart speed was kept at 100 mm/20.

Fourier Transform Infrared Spectroscopy Analysis (FTIR)

Fourier transform infrared (FTIR) spectrum of MEs formulations was recorded by FTIR spectrophotometer by KBr pellet method. The spectrum was scanned in wavelength of 4000 to 400 cm^{-1} at 4 cm^{-1} resolution and the spectrum analysis was done for identification of sample.

In-vitro Drug Release study

In-vitro release of Simvastatin and Alendronate sodium from MEs was determined by the dialysis method. Phosphate-buffered saline (PBS) (pH 7.4) was used as medium along with Tween 80 due to the poor solubility of simvastatin and sink condition requirement of the release test. RPM selected was 50 and Time Interval in the form of 1, 3, 6, 12 and 24 hr respectively.

MEs (Self emulsifying composition) were taken as equivalent to 2 mg of Simvastatin and 2 mg of Alendronate sodium in a dialysis bag and both the end of dialysis bag were closed and placed in beaker containing 50ml release medium. The cumulative amounts of Simvastatin and Alendronate sodium in release medium were determined by LCMS-MS study. All measurements were carried out in duplicate.[14]

Alendronate Quantification in Rat Plasma

METHOD DEVELOPMENT

Rat plasma (180 µL) was spiked with 0.227 to 82.695 ng/mL of Alendronate standard calibrant separately, extracted and analyzed by LCMS. The LCMS response obtained for different levels of calibrant was analyzed by linear regression and regression equation obtained was used for estimating Alendronate concentration in unknown rat plasma samples processed similarly.

Extraction Procedure of Quantification of Alendronate Sodium in Rat Plasma

Thaw the samples to room temperature and vortex and add 100 µL of Rat Plasma sample to the RIA vials and

then add 50 µL of IS solution (Azelnidipine 300ng/mL) to all RIA vials except blank and vortex as well as Add 300 µL of Buffer (100mM Ammonium Acetate) and vortex. Maintain the condition and equilibrate Hipurit wax cartridge (30mg, 1cc) with 1ml methanol followed 1ml HPLC water. Load sample into respective cartridge and wash with 1ml of HPLC water followed by 1ml of methanol and allow it to dry. Elute the cartridge with 300 µL of derivatising agent (0.6 mol/L Trimethyl sily diazo methane in hexane). Then elute with 300 µL of methanol and allow the derivatization to happen for 30 min at room temperature and evaporate to dryness for 40°C at 15 psi. Reconstitute the dried samples with 300 µL mobile phase, vortex and transfer the samples into respective auto sampler vials for LCMS Analysis.[15-17]

Simvastatin Quantification in Rat Plasma

Method Development

Rat plasma (180 µL) was spiked with0.109 to 20.514 ng/mL of Simvastatin standard calibrant separately, extracted and analyzed by LCMS. The LSMS response obtained for different levels of calibrant was analyzed by linear regression and regression equation obtained was used for estimating Simvastatin concentration in unknown rat plasma samples processed similarly.

Extraction Procedure of Quantification of Simvastatin in Rat Plasma

Thaw the samples to room temperature and vortex and Add 100 µL of Rat Plasma sample to the RIA vials and then add 50 µL of IS solution (Telmisartan 13C D3-300ng/mL) to all RIA vials except blank and vortex as well as Add 300 µL of Buffer (100mM Ammonium Acetate) and vortex. Add 2 ml of Ethyl Acetate and vortex the samples on vibramax at 2500 rpm for 140° min and then centrifuge at 4000 rpm for 5 min at 4°C. Transfer 1.7 ml supernatant into respectively labelled RIA vials and evaporate the samples to dryness at 40°C. Reconstitute the samples with 0.2 ml of reconstitution solution, vortex and transfer the samples to respectively labelled shell vials (Auto Sampler) and load onto Auto Sampler. Evaporate to dryness for 40°C at 15 psi and reconstitute the dried samples with 300 µL mobile phase, vortex and transfer the samples into respective auto sampler vials for LCMS Analysis.[18-24] Instrumental and MS conditions were given in Table 2 and 3 whereas Alendronate Quantification with Internal Standard Azelnidipine and Simvastatin Quantification with Internal Standard Telmisartan were given in Table 4 and 5 respectively.

Table 2: Chromatographic conditions of Alendronate Sodium and Simvastatin.

Particulars	Procedure	
	Alendronate Sodium	Simvastatin
Instrument	Shimadzu-HTC	
Column	Kinetex Omega PS-C_{18} 50*4.6mm, 5 µm	Kinetex C_{18} 50*4.6mm, 5 µm
Mobile phase	Acetonitrile:5mM ammonium acetate (80:20v/v)	Acetonitrile:0.1 % Formic acid in water (80:20v/v)
Run Time	6.00 min	3.00 min
Flow rate	0.450 ml/min	
Injection Volume	5 µl	
Auto sampler temp	10°C ± 2°C	
Column oven temp	40°C ± 2°C	

Table 3: MS conditions of Alendronate Sodium and Simvastatin.

Particulars	Procedure	
	Alendronate Sodium	Simvastatin
Instrument	Applied Biosystems MDS Sciex, 4000QTRAP	
Ion Source	Turbo Ion Spray	
Mode	Positive	
CUR	10.00	
CAD	High	
Ion Spray Voltage	5500	
Temperature	450.00°C	
Gas 1 (GS1)	25.00	
Gas 2 (GS2)	10.00	

Table 4: Alendronate Quantification with Internal Standard Azelnidipine.

