Aim and Objective:
The aim of present investigation is to develop a novel emulsifying drug delivery systems (SNEDDS) to enhance the dissolution rate profile by increasing the solubility of Fenofibrate and Simvastatin.

Purpose of study:

The antilipidemic drugs such as Fenofibrate and Simvastatin are selected as a good drug candidate for formulation of self nanoemulsion drug delivery system (SNEDDS) as they are BCS class II compounds having low solubility and low permeability. The low and variable bioavailability of glibenclamide and glimiride are due to low solubility.

The dissolution profile of both the drugs is considered to be a rate limiting step for its absorption. self nanoemulsion drug delivery systems (SNEDDS) are selected to enhance the overall oral bioavailability of Fenofibrate and Simvastatin.

Plan of work:

1. Development of Analytical method for the estimation of Fenofibrate and simvastatin by using Reverse phase-HPLC
2. Solubility studies of Fenofibrate and Simvastatin.
3. Construction of pseudo ternary phase diagram of oil, water and surfactant – co-surfactant mixture to obtain emulsion formation zone.
4. Preparation SNEDDS.
5. Screening of SNEDDS by thermo dynamic stability studies and Accelerated studies Stability studies as per ICH guidelines.
6. Characterization of SNEDDS.
8. In-vitro intestinal permeability studies with marketed product.
3.A.: FENOFIBRATE SELF NANO EMULSION

3.A.1: List of materials:

Table 3. Materials used for the Fenofibrate nano emulsion formulation

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Material</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Fenofibrate</td>
<td>Gift sample from Reddys laboratory (Hyderabad)</td>
</tr>
<tr>
<td>2.</td>
<td>Tween20</td>
<td>Merck specialities pvt. limited (Mumbai)</td>
</tr>
<tr>
<td>3.</td>
<td>Tween80</td>
<td>Merck specialities pvt. limited (Mumbai)</td>
</tr>
<tr>
<td>4.</td>
<td>CremophoreRH40</td>
<td>Gift sample from bright labs (Hyderabad)</td>
</tr>
<tr>
<td>5.</td>
<td>Oleic acid</td>
<td>Merck specialities pvt. limited (Mumbai)</td>
</tr>
<tr>
<td>6.</td>
<td>Soyabean oil</td>
<td>Bright labs (Hyderabad)</td>
</tr>
<tr>
<td>7.</td>
<td>Ethyl alcohol</td>
<td>Changshu Yangyuan chemicals (China)</td>
</tr>
<tr>
<td>8.</td>
<td>Cotton seed oil</td>
<td>Gift Sample from Bright labs (Hyderabad)</td>
</tr>
<tr>
<td>9.</td>
<td>PEG 400</td>
<td>Merck specialties pvt. Limited</td>
</tr>
<tr>
<td>10.</td>
<td>Span 80</td>
<td>SD-Fine chemicals limited , Mumbai</td>
</tr>
<tr>
<td>11.</td>
<td>Propylene glycol</td>
<td>SD-Fine chemicals limited , Mumbai</td>
</tr>
<tr>
<td>12.</td>
<td>Potassium dihydrogen phosphate</td>
<td>SD-Fine chemicals limited , Mumbai</td>
</tr>
<tr>
<td>13.</td>
<td>Meglyoil</td>
<td>Gift sample from bright labs (Hyderabad)</td>
</tr>
</tbody>
</table>
Table 4. Equipments used for Fenofibrate nano emulsion formulation

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Equipment</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Weighing balance SHIMADZU AX200, JAPAN</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Orbital Shaker VIGNAN-0SR30, INDIA</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Magnetic stirrer REMI Equipments, USA</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Dissolution test apparatus II USP LABINDIA DS 8000, INDIA</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Zeta sizer Nano ZS 90, HORIBA, JAPAN</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>PH meter Elico LI127, INDIA</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Conductivity meter LABINDIA pico+, INDIA</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Ultrasonicator CITIZEN CD 4820, INDIA</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>FTIR BROUKER-ALPHA T, GERMANY</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Viscometer BROOKFIELD-DV-II+pro, GERMANY</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Probe sonicator SONICS vibra cell, USA</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>HPLC SHIMADZU SPD20A detector, LC-20AD pumps, DGU-20A3 degasser, USA</td>
<td></td>
</tr>
</tbody>
</table>
3.A.2: DRUG PROFILE:

FENOFIBRATE (Wysocki J et al., 2004 and Keech A et al., 2005):

- **Description**: An antilipemic agent which reduces both cholesterol and triglycerides in the blood.

- **Structure**:

- **Synonyms**:
  - Fenofibrato [INN-Spanish]
  - Fenofibratum [INN-Latin]
  - Fenofibric acid
  - Finofibrate
  - FNF

- **Categories**:
  - Antlipemic Agents
  - Fribic Acid Derivatives

- **Weight**: Average: 360.831
Chemical Formula: C_{20}H_{21}ClO_{4}

IUPAC Name: propan-2-yl 2-{4-[[4-chlorophenyl]carbonyl]phenoxy}-2-methylpropanoate

Pharmacology:

Indication: For use as adjunctive therapy to diet to reduce elevated LDL-C, Total-C, Triglycerides and Apo B, and to increase HDL-C in adult patients with primary hypercholesterolemia or mixed dyslipidemia (Fredrickson Types IIa and IIb).

Pharmacodynamics: Fenofibrate is a lipid regulating agent indicated as adjunctive therapy to diet to reduce elevated LDL-C, Total-C, Triglycerides and Apo B, and to increase HDL-C in adult patients with primary hypercholesterolemia or mixed dyslipidemia (Fredrickson Types IIa and IIb). Fenofibrate is also indicated as adjunctive therapy to diet for treatment of adult patients with hypertriglyceridemia (Fredrickson Types IV and V hyperlipidemia). Fenofibric acid, the active metabolite of Fenofibrate, produces reductions in total cholesterol, LDL cholesterol, apolipoprotein B, total triglycerides and triglyceride rich lipoprotein (VLDL) in treated patients. In addition, treatment with Fenofibrate results in increases in high density lipoprotein (HDL) and apoproteins apoAI and apoAII.

Mechanism of action:

- Fenofibrate exerts its therapeutic effects through activation of peroxisome proliferator activated receptor a (PPARα). This increases lipolysis and elimination of triglyceride-rich particles from plasma by activating lipoprotein lipase and reducing production of apoprotein C-III. The resulting fall in triglycerides produces an alteration in the size and composition of LDL from small, dense particles, to large buoyant particles. These larger particles have a greater affinity for cholesterol receptors and are catabolized rapidly.

- Absorption: Fenofibrate is well absorbed from the gastrointestinal tract. After absorption, Fenofibrate is mainly excreted in the urine in the form of metabolites, primarily fenofibric acid and fenofibric acid glucuronide.
- **Volume of distribution**: 95 L [moderate renal impairment (creatinine clearance of 50 to 90 mL/min)] 30 L [healthy adults]

- **Protein binding**: 99% (Serum protein binding)

- **Route of elimination**: Fenofibric acid is primarily conjugated with glucuronic acid and then excreted in urine. Following oral administration in healthy volunteers, approximately 60% of a single dose of radiolabelled Fenofibrate appeared in urine, primarily as fenofibric acid and its glucuronate conjugate and 25% was excreted in the feces.

- **Half life**: 20 hours

- **Clearance**: 1.2 L/h [Eldery]

- **Toxicity**: LD$_{50}$=1600 mg/kg (Oral, in mice); Investigated as a teratogen and reproductive hazard.
  
  Affected organisms: Humans and other mammals

- **Properties**:
  
  **State**: solid
  
  Melting point: 80.5 °C

- **Experimental Properties**

  Water solubility: 0.25mg/ml at 25 °C
EXCIPIENT PROFILE:

3.A.3.MEGLYOL OIL (Raymond C Rowe et al., 2006):

- **Nonproprietary Names**
  - BP: Fractionated Coconut Oil
  - PhEur: Medium Chain Triglycerides
  - USPNF: Medium-Chain Triglycerides

- **Synonyms**
  - Crodolene; Crossential 094; elaic acid; Emersol; Glycon; Groco; Hy-Phi; Industrene; Metaupon; Neo-Fat; cis-9-octadecenoic acid; 9,10-octadecenoic acid; oleinic acid; Priolene.

- **Chemical Name and CAS Registry Number**
  - Caprylic/Capric Triglyceride, 37332-31-3

- **Empirical Formula and Molecular Weight**
  - Molecular Formula: C_{29}H_{54}O_{6}
  - Molecular Weight: 498

- **Functional Category**
  - Excellent spreadability on the skin and good skin absorption.

- **Applications in Pharmaceutical Formulation or Technology**

  **Oral Products:** Tablets, dragees: Anti-sticking, polishing agents.
  Soft gelatin capsules: Chemically neutral, low-viscosity carrier oil, absorption promoter.
  Drops: Carrier, solvent, and absorption promoter.
  Suspensions, syrups: Carrier and absorption promoter for antibiotics etc.
  Aerosol products: Carrier and solvent (nitroglycerine) etc.
Parenteral Products

Intravenous: Meglyoil 810 and 812 as part of fatty emulsions for parenteral nutrition.

Intramuscular injections: Carrier and solvent.

Topical therapeutics

Psoriasis Treatment and Antipruritics: absorbent, scale-detaching and keratin-softening oil component, particularly in combination with Vitamin A.

Rectal products

Anti-nucleating and dispersing aid for active ingredients in Hard Fat (WITEPSOL)-suppositories.

Cosmetic Skin care cosmetics

Creams and lotions: Non-greasy emollient oil components with very good spread ability.

- Description

Meglyoil neutral oils are clear, virtually colorless liquids of neutral odor and taste. Meglyoil neutral oils are very pure because of their carefully selected raw materials. As a result of tightly controlled manufacturing process, they contain very few microorganisms and are free of additives such as antioxidants, solvents and catalyst residues.

- Typical Properties

Acidity/alkalinity: pH = 4.4 (saturated aqueous solution)
Flash point: 240°C

Melting point: -10°C

Refractive index: \( n_{26D} = 1.448 - 1.451 \)

- **Solubility:**

Meglyoil neutral oils are soluble at 20 °C in the following solvents:

Hexane, toluene, diethyl ether, ethyl acetate, acetone, isopropanol, and ethanol 96%.

Neutral oils are miscible in all ratios with paraffin hydrocarbons and natural oils.

Vapor pressure: 0.133(100°C).

Viscosity (dynamic): 27-33 mPa at 25°C.

- **Stability and Storage Conditions**

Meglyoil neutral oils are not heat-sensitive. Even in hot climates cooling is not necessary. At low temperatures parts of the triglycerides may crystallize. This phenomenon is completely reversible. Meglyoil neutral oils have a very low water content, and are therefore not sensitive to hydrolytic and microbial splitting. If stored in tightly closed containers, protected from moisture and light, the shelf life for meglyoil types 810, 812, 829 and 840 is at least 3 years, for meglyoil 818 one year.

- **Incompatibilities**

Incompatible with aluminum, calcium, heavy metals, iodine solutions, perchloric acid, and oxidizing agents.

- **Method of Manufacture** Meglyol oil is an edible oil extracted from the kernel or meat of matured coconut harvested from the coconut palm (*Cocos nucifera*). Throughout the tropical world, it has provided the primary source of fat in the diets of millions of people for
generations. It has various applications in food, medicine, and industry. Coconut oil is very heat-stable, which makes it suited to methods of cooking at high temperatures like frying.

- **Safety**

  Meglyoil neutral oils are readily biodegradable. Therefore they have the same behaviour as natural fat products with a triglyceride structure.

  Meglyoil is used in oral and topical pharmaceutical formulations.

  - LD50 (mouse, IV): 2 g/kg
  - LD50 (rat, IV): 2.4 mg/kg
  - LD50 (rat, oral): 10.4 g/kg

- **Handling Precautions**

  Observe normal precautions appropriate to the circumstances and quantity of material handled. Gloves and eye protection are recommended.

- **Regulatory Status**

  GRAS listed. Included in the FDA Inactive Ingredients Guide (inhalation and nasal aerosols, tablets, topical and transdermal preparations). Included in non parenteral medicines licensed in the UK. Included in the Canadian List of Acceptable Non-medicinal Ingredients.

3.4.4. TWEEN 80 (Raymond C Rowe et al., 2006):

- **Names Nonproprietary**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Common Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>Polysorbate 80</td>
</tr>
<tr>
<td>PhEur</td>
<td>Polysorbatum 80</td>
</tr>
<tr>
<td>USPNF</td>
<td>Polysorbate 80</td>
</tr>
</tbody>
</table>
Synonyms

Armotan PML 80, Capmul POE-L, Campul POE-L Low PV, Crillet 1, Drewmulse, E432, Durfax 80, E432, Sorbax PML-80, sorbitan monododecanoate, Sorgen TW-80, T-Maz 20, T-Maz 20K, poly(oxy-1,2-ethanediyl) derivatives, polyoxyethylene 80 laurate, Protasorb 1-20, Tego SML 20, Tween 80.

➢ Chemical Names and CAS Registry Number

Polyoxyethylene 80 sorbitan monolaurate [9005-64-5]

➢ Empirical Formula and Molecular Weight

C_{64}H_{120}O_{26}; 1310

➢ Functional Category: Emulsifying agent, nonionic surfactant; solubilizing agent; wetting, dispersing/suspending agent.

Applications in Pharmaceutical Formulation or Technology: Polyoxyethylene sorbitan fatty acid esters (polysorbates) are a series of partial fatty acid esters of sorbitol and its anhydrides copolymerized with approximately 20, 5, or 4 moles of ethylene oxide for each mole of sorbitol and its anhydrides. The resulting product is therefore a mixture of molecules of varying sizes rather than a single uniform compound. Polysorbates containing 80 units of oxyethylene are hydrophilic nonionic surfactants that are used widely as emulsifying agents in the preparation of stable oil-in-water pharmaceutical emulsions. They may also be used as solubilizing agents for a variety of substances including essential oils and oil-soluble vitamins, and as wetting agents in the formulation of oral and parenteral suspensions. They have been found to be useful in improving the oral bioavailability of drug molecules that are substrates for p-glycoprotein.
➢ **Description:** Polysorbates have a characteristic odor and a warm, somewhat bitter taste and yellow in colour

➢ **Typical Properties:**

   - **Hydroxyl value:** 96–108
   - **Moisture content:** 3.0
   - **Saponification value:** 40-50
   - **Acidity/alkalinity:** pH = 6.0–8 for a 5% w/v aqueous
   - **Flash point:** 149°C
   - **HLB value:** 16.7

➢ **Solubility:** Soluble in ethanol and water, insoluble in vegetable oils.