Compound Name	*m/z* (Q1/Q3)	DP	CE	EP	CXP
Alendronate	348.1/163.1	49	54.5	14.3	13
Azelnidipine	590.4/167.2	58.5	52.5	14.3	13

Table 5: Simvastatin Quantification with Internal Standard Telmisartan.

Compound Name	*m/z* (Q1/Q3)	DP	CE	EP	CXP
Simvastatin	419.3/285.4	49	32.2	14.5	10
Telmisartan 13C D3	348.1/163.1	86	29.8	14.1	10

In-vitro Cytotoxicity using MTT assay

Briefly, 100µL of cell suspension was added to each well of the 96 well microtiter plates (50,000 cells/well). After 24 h incubation, the supernatant from each well was replaced with 100µL of different concentrations of test drugs. The plates were then incubated at 37°C for 24h in a 5% CO_2 atmosphere. After incubation, the test solutions in the wells were replaced with 100µL of MTT solution (0.05mg) and plates were incubated at 37°C in a 5% CO_2 atmosphere for 4h. The MTT solution was replaced with DMSO (100µL) and plates were gently shaken to solubilize the formed formazan crystals. The absorbance was measured using a microplate reader at a wavelength of 590nm. The % growth inhibition was calculated, and the concentration of test drug needed to inhibit 50% cell growth (IC_{50}) is generated from the dose-response curves for each cell line.[25,26]

Cell Cycle arresting behaviour using FACS

1×10^6 cells were seeded and cultured for 24h in a 6-well plate containing 2mL of media. Cells were then incubated with drug solutions (2mL) prepared in complete media for 24h. Cells were then harvested, centrifuged at 2000rpm for 5 min at room temperature and the supernatant was discarded carefully retaining the cell pellet. The cell pellet was washed twice by resuspending in 2mL of 1X PBS. Cells were then fixed by resuspending in 300µL of sheath fluid followed by the addition of 1mL of chilled 70% EtOH drop by drop with continuous gentle shaking, and another 1mL of chilled 70% EtOH was added at once. The cells were then stored at 4°C overnight, centrifuged at 2000rpm for 5 min and the pellet was washed twice with cold 1X PBS (2mL). The cell pellet was then resuspended in 450µL of sheath fluid containing 0.05mg/mL propidium iodide (PI) and 0.05mg/mL RNase A and incubated for 15 min in dark. The percentage of treated and untreated cell populations in various stages of the cell cycle was determined using FACS Caliber (BD Biosciences, San Jose, CA). The standard Colchicine (25µM) was used as a positive control and a minimum of 10,000 cells were acquired for each sample.[26,27]

Apoptosis Study

1×10^6 cells per well were seeded into a 6-well plate. After 24h, the floating (dead) cells were transferred into 15mL tubes. The cell suspension was then centrifuged, cells were washed twice with cold PBS and thenold culture medium with a new medium of the same volume containing drug solutions. After 24h of incubation, the culture medium along with the Binding Buffer at a concentration of ~1×10^6 cells/mL. Then, 500µL of cell suspension was aliquot and 10µL of PI and 5µL Annexin V were added. The suspension was then incubated for 15 min at room temperature in the dark. Post incubation, the cells were analyzed by flow cytometer as soon as possible (within 1h). The standard Doxorubicin (25µM) was used as a positive control and a minimum of 10,000 cells were acquired for each experimental set up.[27,28]

Stability Study

Optimized batch of prepared MEs were subjected to stability testing as per ICH guidelines. The preparation was stored in air-tight glass containers and protected from light. Samples maintained in a stability chamber (Remi CHM-6) under refrigerated condition (2-8°C), long term condition (25±2°C/60±5%RH) and accelerated conditions (40±2°C, 75±5% RH) with humidity and temperature control at 0, 1, 2 and 3 month. Samples were observed visually for phase separation. During monthly interval, sample was analyzed for particle size and entrapment efficiency.[29,30]

RESULTS AND DISCUSSION

Poloxamer 407 (P-407), a US FDA-approved amphiphilic block copolymer of poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO), is most attractive due to its biocompatibility and low toxicity. The PPO forms the hydrophobic core and solubilises the hydrophobic drug in aqueous media, while the hydrophilic PEO corona maintains the dispersion stability. TPGS, an amphiphilic block copolymer derived from Vitamin E (α-tocopherol) and polyethylene glycol 1000, has been widely used in the pharmaceutical field as a solubilizer, absorption enhancer and a vehicle for lipid-based drug delivery formulations.

Solubility Study

Various oils, surfactants and co-surfactants were screened by performing solubility study as per the method described. Solubility of Simvastatin in different vehicles was determined using calibration curve in methanol (Figures 1 and 2). As per solubility data of Simvastatin in different oils, maximum amount of simvastatin dissolves in soya bean oil. So soya bean oil was selected as oil having solubility of 62.54±0.356 mg/ml.