➢ **Stability and Storage Conditions:**

Polysorbates are stable to electrolytes and weak acids and bases; gradual saponification occurs with strong acids and bases. The oleic acid esters are sensitive to oxidation. Polysorbates are hygroscopic and should be examined for water content prior to use and dried if necessary. Also, in common with other polyoxyethylene surfactants, prolonged storage can lead to the formation of peroxides. Polysorbates should be stored in a well-closed container, protected from light, in a cool, dry place.

➢ **Incompatibilities:**

   Discoloration and/or precipitation occur with various substances, especially phenols, tannins, tars, and tarlike materials. The antimicrobial activity of paraben preservatives is reduced in the presence of polysorbates.

➢ **Safety:**

Polysorbates are widely used in cosmetics, food products, and oral, parenteral, and topical pharmaceutical formulations and are generally regarded as nontoxic and nonirritant...
materials. There have, however, been occasional reports of hypersensitivity to polysorbates following their topical and intramuscular use. Polysorbates have also been associated with serious adverse effects, including some deaths; in low-birthweight infants intravenously administered a vitamin E preparation containing a mixture of polysorbates 20 and 80. When heated to decomposition, the polysorbates emit acrid smoke and irritating fumes. The WHO has set an estimated acceptable daily intake for polysorbates 20, 40, 60, 65, and 80, calculated as total polysorbate esters, at up to 25 mg/kg body-weight Polysorbate 80:

- LD$_{50}$ (hamster, oral): 18 g/kg
- LD$_{50}$ (mouse, IV): 1.42 g/kg
- LD$_{50}$ (rat, oral): 37 g/kg:

Handling Precautions:

Observe normal precautions appropriate to the circumstances and quantity of material handled. Eye protection and gloves are recommended.

Regulatory Status: Polysorbates 60, 65, and 80 are GRAS listed. Polysorbates 20, 40, 60, 65, and 80 are accepted as food additives in Europe. Polysorbates 20, 40, 60, and 80 are included in the FDA Inactive Ingredients Guide (IM, IV, oral, rectal, topical, and vaginal preparations). Polysorbates are included in parenteral and nonparenteral medicines licensed in the UK. Polysorbates 20, 21, 40, 60, 61, 65, 80, 81, 85, and 120 are included in the Canadian List of Acceptable Non-medicinal Ingredients.
3.A.5. POLYETHYLENE GLYCOL 400 (Raymond C Rowe et al., 2006):

➢ **Nonproprietary Names**

BP : Macrogol

JP: Macrogol 400, Macrogol 1500, Macrogol 4000, Macrogol 6000

PhEur : Macrogols

USP-N: Polyethylene Glycol

➢ **Synonyms**

Polyethylene oxide, polyoxyethylene, Macrogol, Polyoxyethlene, Aquaffin, Nycoline, alpha-hydro-omega-hydroxypoly(oxy-1,2-ethanediyl), Npolyethylene glycols, Poly Ethylene Oxide, Polyoxyethylene, Polyglycol, 1,2-ethanediol Ehoxylated, Polyoxyethylene ether.

➢ **Chemical Name and CAS Registry Number**

Polyethylene glycol, 25322-68-3

➢ **Empirical Formula and Molecular Weight**

MolecularFormula : C_{2n}H_{4n+2}O_{n+1}, n=8.2to9.1

Molecular Weight : 380-420 g/mol

➢ **Functional Category**

Ointment base, plasticizer, solvent, suppository base, tablet and capsule lubricant.

➢ **Applications in Pharmaceutical Formulation or Technology**

PEG - 400 is harmless towards skin; easily soluble in water and faintly sweet in taste. This makes it an attractive ingredient in cosmetics such as creams, jellies and lotions. PEG-400 is added to lotions intended to have a mild relaxing effect on skin. It is easily absorbed by the skin acting as solution promoter for the therapeutic agents which can be added to the
lotion. It imparts smoothness to skin but does not have a strong dehydrating effect as glycerine.

**Description**

PEG 400 (polyethylene glycol 400) is a low molecular weight grade of polyethylene glycol. It is a clear, colorless, viscous liquid. Due in part to its low toxicity, PEG 400 is widely used in a variety of pharmaceutical formulations.

**Typical Properties**

- **Acidity/alkalinity**: pH = 4.4 (saturated aqueous solution)
- **Auto ignition temperature**: 363°C
- **Boiling point**: 260-280°C
- **Density**: 1.128 g/cm³
- **Flash point**: 238 °C
- **Melting point**: 4-8 °C
- **Refractive index**: n²⁰/D1.471

**Solubility:**

All grades of polyethylene glycol are soluble in water and miscible in all proportions with other polyethylene glycols (after melting, if necessary). Aqueous solutions of higher molecular weight grades may form gels. Liquid polyethylene glycols are soluble in acetone, alcohols, benzene, glycerin, and glycols. Solid polyethylene glycols are soluble in acetone, dichloromethane, ethanol (95%), and methanol; they are slightly soluble in aliphatic hydrocarbons and ether, but insoluble in fats, fixed oils, and mineral oil.

- **Vapor pressure**: 0.133(100°C)
- **Viscosity (dynamic)**: 90.0 cSt at 25 °C, 7.3 cSt at 99 °C
➤ **Incompatibilities**

Glycols are not compatible with Penicillin, Bicitracine, Iodine, Potassium Iodide, Sorbitol, Tannic Acid, Bismuth salts. Glycols are also not suitable with Polyethylene, Backlite & celluloids.

➤ **Method of Manufacture**

Polyethylene glycol polymers are formed by the reaction of ethylene oxide and water under pressure in the presence of a catalyst.

➤ **Safety**

Polyethylene glycols are widely used in a variety of pharmaceutical formulations. Generally, they are regarded as nontoxic and nonirritant materials. Adverse reactions to polyethylene glycols have been reported, the greatest toxicity being with glycols of low molecular weight. However, the toxicity of glycols is relatively low. Polyethylene glycols administered topically may cause stinging, especially when applied to mucous membranes. Hypersensitivity reactions to polyethylene glycols applied topically have also been reported, including urticaria and delayed allergic reactions. The most serious adverse effects associated with polyethylene glycols are hyperosmolarity, metabolic acidosis, and renal failure following the topical use of polyethylene glycols in burn patients. Topical preparations containing polyethylene glycols should therefore be used cautiously in patients with renal failure, extensive burns, or open wounds.

➤ **Handling Precautions**

Observe normal precautions appropriate to the circumstances and quantity of material handled. Eye protection is recommended.
> **Regulatory Status**: Included in the FDA Inactive Ingredients Database (dental preparations; IM and IV injections; ophthalmic preparations; oral capsules, solutions, syrups, and tablets; rectal, topical, and vaginal preparations). Included in nonparenteral medicines licensed in the UK. Included in the Canadian List of Acceptable Non-medicinal Ingredients.

3.A.6 **Fenofibrate method development by using RP-HPLC (Jain N et al., 2009):**

i) **Preparation of standard solution**: A standard stock solution was prepared by accurately weighing about 10 mg of Fenofibrate standard and transferred into 10 ml volumetric flask; added mobile phase methanol-0.2M phosphate buffer pH 7.4 as the mobile phase and injected in 20μl port. and sonicated for 5 minutes to dissolve and made up to volume with mobile phase to obtain a final concentration of 1 mg/ml.

ii) **Calibration curve**: The required test samples were prepared freshly using the stock solution in the range of 1-2μg/ml. Triplicate 20μL injections were made for each concentration and were analyzed under the conditions prescribed chromatographic conditions. A calibration curve was obtained by plotting the response (peak area) versus concentration of drug and represented. Regression equation was calculated. The method was found linear over a concentration range 1 to 2μg/ml.