As per solubility data of Simvastatin in different surfactants, maximum amount of simvastatin dissolved in Tween 80 and Span 80. So Tween 80 and Span 80 were screened as surfactant/co-surfactant having solubility of 77.05±0.620 and 85.32±0.620 mg/ml

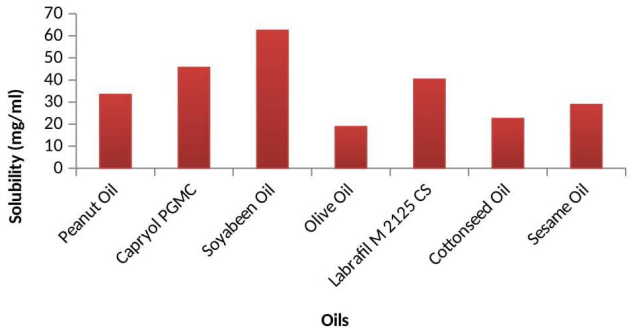

Figure 1: Solubility of Simvastatin in various Oils.

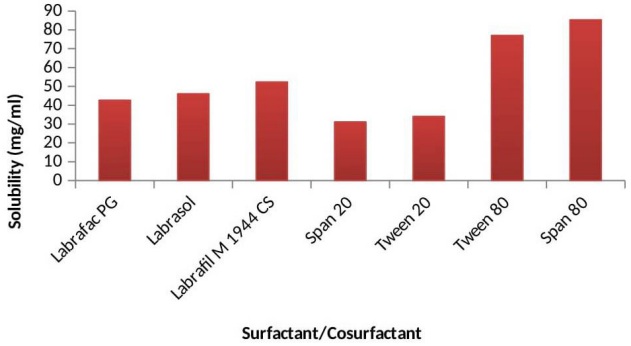

Figure 2: Solubility of Simvastatin in various Surfactants/ Co-surfactants.

respectively. Tween 80 and Span 80 have good ability to emulsify soya bean oil; even though number of inversions required for formation of uniform emulsion with Tween 80 and Span 80 was less with high % transmittance. So Tween 80 as surfactant and Span 80 as co-surfactant were confirmed.

Screening of surfactants for emulsifying ability with Soya bean Oil

The % transmittance values and number of inversions required for uniform emulsion of various dispersions were given in Table 6.

From the results, it was observed that Simvastatin was found to have good solubility in Span 80 and Tween 80. Soyabean Oil shows highest transmittance with Span 80 and Tween 80 as compared with Labrasol and Labrafil M 1944CS which has good ability to emulsify soyabean oil and number of inversions required for formation of uniform emulsion is less. Therefore Span 80 and Tween 80 were selected for further study.

Preparation of Multiple/Double Emulsions

In the current study, Multiple Emulsions was prepared by using TPGS, Poloxamer 407, Soyabean oil, Span 80 and Tween 80 with varying concentration. The oil and surfactant/co-surfactant were selected on the basis of solubility study.

Table 6: Emulsification efficacy of surfactant with Soyabean Oil.

Surfactants	% Transmittance	Number of Inversions
Tween 80	98.2	07
Labrasol	91.3	15
Span 80	99.3	05
Labrafil M 1944CS	93.2	12

Table 7: Turbidity Measurement.

Batch	F1	F2	F3
Turbidity Value (NTU)	1.45±0.12	0.94±0.26	0.82±0.18

Characterization of prepared Multiple/Double Emulsion

Visual Assessment

The prepared MEs containing Simvastatin and Alendronate sodium was visually observed for any changes or phase separation. There was no change in the preparation. The upper limit for formation of transparent MEs was set as 1 min, since when emulsion get occurs slowly in more than 1 min.

Turbidity Measurement

It was observed that, by increasing ratio of Tween 80 and Span 80, there will be decrease in the turbidity value due to better solubility of the drug which were presented in Table 7. In the formulation of F1 to F3, due to increase surfactant concentration of Span 80 and Tween 80, So there will be decrease in turbidity value from 1.45±0.12 to 0.82±0.18.

Viscosity Determination

Prepared batches of Multiple Emulsions exhibited Non Newtonian shear thinning pseudo plastic flow behaviour with viscosity of the system decreasing with increasing shear rate. As a result, prepared multiple emulsions showed shear thinning behaviour and apparent viscosity which decreases with increase in shear rate. Furthermore it was observed that viscosity increases with increasing concentrations of Span 80 (Figure 3).

Particle Size and Zeta Potential

The F2 batch of prepared MEs showed single larger particle size peak (848.6±4.90 PDI: 0.542) before High pressure homogenization whereas after High pressure homogenization of the same batch, the small and single

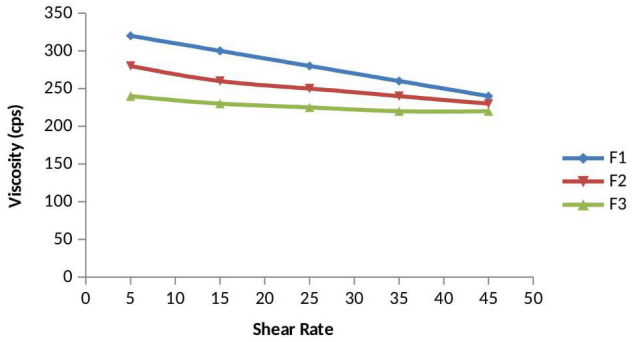

Figure 3: Rheological Study of prepared Multiple Emulsions (MEs).

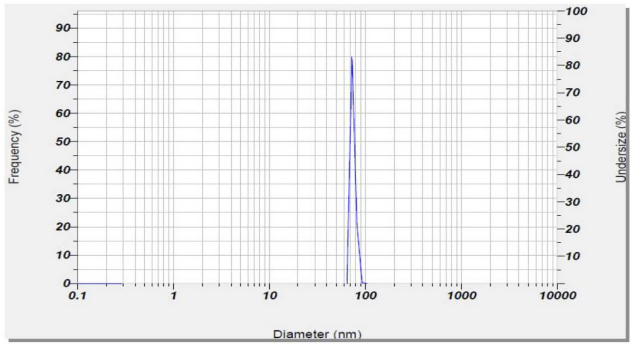

Figure 4: Particle Size of optimized batch F2.

Table 8: Particle Size of Batch F1 to F3 before and after High Pressure Homogenization.

Batch	Before HPH Particle Size (nm)	After HPH Particle Size (nm)
F1	938.5±5.12	78.9±3.27
F2	848.6±4.90	71.8±2.69
F3	992.7±4.07	80.5±3.15

particle size peak was obtained (71.8±2.69 PDI: 0.327) (Figure 4). While comparing F2 Batch with F1 and F3, F2 showed single and small particle size peak (Table 8). Besides, the F2 batch showed average zeta potential of -28.7±3.21mV before High pressure homogenization whereas the same batch after High pressure homogenization showed average zeta potential of -13.7±1.36mV (Figure 5) and is found increased in Batch F1 and F3 (Table 9).

Transmission Electron Microscopy (TEM) Analysis

The objective of the TEM analysis was to confirm the small spherical shaped droplets formation of the optimized batch of prepared MEs. The TEM image of optimized batch confirmed the small spherical shape formation of the developed system of MEs which was

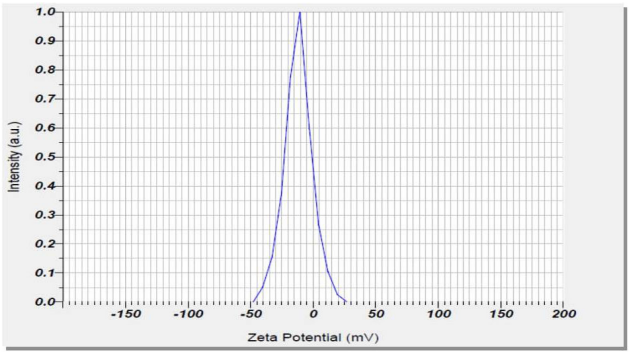

Figure 5: Zeta Potential of optimized batch F2

Table 9: Zeta Potential of Batch F1 to F3 before and after High Pressure Homogenization.

Batch	Before HPH (mV)	After HPH (mV)
F1	-29.3±1.96	-12.2±1.14
F2	-28.7±3.21	-13.7±1.36
F3	-32.2±2.44	-11.6±1.88

Values presented are mean ± SD, n=3

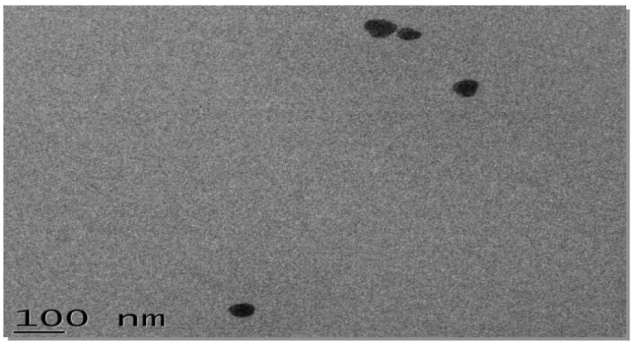

Figure 6: Transmission Electron Microscopy (TEM) Analysis of Optimizied Batch.

shown in Figure 6. It confirmed fine dispersed emulsion without any signs of clumps or aggregation.

Crystallinity Study by X-Ray Diffraction (XRD)

The X-Ray Diffractogram of pure simvastatin, sharp peaks were observed at 2000 and 2300 intensity whereas pure alendronate sodium, sharp peaks were observed at 1600 and 1900 intensity which revealed crystalline nature of drug. The X-Ray Diffractogram of prepared MEs showed sharp peaks at 1300 and 1600 which revealed that there was conversion of crystalline to amorphous form of the drug which was shown in Figure 7. Drugs get disordered crystalline phase in the oily inner core.

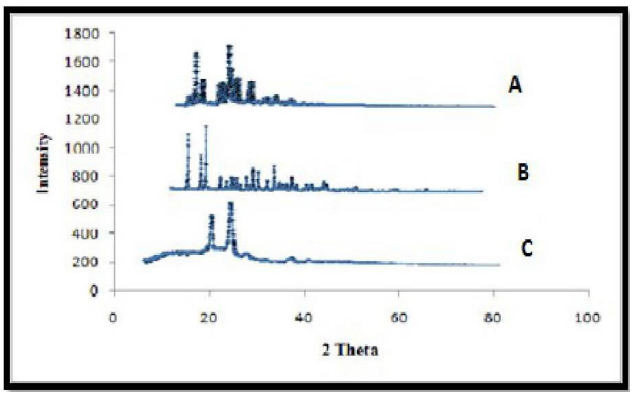

Figure 7: X-Ray Diffractogram of Simvastatin (A), Alendronate Sodium (B) and prepared MEs (C).