<table>
<thead>
<tr>
<th>Column</th>
<th>Phenomenex Luna C18 5μm 4.6x250mm(i.d) column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>A : 0.2M Phosphate buffer (PH 7.4)</td>
</tr>
<tr>
<td></td>
<td>B : Methanol</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1000 μL/min</td>
</tr>
<tr>
<td>Detector</td>
<td>UV, D2 lamp, 287 nm</td>
</tr>
<tr>
<td>Column temp.</td>
<td>controlled room temperature (25°C)</td>
</tr>
<tr>
<td>Injection</td>
<td>20 μL sample loop</td>
</tr>
</tbody>
</table>
iii) **Method Validation:**

**a) Linearity**

Drug solutions were prepared in the concentration range of 1 to 2 μg/ml for Fenofibrate. The solutions were injected in triplicate into the HPLC column using methanol-0.2M phosphate buffer pH 7.4 as the mobile phase and injected in 20μl port.

**b) Precision**

Six injections, of three different concentrations for were given on the same day and the values of relative standard deviation were calculated to determine intra-day precision. These studies were repeated on different days to determine inter-day precision. Intermediate precision was established through separation studies on another chromatographic system by another analyst.

**c) Accuracy**

The accuracy of the method was determined by recovery experiments. Known concentration of working standard was added to the fixed concentration of the pre-analyzed micro emulsion. Percent recovery was calculated by comparing the area before and after the addition of working standard. The recovery studies were performed in triplicate. The standard addition method was performed at 50%, 100%, 150% level and the percentage recovery was calculated.

**d) Limit of Detection and Limit of Quantification**

Standard stock solutions of Fenofibrate (1mg/ml) is prepared. Standard solutions of Fenofibrate were prepared by diluting the standard stock solutions with mobile phase. The LOD and LOQ for Fenofibrate under the present chromatographic conditions were estimated at a signal-to-noise ratio (S/N) of 3:1 and 10:1 respectively, by injecting a series of diluted solutions with known concentrations.
e) Robustness

Robustness of the method was checked by making slight changes in chromatographic conditions like mobile phase ratio, pH of buffer, flowing rate.

3.A.7: Solubility studies:

The solubility study was used to identify the suitable oil and surfactant that possess good solubilizing capacity for Fenofibrate. One ml of each of the selected vehicle was added to each capped vial containing an excess of Fenofibrate. After sealing the mixture was shaken well until suspension was formed. Formed suspensions were then shaken by using orbital shaking incubator (VIGNAN-0SR30) at 25°C for 48hrs after shaking the solution was filtered using a membrane filter (0.45μ, 13 mm, Whatman, USA) and filtrates were centrifuged at 5000rpm for 1hr. The concentration of Fenofibrate in each vehicle and their ratios were quantified by HPLC.

3.A.8: Drug-Excipients compatibility studies:

Compatibility studies for drug and excipients were studied by using FTIR (BRUKER-ALPHA T). It is the most widely used method in pre-formulation studies since it can indicate the existence of possible drug-excipients interactions in the formulation. In this study FTIR instrument was used. FTIR spectra for the drug and the excipients of the optimized formulations were obtained. One drop of optimized formulation is mixed with KBr and used for the analysis of FTIR spectrum. Pure drug was also mixed with KBr and spectrum was obtained. Both spectra were compared for possible deviations RP-HPLC and were graphically represented. UV detector is used by keeping λmax at 287nm.


The ratio of surfactant to co-surfactant ratios was on the weight basis. The mixture of surfactant and co-surfactant is referred to as “surfactant phase” in the following discussion. Six types of surfactant phases were prepared: Tween80+peg400(1:1,2:1,3:1), Tween80+alcohol(1:1,2:1,3:1), Cremophor RH40 + PEG400(1:1,2:1,3:1), Cremophor RH40 + Alcohol(1:1,2:1,3:1), The megloil oil was mixed with Deionized water of 1:9, 2:8, 3:7, 4:6, 5:5,
A titration technique was employed for the preparation of the pseudo ternary phase diagrams. Surfactant and cosurfactant mixture was added in small increments (less than 5% w/w) to the mixture of megiol oil/water phase at room temperature. After each Surfactant and cosurfactant addition, the mixture was stirred in a beaker for 2-3 min using a stirring bar and a magnetic stirring plate.

3.A.10: Method of preparation:

A series of nanoemulsion were prepared in each of the twenty formulations with varying concentrations of oil, surfactant, and co-surfactant. In all the formulations, the level of Fenofibrate was constant (i.e. 50 mg). The formulations are prepared by initially dissolving the formulation amount of Fenofibrate was dispersed in surfactant and co-surfactant by using magnetic stirrer. Then add oil phase and water phase until get homogenization for 15min. The formulated emulsion was probe sonicated for size reduction. The prepared formulations are equilibrated at room temperature for at least 48hrs and observed or examined for signs of turbidity or phase separation prior to further studies.

**TABLE 5: Different Formulations**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenofibrate(mg)</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Meglyoil (% w/w)</td>
<td>28.46</td>
<td>19.16</td>
<td>29.4</td>
<td>12.88</td>
<td>28.57</td>
</tr>
<tr>
<td>Tween 80 (%w/w)</td>
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<td>57.1</td>
<td>65.97</td>
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<td>10.26</td>
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<td>13.42</td>
<td>21.13</td>
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<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
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<tr>
<td>Meglyoil (% w/w)</td>
<td>28.14</td>
<td>27.13</td>
<td>20.6</td>
<td>31.69</td>
<td>15.04</td>
</tr>
<tr>
<td>Tween 80 (%w/w)</td>
<td>51.85</td>
<td>54.26</td>
<td>57.25</td>
<td>52.8</td>
<td>53.09</td>
</tr>
<tr>
<td>PEG 400 (% w/w)</td>
<td>20.00</td>
<td>18.60</td>
<td>22.13</td>
<td>15.49</td>
<td>31.85</td>
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### Materials and Methods

#### Ingredients

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<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
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<tr>
<td>Meglyoil (% w/w)</td>
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<td>PEG 400 (% w/w)</td>
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<th>F18</th>
<th>F19</th>
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<tr>
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<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
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<tr>
<td>Meglyoil (% w/w)</td>
<td>25.14</td>
<td>23.13</td>
<td>24.6</td>
<td>37.69</td>
<td>11.04</td>
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<tr>
<td>Tween 80 (%w/w)</td>
<td>54.85</td>
<td>56.26</td>
<td>55.25</td>
<td>50.8</td>
<td>51.09</td>
</tr>
<tr>
<td>PEG 400 (% w/w)</td>
<td>20.00</td>
<td>20.60</td>
<td>20.13</td>
<td>13.49</td>
<td>29.85</td>
</tr>
</tbody>
</table>

**Prepared SNEDDS formulations**

**3.A.11 Characterization:**

**i) Thermodynamic Stability Studies (S. Shafiq, et al., 2007):**

Selected formulations were subjected to different thermodynamic stability tests to assess their physical stability.
1. Heating–cooling cycle: Six cycles between refrigerator temperature (4°C) and 45°C with storage at each temperature of not less than 48 h were conducted, and the formulations were examined for stability at these temperatures.