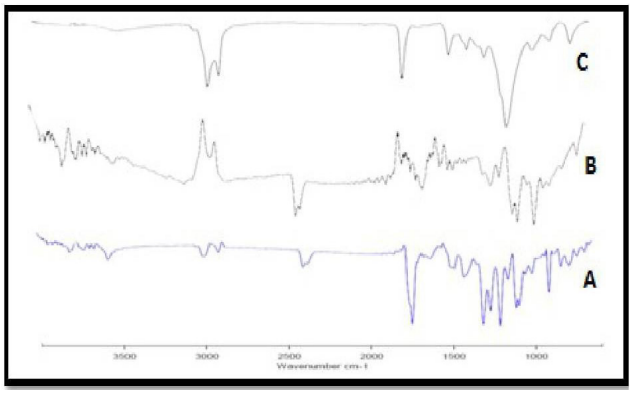

Figure 8: FTIR Studies of Simvastatin (A), Alendronate Sodium (B) and prepared MEs (C).

Fourier Transform Infrared Spectroscopy (FTIR) study

While comparing individual spectra of simvastatin (A) and alendronate sodium (B) with prepared Multiple Emulsions (MEs), it was observed that no any major changes observed in the functional groups which were shown in Figure 8.

In-vitro Drug Release Profile of Plain Simvastatin, Alendronate Sodium and prepared Multiple Emulsions (MEs)

In-vitro drug release was studied by using Simvastatin and Alendronate Sodium as plain drug and prepared Self emulsifying composition. It has been observed that, plain simvastatin showed drug release 42.51±2.35 in 24hrs whereas from prepared self-emulsifying composition in the form MEs, it was 53.54±3.34. Plain Alendronate Sodium showed drug release 98.05±1.11 in 3hr whereas from self-emulsifying composition in the form MEs, it was 44.26±1.20 in 24hr (Figure 9). SVS and ADS in SEDDS in the form of MEs showed narrow release pattern as compared with plain drugs.

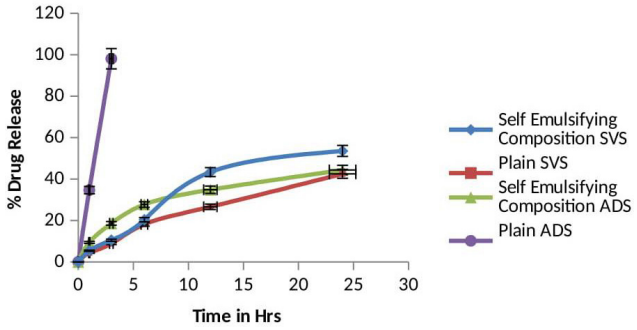

Figure 9: Comparative *in-vitro* Drug Release profile of Plain Simvastatin, Alendronate Sodium and SEC Containing both Simvastatin and Alendronate Sodium.

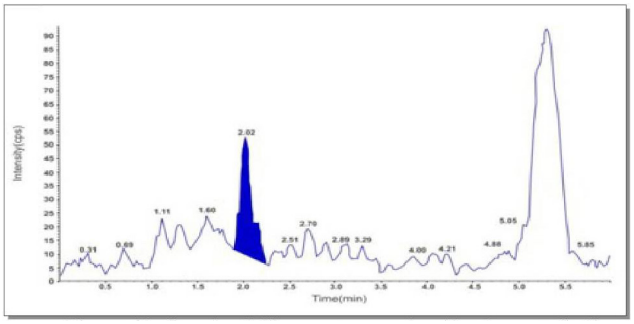

Figure 10: Standard Chromatogram for Alendronate Sodium.

So the simultaneous delivery of both the drugs like Simvastatin and Alendronate sodium can be achieved.

Alendronate and Simvastatin Quantification in Rat Plasma

Standard chromatogram of Alendronate and Internal Standard (IS)

The Pharmacokinetic data of Alendronate shows C_{max} of 51.14 and 2.19 ng/mL for Oral and IV route of administration respectively. T_{max} for both shows around 12 hr for oral and 6 hrs for IV administration. The Area under curve (AUC) for IV administration shows 42.308 and for Oral it shows around 710.01. Standard Chromatogram for Alendronate, Standard Chromatogram for Azelnidipine Internal standard and Linearity Graph of Alendronate Sodium Calibrant were shown in Figures 10 to 12 respectively.

Regression Equation was used for calculating Alendronate in unknown samples:

$$Y=0.0016\ x + 0.000318$$

Where Y = Peak area of Alendronate and

X is the concentration of Alendronate in ng/mL of plasma samples

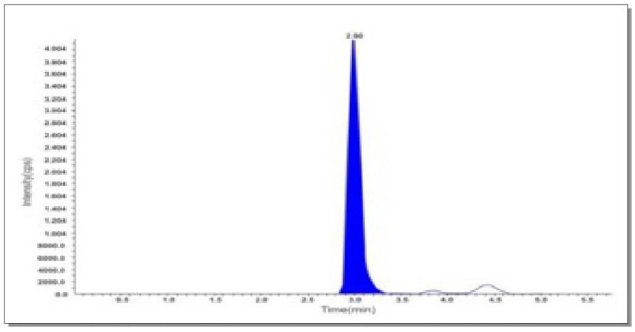

Figure 11: Standard Chromatogram for Azelnidipine Internal standard.