2. Centrifugation test: Formulations were centrifuged at 3,500 rpm for 30 min, and we looked for phase separation.

3. Freeze–thaw cycle: Three freeze–thaw cycles between −21°C and +25°C, with formulation storage at each temperature for not less than 48 h, were performed.

The selected formulations are subjected to stability studies.

ii) Globule size measurement (Patil p et al., 2007):

The mean particle size and polydispersity index of the size distribution of emulsion globules was determined by using photon correlation spectroscopy (which analyses the fluctuations in light scattering due to brownian motion of the particles) using Nano Zeta sizer (Horiba Instruments, Japan) able to measure sizes between 10-3000nm. Light scattering was monitored at 25°C at a 90° angle. The dispersed formulations were measured after dilution (1:100) to produce the required count rate (50-200) to enable the accurate measurement.

iii) Polydispersity:

Polydispersity is the ratio of standard deviation to mean droplet size, so it indicates the uniformity of droplet size within the formulation. The higher the polydispersity, the lower the uniformity of the droplet size in the formulation.

iv) Zeta potential (Craig et al., 1995):

The zeta potential of nanoemulsion were determined using Nano Zeta sizer (Horiba Instruments, Japan). Charge on emulsion droplets and their mean Zeta potential values (±SD) were obtained from the instruments.

v) Viscosity determination (Craig et al., 1995):

The viscosity of nanoemulsion formulation generally was very low. This was expected, because one of characteristics of nanoemulsion formulation is lower viscosity.
The viscosity of formulation (0.5 g) was determined without dilution using BROOKFIELD-DV-II+pro viscometer using spindle 00 UV adaptor at 25+_0.5 °C.

vi) Refractive index (Craig et al., 1995):

Refractive index of placebo formulations (without drugs), drug loaded formulation and one year old formulation was using Abbes type refractometer. When the refractive index values for formulation were compared with those of placebo and one year old formulation, it was found that there were no significant differences between the values, therefore it can be concluded that the nanoemulsion formulation were not only thermodynamically stable but also chemically stable and remain isotropic; thus there were no interaction between nanoemulsion excipients and drug.

vii) Conductivity determination:

A conductometer (LABINDIA pico+) was used in non-linear temperature compensation mode, according to EN 27888 conductivity was determined during heat between 45 & 90°C under magnetic stirring at an agitation of 250 rpm. This temperature ranges permit a steady state to be achieved, either as an emulsion o/w (high steady state) or as an emulsion w/o (low steady state) in different condition tested. The recording of conductivity relative to temperature permits the determination of phase inversion temperature. Conductivity values lower than 10 micro cm\(^{-1}\) means that the continuous phase is oil, where as a highly steady state shows that water is the continuous phase.

viii) Entrapment efficiency:

A weighed quantity of SNEDDS were added to 100mL of phosphate buffer of pH 7.4. The resulting mixture was keep for 24 hours at dark place. Then the solution was filtered through membrane filter of 0.45 µm pore size and 1mL of this solution was diluted to 10 mL using phosphate buffer of pH 7.4. After further suitable dilution, the samples were analyzed by HPLC for the drug content at 287 nm. The drug entrapment efficiency was determined using the relationship:

\[
\text{Drug entrapment efficiency} = \frac{\text{Experimental drug content} \times 100}{\text{Theoretical drug content}}
\]
x) Transmission electron microscopy (TEM)

The morphology and structure of the drug loaded oil droplets in the nanoemulsion formulations were visualized with TEM analysis. It is also important to visualize any precipitation of the drug upon addition of the aqueous phase. Briefly, a drop of each sample was placed over the formvar coated grid. The samples were then negatively stained with phosphotungstic acid (1% w/v) solution for 10 min at room temperature. Excess liquid was blotted with a piece of Whatman filter paper. The samples were then observed with TEM (Hitachi, H-7500, Tokyo, Japan) operated at 80 kV.

3.A.12 In vitro drug release studies (Buch P et al., 2010)

Drug release tests on each batch of the nanoemulsions were carried out using a USP II dissolution rate test apparatus at a stirring speed of 50 rpm and temperature of 37 ± 0.5°C. An amount of the dry emulsion equivalent to 10mg of drug was filled in a hard gelatin capsule (Size no.1) and was placed in the dissolution medium containing 900mL of phosphate buffer pH 7.4. A 5mL quantity of the dissolution medium was sampled at predetermined time intervals, and fresh dissolution medium was simultaneously used to replenish the dissolution medium on each occasion to keep the volume constant. The sample was filtered through filter disc and the filtrate was diluted with fresh dissolution medium if necessary. The samples were analyzed using RP-HPLC UV detector at 287nm.

3.A.13 In Vitro Bio-equivalence Studies:

The Bio-equivalence studies were performed by comparing the rate and extent of drug release from the developed formulation and the commercial tablet formulation (Lofibre 50mg). The release of Finofibrate from the NE formulation and commercial capsule formulation was determined according to USP dissolution apparatus type-II.

3.A.14 In Vitro Intestinal Permeation Studies (Buch P et al., 2010):

The methods employed were modified from experimental procedures well described in the literature. Male Sprague-Dawley rats (250-300g) were killed by overdose with pentobarbitone administered by intravenous injection. To check the intra duodenal permeability, the duodenal
part of the small intestine was isolated and taken for the in vitro diffusion study. Then this tissue was thoroughly washed with cold Ringer’s solution to remove the mucous and lumen contents. The SNEDDS sample was diluted with 1 mL of distilled water (outside mixing for 1 minute by vortex mixer), and for the tablet sample a suspension of tablet was made in distilled water. The resultant sample (1 mg/mL) was injected into the lumen of the duodenum using a syringe, and the 2 sides of the intestine were tightly closed. Then the tissue was placed in a chamber of organ bath with continuous aeration and a constant temperature of 37°C. The receiver compartment was filled with 30mL of phosphate-buffered saline (pH 5.5). At predetermined time intervals of 5min (up to 1 hour), 2 ml of the samples were withdrawn and the drug concentration was determined by HPLC at maximum wavelength 287nm. The percent diffusion of drug was calculated against time and plotted on a graph.

Figure: 32. In Vitro Intestinal Permeation Studies. A) tightly closing the duodenum filled with diluted emulsion, B) placing the tissue on organ bath. C) tissue mounted in an organ bath.
3.B: Simvastatin Nanoemulsions

3.B.1: List of materials & Equipments:

Table 6: List of Materials used for Simvastatin nano emulsion:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Simvastatin</td>
<td>Gift sample from Bright labs (Hyderabad)</td>
</tr>
<tr>
<td>2</td>
<td>Tween20</td>
<td>Merck specialities pvt limited (Mumbai)</td>
</tr>
<tr>
<td>3</td>
<td>Tween80</td>
<td>Merck specialities pvt limited (Mumbai)</td>
</tr>
<tr>
<td>4</td>
<td>CremophoreRH40</td>
<td>Gift sample from bright labs (Hyderabad)</td>
</tr>
<tr>
<td>5</td>
<td>Oleic acid</td>
<td>Merck specialities pvt limited (Mumbai)</td>
</tr>
<tr>
<td>6</td>
<td>Soyabean oil</td>
<td>Gift Sample from Bright labs (Hyderabad)</td>
</tr>
<tr>
<td>7</td>
<td>Ethyl alcohol</td>
<td>Changshu Yangyuan chemicals (China)</td>
</tr>
<tr>
<td>8</td>
<td>Sunflower oil</td>
<td>Gift Sample from Bright labs (Hyderabad)</td>
</tr>
<tr>
<td>9</td>
<td>Span20</td>
<td>Sd-Fine chemicals limited, Mumbai</td>
</tr>
<tr>
<td>10</td>
<td>Span 80</td>
<td>Sd-Fine chemicals limited, Mumbai</td>
</tr>
<tr>
<td>11</td>
<td>Propylene glycol</td>
<td>Sd-Fine chemicals limited, Mumbai</td>
</tr>
<tr>
<td>12</td>
<td>Potassium dihydrogen phosphate</td>
<td>Sd-Fine chemicals limited, Mumbai</td>
</tr>
<tr>
<td>13</td>
<td>HPLC grade Acetonitrile</td>
<td>Sd-Fine chemicals limited, Mumbai</td>
</tr>
<tr>
<td>14</td>
<td>Meglyoil</td>
<td>Gift sample from bright labs (Hyderabad)</td>
</tr>
<tr>
<td>15</td>
<td>Transcutol p</td>
<td>Sd-Fine chemicals limited, Mumbai</td>
</tr>
</tbody>
</table>