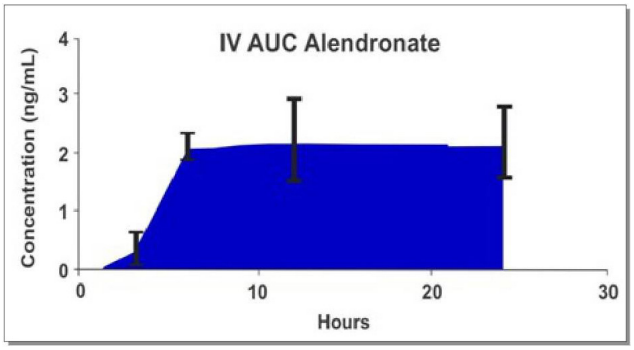

Figure 13: Area under the Curve (AUC) of Alendronate (IV).

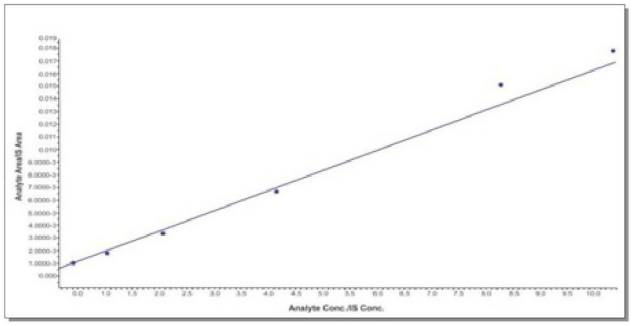

Figure 12: Linearity Curve of Alendronate Sodium.

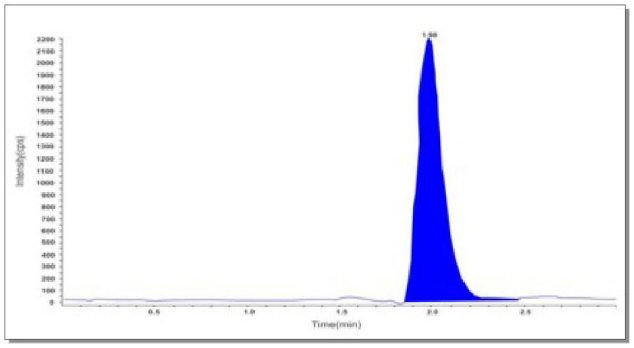

Figure 14: Area under the Curve (AUC) of Alendronate (Oral).

Plasma Alendronate levels (Area under the curve) in the form of IV and Oral administration were presented in Figures 13 and 14.

Standard chromatogram of Simvastatin and Internal Standard (IS)

The Pharmacokinetic data of Simvastatin shows C_{max} of 3.4283 and 4.1137 ng/mL for Oral and IV route of administration. T_{max} for both shows around 1 hr for oral and IV administration. The Area under Curve (AUC) for IV administration shows 28.902 and for Oral it shows around 14.413. Standard Chromatogram for Simvastatin, Standard Chromatogram for Telmisartan Internal standard and Linearity Graph of Simvastatin Calibrant were shown in Figures 15 to 17 respectively. Regression Equation was used for calculating Simvastatin in unknown samples:

$$Y= 0.0708 x + 0.0099$$

Where Y = Peak area of Simvastatin and

X is the concentration of Simvastatin in ng/mL of plasma samples

Plasma Simvastatin levels (Area under the curve) in the form of IV and Oral administration were presented in Figures 18 and 19. Summary of AUC, C_{max} and T_{max} was given in Table 10.

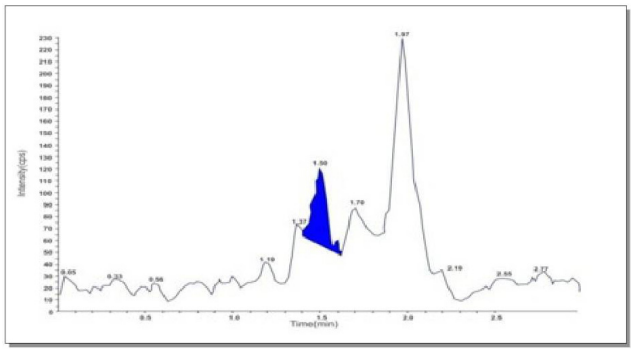

Figure 15: Standard Chromatogram for Simvastatin.

In-vitro Cytotoxicity Study

The effect of Simvastatin (SVS), Alendronate Sodium (ADS) and prepared MEs on % cell growth was checked. All tested formulations caused concentration dependent cell growth inhibition against all cell lines tested. The A549 cells are found significantly more sensitive to SVS treatment than ADS treatment as compared to other two cells tested. The MEs prepared by the use of both hydrophilic alendronate sodium and lipophilic simvastatin significantly inhibited the growth of all cells as compared to all other treatments. The IC_{50} values of all formulations against tested cell lines are presented in Table 11.

Table 10: Summary of AUC, C_{max} and T_{max}.

Samples	Oral		IV	
	ADS	SVS	ADS	SVS
AUC ng/mL * h	710.01	14.413	42.308	28.902
C_{max} ng/ mL	51.14	3.4283	2.19	4.1137
T_{max} Hour	12	1	12	1

Table 11: IC_{50} Value obtained after 24-h treatment with test substances.