All other ingredient used was of analytical grades.
### Table 7: Equipments used for formulation

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Equipment</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Weighing balance</td>
<td>SHIMADZU AX200, JAPAN</td>
</tr>
<tr>
<td>2</td>
<td>Orbital Shaker</td>
<td>VIGNAN-0SR30, INDIA</td>
</tr>
<tr>
<td>3</td>
<td>Magnetic stirrer</td>
<td>REMI Equipments, USA</td>
</tr>
<tr>
<td>4</td>
<td>UV–Visible Spectrophotometer</td>
<td>LABINDIA 3200, INDIA</td>
</tr>
<tr>
<td>5</td>
<td>Dissolution test apparatus II USP</td>
<td>LABINDIA DS 8000, INDIA</td>
</tr>
<tr>
<td>6</td>
<td>Zeta sizer</td>
<td>Nano ZS 90, HORIBA, JAPAN</td>
</tr>
<tr>
<td>7</td>
<td>PH meter</td>
<td>Elico LI127, INDIA</td>
</tr>
<tr>
<td>8</td>
<td>Conductivity meter</td>
<td>LABINDIA pico+, INDIA</td>
</tr>
<tr>
<td>9</td>
<td>Ultrasonicator</td>
<td>CITIZEN CD 4820, INDIA</td>
</tr>
<tr>
<td>11</td>
<td>Viscometer</td>
<td>BROOKFIELD-DV-II+pro, GERMANY</td>
</tr>
<tr>
<td>12</td>
<td>FTIR</td>
<td>BRUKER-ALPHA T, GERMANY</td>
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<tr>
<td>14</td>
<td>Probe sonicator</td>
<td>SONICS vibra cell, USA</td>
</tr>
<tr>
<td>15</td>
<td>HPLC</td>
<td>SHIMADZU SPD20A detector, LC-20AD pumps, DGU-20A3 degasser, JAPAN</td>
</tr>
</tbody>
</table>
3.B.2. SIMVASTATIN (Wolozin B et al., 2007)

- **Description**: A derivative of lovastatin and potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (hydroxymethylglutaryl COA reductases), which is the rate-limiting enzyme in cholesterol biosynthesis. It may also interfere with steroid hormone production. Due to the induction of hepatic LDL receptors, it increases breakdown of LDL cholesterol.

- **Structure**:

- **Synonyms**:
  - Simvastatin [Usan:Ban:Inn]
  - Simvastatina [Spanish]
  - Simvastatine [French]
  - Simvastatinum [Latin]

- **Categories**:
  - Anticholesteremic Agents
  - Antilipemic Agents
  - Hydroxymethylglutaryl-CoA Reductase Inhibitors
Materials and Methods

- **Weight:** Average: 418.5662
  Monoisotopic: 418.271924326

**Chemical Formula:** CC$_{25}$H$_{38}$O$_5$

- **IUPAC Name:** (1S,3R,7S,8S,8aR)-8-{2-[(2R,4R)-4-hydroxy-6-oxooxan-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8a-hexahydonaphthalen-1-yl 2,2-dimethylbutanoate

**Pharmacology:**

Indication: For the treatment of hypercholesterolemia, primary hypercholesterolemia or mixed dyslipidemia (Fredrickson Types IIa and IIb)

- **Pharmacodynamics:** Simvastatin, the methylated form of lovastatin, is an oral antilipemic agent which inhibits HMG-CoA reductase. Simvastatin is used in the treatment of primary hypercholesterolemia and is effective in reducing total and LDL-cholesterol as well as plasma triglycerides and apolipoproteinB.

- **Mechanism of action:** The 6-membered lactone ring of simvastatin is hydrolyzed *in vivo* to generate the beta, delta-dihydroxy acid, an active metabolite structurally similar to HMG-CoA (hydroxymethylglutaryl CoA). Once hydrolyzed, simvastatin competes with HMG-CoA for HMG-CoA reductase, a hepatic microsomal enzyme. Interference with the activity of this enzyme reduces the quantity of mevalonic acid, a precursor of cholesterol.

- **Absorption** Absorption of simvastatin, estimated relative to an intravenous reference dose, in each of two animal species tested, averaged about 85% of an oral dose. In animal studies, after oral dosing, simvastatin achieved substantially higher concentrations in the liver than in non-target tissues.

- **Protein binding:** Both simvastatin and its b-hydroxyacid metabolite are highly bound (approximately 95%) to human plasma proteins.

**Route of elimination:**

- **Metabolism:** Hepatic, simvastatin is a substrate for CYP3A4.
➢ **Route of elimination**

Following an oral dose of 14C-labeled simvastatin in man, 13% of the dose was excreted in urine and 60% in feces.

➢ **Half life:** 3 hours

➢ **Clearance:** 1.2 L/h [Eldery]

➢ **Toxicity:** LD$_{50}$=1600 mg/kg (Oral, in mice); Investigated as a teratogen and reproductive hazard.

➢ **Affected organisms:** Humans and other mammals

➢ **Properties:** State: solid

Melting point: 135-138 °C

**Drug Interactions:**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amprenavir</td>
<td>Amprenavir may increase the effect and toxicity of simvastatin. Concomitant therapy is contraindicated.</td>
</tr>
<tr>
<td>Atazanavir</td>
<td>Increased risk of myopathy / rhabdomyolysis</td>
</tr>
<tr>
<td>Bosentan</td>
<td>Bosentan may decrease the serum concentration of simvastatin by increasing its metabolism. Monitor for changes in the therapeutic and adverse effects of simvastatin if bosentan is initiated, discontinued or dose changed.</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Carbamazepine, a p-glycoprotein inducer, may decrease the effect of simvastatin by increasing its efflux. Monitor for changes in the therapeutic and adverse effects of simvastatin if carbamazepine is initiated, discontinued or dose changed.</td>
</tr>
</tbody>
</table>
Materials and Methods

Clarithromycin

The macrolide, clarithromycin, may increase the toxicity of the statin, simvastatin.

Colchicine

Increased risk of rhabdomyolysis with this combination.

Cyclosporine

Possible myopathy and rhabdomyolysis.

Delavirdine

Delavirdine, a strong CYP3A4 inhibitor, may increase the serum concentration of simvastatin by decreasing its metabolism. Monitor for changes in the therapeutic and adverse effects of simvastatin if delavirdine is initiated, discontinued or dose changed.

FOOD INTERACTIONS

- Avoid alcohol.
- Avoid drastic changes in dietary habit.
- Avoid taking with grapefruit juice.

3.B.3: CREMOPHORE RH 40 (Raymond C Rowe et al., 2006)

Generic name: Polyoxyl 40 Hydrogenated Castor Oil (USP/NF).

Macrogol-Glycerolhydroxystearat (DAB).

Polyoxyethylenglyceroltrihydroxystearat (DAC).

Chemical nature: Cremophor RH 40 is a nonionic solubilizer and emulsifying agent obtained by reacting 45 moles of ethylene oxide with 1 mole of hydrogenated castor oil.

Properties: Cremophor RH 40 is a white to yellowish thin paste at 20 °C. The HLB value lies between 14 and 16. The solidification point is 20 – 28 °C, saponification value is 50 – 60, hydroxyl value is 60 – 75, acid value is #1, and iodine value is #1.
Materials and Methods

Water content, K. Fischer  # 2%
Ash  # 0.25%
Heavy metals  # 10 ppm

**Solubility:** Cremophor RH 40 forms clear solutions in water, ethanol, 2-propanol, n-propanol, ethyl acetate, chloroform, carbon tetrachloride, toluene and xylene. Solutions become cloudy as the temperature increases.

**Stability:** Pure Cremophor RH 40 is chemically very stable. Prolonged exposure to heat can cause physical separation into a liquid and a solid phase on cooling but the product can be restored to its original form by homogenization. Cremophor RH 40 is stable in aqueous alcohol and purely aqueous solutions. However, it must be noted that strong bases or acids should not be added, as otherwise the ester components may be saponified. decrease in the pH value.

**Applications in Pharmaceutical Formulation or Technology**
Polyoxyethylene castor oil derivatives are nonionic surfactants used in oral, topical, and parenteral pharmaceutical formulations. Cremophor RH emulsifies or solubilizes the fat-soluble vitamins A, D, E, and K in aqueous solutions for oral and topical administration. It has also been used in an aqueous mixture together with caprylic/capric glyceride for mucosal vaccination, providing a potential alternative to parenteral vaccination. It has also been used to enhance the permeability of peptides across monolayers of Caco-2 cells by inhibiting the apically polarized efflux system, enhancing intestinal absorption of some drugs. Cremophor has been used as a vehicle for boron neutron-capture therapy in mice; which is a form of radiation therapy used in the treatment of glioblastoma multiforme.
Cremophore castor oil is also used in the production of glycerin suppositories.

**Handling Precautions:**
Observe normal precautions appropriate to the circumstances and quantity of material handled. Eye protection and gloves are recommended.
Regulatory Status:
Included in the FDA Inactive Ingredients Guide (IV injections and ophthalmic solutions). Included in parenteral medicines licensed in the UK. Included in the Canadian List of Acceptable Non-medicinal Ingredients.

3.A.4: OLEIC ACID  (Raymond C Rowe et al., 2006):

- **Nonproprietary Names**  
  BP: Oleic acid  
  PhEur: Acidum oleicum  
  USPNF: Oleic acid

- **Synonyms:**  
  Crodolene; Crossential 094; elaic acid; Emersol; Glycon; Groco; Hy-Phi; Industrene; Metaupon; Neo-Fat; cis-9-octadecenoic acid; 9,10-octadecenoic acid; oleinic acid; Priolene.

- **Chemical Name and CAS Registry Number**: (Z)-9-Octadecenoic acid [112-80-1]

- **Empirical Formula and Molecular Weight**: \( \text{C}_{18}\text{H}_{34}\text{O}_2 \) and 282.4

- **Functional Category**: Emulsifying agent; skin penetrant.

- **Applications in Pharmaceutical Formulation or Technology**:  
  Oleic acid is used as an emulsifying agent in foods and topical pharmaceutical formulations. It has also been used as a penetration enhancer in transdermal formulations. Oleic acid labeled with 131I and 3H is used in medical imaging.

- **Description**: A yellowish to pale brown, oily liquid with a characteristic lard like odor and taste.

- **Typical Properties**:
  - Acidity/alkalinity: \( \text{pH} = 4.4 \) (saturated aqueous solution)
  - Autoignition temperature: 363°C
  - Boiling point: 2868°C at 13.3 kPa (100 mmHg)
  - Density: 0.895 g/cm³
  - Flash point: 189°C
Melting point: 48°C

- **Solubility:** miscible with benzene, chloroform, ethanol (95%), ether, hexane, and fixed and volatile oils; practically insoluble in water.

- **Stability and Storage Conditions:** On exposure to air, oleic acid gradually absorbs oxygen, darkens in color, and develops a more pronounced odor. At atmospheric pressure, it decomposes when heated at 80–100°C.

Oleic acid should be stored in a well-filled, well-closed container, protected from light, in a cool, dry place.

- **Incompatibilities:**
  Incompatible with aluminum, calcium, heavy metals, iodine solutions, perchloric acid, and oxidizing agents. Oleic acid reacts with alkalis to form soaps.

- **Safety:**
  Oleic acid is used in oral and topical pharmaceutical formulations. In vitro tests have shown that oleic acid causes rupture of red blood cells (hemolysis), and intravenous injection or ingestion of a large quantity of oleic acid can therefore be harmful. The effects of oleic acid on alveolar and buccal epithelial cells in vitro have also been studied; the in vitro and in vivo effects of oleic acid on rat skin have been reported. Oleic acid is a moderate skin irritant; it should not be used in eye preparations. An acceptable daily intake for the calcium, sodium, and potassium salts of oleic acid was not specified by the WHO since the total daily intake of these materials in foods was such that they did not pose a hazard to health.

- **Handling Precautions:** Observe normal precautions appropriate to the circumstances and quantity of material handled. Gloves and eye protection are recommended.

- **Regulatory Status:** GRAS listed. Included in the FDA Inactive Ingredients Guide (inhalation and nasal aerosols, tablets, topical and transdermal preparations). Included in nonparenteral medicines licensed in the UK. Included in the Canadian List of Acceptable Nonmedicinal Ingredients.
3.B.5: TRANSCUTOL P (Raymond C Rowe et al., 2006)

- **Name:** Diethylene Glycol Monoethyl Ether
  
  **Synonyms:** Seahosol DG-L; Shihozoru DG; Solvolsol; Transcutol; Transcutol CG; Transcutol HP; Transcutol P; Trivalin SF.
  
- **Chemical name:** Diethylene glycol monoethyl ether, 3,6-Dioxa-1-octanol.
  
- **CAS Registry Number:** 111-90-0
  
  **Molecular Formula:** C₆H₁₄O₃
  
  **Molecular Weight:** 134.17
  
  **Physical form:** Liquid with a mild, pleasant odour; hydroscopic.
  
- **Solubility** In water: Miscible
  
- **Partition coefficient (Log Pow):** Log Pow: - 0.54
  
- **Physical and chemical specifications**
  
  Appearance: colourless liquid
  
  Melting point: - 76 °C
  
  Boiling point: 197 – 205 °C
  
  Density: 0.988
  
  Vapour Pressure: 0.19 hPa
  
- **Function and uses:** It is used in the chemical and paint industries as a solvent for nitrocellulose, resins, and dyes. DEGEE is not used in food or detergent products.
  