Formulation	IC$_{50}$ Value (µg/mL)		
	A-549	MDAMB-231	PC-3
SVS	1.163±0.079	1.348±0.086	1.397±0.091
ADS	1.211±0.113	1.396±0.182	1.702±0.216
SA-MEs	0.030±0.014	0.088±0.013	0.019±0.002

Values presented are mean ± SD, *n*=3

Table 12: Apoptosis Study of Self Emulsifying Composition (MEs) on PC-3, MDAMB-231 and A-549 Cell Line.

Cell Lines	Viable cells	Early Apoptotic	Late Apoptotic	Necrotic cells
PC-3	47.52	18.54	11.77	22.16
A-549	49.18	00	9.44	41.37
MDAMB-231	46.44	0.035	28.19	25.33

Apoptosis Study

In the present study, apoptotic activity of MEs was determined by using ANNEXIN V FITC and Propidium Iodide staining method. The MEs treatment resulted in significantly more MDAMB-231 cells in the late apoptotic phase as compared to other cell lines whereas the treatment caused about increased in the necrotic cells in case of A-549 cell line (Table 12).

Cell Cycle arresting behaviour using FACS

In the present study, The MEs treatment resulted about more number of cells are gated in S phase (A-549: 49.155±4.6) as compared with other two cell lines (PC-3: 47.75±0.24 and MDAMB-231: 48.55±3.68). Also significant effect was observed about arresting more number of cells in G0/G1 phase (A-549: 46.94±3.83) as compared with other two cell lines (PC-3: 44.29±2.1 and MDAMB-231: 46.21±3.76). It was confirmed that MEs treatment caused significant cell cycle arresting against all cell lines where A-549 cells are found somewhat more sensitive to MEs treatment as compared to PC-3 and MDAMB-231 (Table 13).

Table 13: Cell Cycle Analysis of Self Emulsifying Composition (MEs) on PC-3, MDAMB-231 and A-549 Cell Line.

Cell Line	Sub G0	G0/G1	S	G2M
Control	0.075±0.007	77.775±0.5	7.865±0.6	14.55±0.12
PC-3	0.01±0.01	44.29±2.1	47.75±0.24	8.31±0.4
A-549	3.01±0.04	46.94±3.83	49.155±4.6	2.375±0.4
MDAMB-231	4.76±0.05	46.21±3.76	48.55±3.68	2.14±0.03

Table 14: Stability Study of prepared MEs at different storage conditions.

Condition	Particle Size (nm)				Entrapment Efficiency (%)			
	0 Month	1 Month	2 Month	3 Month	0 Month	1 Month	2 Month	3 Month
2-8^{0}C	71.8	71.8	71.7	71.7	95.7	95.7	95.6	95.5
25±2^{0}C/ 60±5%RH	71.8	71.1	69.2	68.4	95.7	94.6	93.4	92.9
40±2^{0}C, 75±5% RH	71.8	71.7	72.2	74.3	95.7	95.5	94.2	92.4

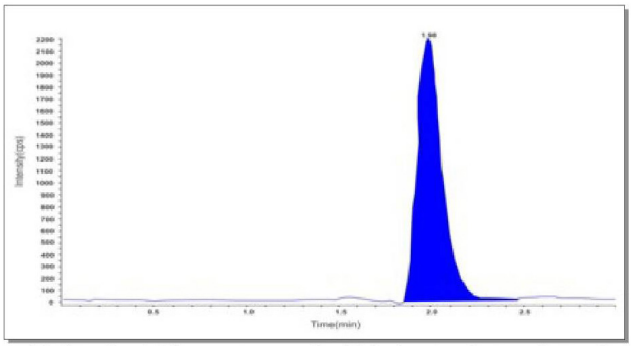

Figure 16: Standard Chromatogram for Telmisartan Internal standard.

Stability Study

Influence of optimized batch preparation of Multiple/ Double emulsion at different storage condition on stability was assessed by visually and through particle size and entrapment efficiency which was shown in Table 14.

a) The formulations confirmed adequate physical as well as chemical stability when stored under refrigerated condition.

b) Decrease in the particle size and entrapment efficiency was observed on storage at 25±2^{0}C/60±5%RH which might be due to breaking of MEs into simple emulsion.

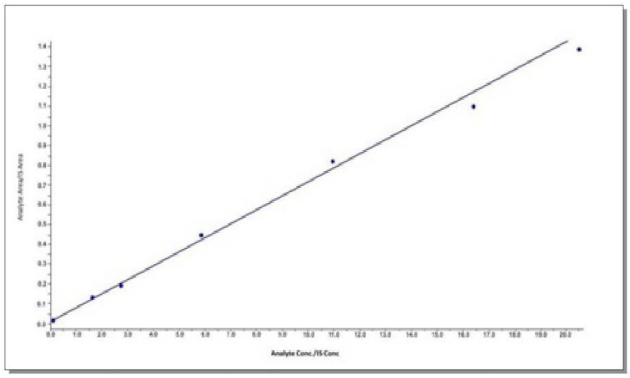

Figure 17: Linearity curve of Simvastatin.

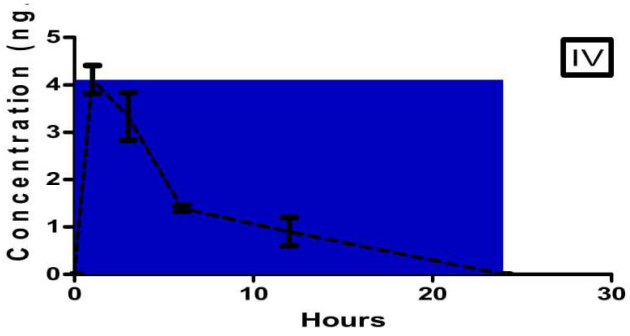

Figure 18: Area under the Curve (AUC) of Simvastatin (IV).

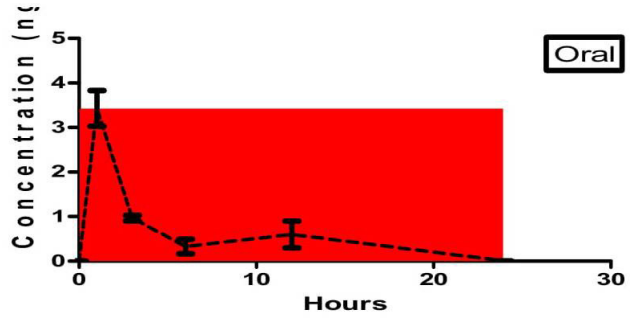

Figure 19: Area under the Curve (AUC) of Simvastatin (Oral).

c) Extreme increase in particle size and decrease in the EE was observed under accelerated condition at 3 months which might be due to coalescence of the globules and loss of entrapped drug from MEs leads to decrease in EE.

CONCLUSION

In the present study (preliminary examination), self double emulsifying drug delivery system in the form of w/o/w emulsion was developed successfully for simultaneous oral bioavailability of very poorly bio-available both highly hydrophilic (ADS) and highly lipophilic (SVS) which improved the oral therapeutic efficacy of this combination therapy. The prepared composition significantly increases *in-vitro* anticancer activity in the form of cell cycle analysis, cytotoxicity study and apoptosis activity, thus indicates the importance of simultaneous delivery of Lipophilic and Hydrophilic drug. *In-vivo* Pharmacokinetic study results revealed superior oral bioavailability of both SVS and ADS as compared to reported bioavailability of both the drugs. Further IV administration of prepared MEs at dose equivalent to half of the orally administered dose resulted in required pharmacokinetic profile for both SVS and ADS indicating its suitability for IV administration.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

MEs: Multiple Emulsions; **SVS:** Simvastatin; **ADS:** Alendronate Sodium; **TEM:** Transmission Electron Microscopy; **FTIR:** Fourier Transform Infrared Spectroscopy; **NBPs:** Nitrogen Containing Bisphosphonates; **TPGS:** D-α-Tocopherol polyethylene glycol 1000 succinate; **HPH:** High Pressure Homogenization; **PBS:** Phosphate Buffer Saline; **LCMS:** Liquid Chromatography Mass Spectroscopy; **FACS:** Fluorescence activated cell sorting; **EE:** Entrapment Efficiency; **AUC:** Area under Curve; **IS:** Internal Standard.

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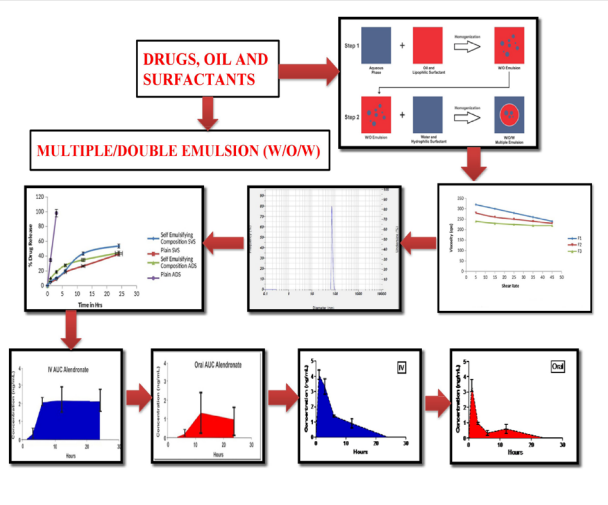
PICTORIAL ABSTRACT



SUMMARY

The Self Emulsifying Drug Delivery System in the form of MEs was prepared in the form of primary and secondary emulsification process. The Batches were prepared with variation in the concentration of Oil and Surfactants ratio and characterized in the form of Visual Assessment, Turbidity measurement, Drug Content and Viscosity. Particle Size and Zeta Potential was determined before and after High Pressure Homogenization for checking the effect. *In-vitro* Drug release study was performed in case of plain drugs and prepared MEs. The study showed narrow release pattern as compared with plain drugs. Controlled release was observed due to high surfactant concentration in Self Emulsifying composition, so simultaneous delivery of both the drugs can be achieved. SA-MEs retarded the growth of cells with low IC_{50} value against all the cells. Further SA-MEs treatment significantly retarded cell multiplication in S phase and developed in high concentration of late apoptotic and necrotic cells at low concentration. It reveals that SA-MEs could be an alternative for quick effect against all screened cell lines. The formulations confirmed adequate physical as well as chemical stability when stored under refrigerated condition.

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