Purified DEGEE (>99%) is used in cosmetics and dermatological preparations and as solvent in some medicine products. DEGEE enhance the percutaneous absorption through the skin and mucosal barriers. It is used in some drugs to enhance absorption.
3.B.6: Simvastatin method development by using RP-HPLC (Nazzal S et al., 2009):

**Instrumentation:**

Chromatographic separation was performed on a chromatographic system equipped with a LC-20AD pump; variable wavelength programmable UV/VIS detector, SPD-20A and rheodyne injector with 20μl fixed loop (LC-20AD Shimadzu).

**Chromatographic conditions:**

- **Columns**: Phenomenex Luna C18 5µm 4.6x250mm(i.d) column
- **Mobile phase**: 
  - A: 0.1% Triethylamine buffer (PH 7.4)
  - B: Acetonitrile
- **Isocratic**: 20:80 v/v
- **Flow rate**: 1000 μL/min
- **Detector**: UV, D2 lamp, 238 nm
- **Column temp.**: controlled room temperature (25°C)
- **Injection**: 20 μL sample loop.

Figure 3: Shimadzu chromatographic system equipped with a LC-20AD pump; variable wavelength programmable UV/VIS detector, SPD-20A and rheodyne injector with 20μl fixed loop.
i) **Preparation of standard solution**: A standard stock solution was prepared by weighing about 100 mg of Simvastatin standard and transferred into 100 ml volumetric flask; to the contents 25 ml of mobile phase was added and sonicated for about 5 minutes for complete solubility and was made up to volume with mobile phase to obtain a final concentration of 1mg/ml of Simvastatin.

ii) **Calibration curve**: From the stock solution, measured volumes of working standards were prepared in the concentration range of 200 – 600 ng/ml. 20μL injections were made for each concentration in triplicate and were analyzed under optimized chromatographic conditions. A calibration curve was plotted by using the response (peak area) versus concentration of drug. Regression equation was calculated.

iii) **Method Validation**:
   a) **Linearity**
   Drug solutions were prepared in the concentration range of 200 to 600 ng/ ml for Simvastatin. The solutions were injected in triplicate into the HPLC column using methanol-0.1% Triethylamine buffer( PH 7.4) as the mobile phase and injected in 20μl port.

   b) **Precision**
   Six injections, of three different concentrations for were given on the same day and the values of relative standard deviation were calculated to determine intra-day precision. These studies were repeated on different days to determine inter-day precision. Intermediate precision was established through separation studies on another chromatographic system by another analyst.

   c) **Accuracy**
   The accuracy of the method was determined by recovery experiments. Known concentration of working standard was added to the fixed concentration of the pre-analyzed micro emulsion. Percent recovery was calculated by comparing the area before and after the addition of working standard. The recovery studies were performed in triplicate. The standard addition method was performed at 50%, 100%, 150% level and the percentage recovery was calculated.
Materials and Methods

d) Limit of Detection and Limit of Quantification

Standard stock solutions of simvastatin (1mg/ml) is prepared. Standard solutions of simvastatin were prepared by diluting the standard stock solutions with mobile phase. The LOD and LOQ for Simvastatin under the present chromatographic conditions were estimated at a signal-to-noise ratio (S/N) of 3:1 and 10:1 respectively, by injecting a series of diluted solutions with known concentrations.

e) Robustness

Robustness of the method was checked by making slight changes in chromatographic conditions like mobile phase ratio, pH of buffer, flowing rate.

3.A.7: Solubility studies:

The solubility of simvastatin in various oils, surfactants, and cosurfactants was determined, respectively. 2 ml of each of the selected vehicle were added to each cap vial containing an excess of simvastatin (10 mg). After sealing, the mixture was heated at 40°C in a water-bath to facilitate the solubilization using a vortex mixer. Mixtures were shaken with shaker at 25°C for 48 h. After reaching equilibrium, each vial was centrifuged at 3000 rpm for 5 min, and excess insoluble simvastatin was discarded by filtration using a membrane filter (0.45µm,13mm, Whatman, USA). The concentration of simvastatin was quantified by HPLC. The solubility of simvastatin in various oils and surfactants were represented in figure.

3.A.8: Drug-Excipients compatibility studies: Compatibility studies for drug and excipients were studied by using FTIR (BRUKER-ALPHA T). It is the most widely used method in pre-formulation studies since it can indicate the existence of possible drug-excipients interactions in the formulation. In this study FTIR instrument was used. FTIR spectra for the drug and the excipients of the optimized formulations were obtained. One drop of optimized formulation is mixed with KBr and used for the analysis of FTIR spectrum. Pure drug was also mixed with KBr and spectrum was obtained. Both spectra were compared for possible deviations.
The pseudo-ternary phase diagrams of oil, surfactant: cosurfactant, and water were developed using surfactant titration method: the mixtures of oil and water at certain weight ratios were titrated with surfactant/co-surfactant mix in a dropwise manner. Six types of surfactant phases were prepared: Tween80+PEG400 (1:1,2:1,3:1), Cremophore RH40 + Transcutol p (1:1,2:1,3:1), For each phase diagrams at a specific ratio of surfactant/cosurfactant transparent and homogenous mixture of oil and drug was formed under the mixing by magnetic stirring. Then, visually observed for phase clarity and flow ability. After the identification of self-nanomulsion region in the phase diagrams, the SNEDDS formulations were selected at desired component ratios. In order to form the SNEDDS.

3.A.10: Formulation design of SNEDDS containing simvastatin

Various formulations of SNEDDS had been developed using different ratios of oil, surfactant-cosurfactant and water with constant amount of drug. A series of SNEDDS were prepared in each of the twenty formulations with varying concentrations of oil, surfactant, and co surfactant. In all the formulations, the level of Simvastatin was constant (i.e. 10 mg). The formulations are prepared by initially dissolving the formulation amount of Simvastatin was dispersed in surfactant and co surfactant by using magnetic stirrer. Then add oil phase and water phase until get homogenization for 15min. The formulated emulsion was probe sonicated for size reduction. The prepared formulations are equilibrated at room temperature for atleast 48hrs and observed or examined for signs of turbidity or phase separation prior to further studies.

Table 8: Various simvastatin formulations

<table>
<thead>
<tr>
<th>Ingredients (%w/w)</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simvastatin</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>38.85</td>
<td>36.32</td>
<td>33.59</td>
<td>29.75</td>
<td>24.12</td>
</tr>
<tr>
<td>Cremophore RH-40</td>
<td>47.87</td>
<td>49.83</td>
<td>52.29</td>
<td>56.80</td>
<td>61.42</td>
</tr>
</tbody>
</table>
3.B.11: Stability and Characterization:

i) Thermodynamic Stability Studies (S. Shafiq, et al., 2007):

Selected formulations were subjected to different thermodynamic stability tests to assess their physical stability.
1. **Heating–cooling cycle:** Six cycles between refrigerator temperature (4°C) and 45°C with storage at each temperature of not less than 48 h were conducted, and the formulations were examined for stability at these temperatures.

2. **Centrifugation test:** Formulations were centrifuged at 3,500 rpm for 30 min, and we looked for phase separation.

3. **Freeze–thaw cycle:** Three freeze–thaw cycles between −21°C and +25°C, with formulation storage at each temperature for not less than 48 h, were performed.

**ii) Globule size measurement (Patil P et al., 2007):**

The mean particle size and poly dispersity index of the size distribution of emulsion globules was determined by using photon correlation spectroscopy (which analyses the fluctuations in light scattering due to brownian motion of the particles) using Nano Zeta sizer (Horiba Instruments, Japan) able to measure sizes between 10-3000nm. Light scattering was monitored at 25°C at a 90° angle. The dispersed formulations were measured after dilution (1:100) to produce the required count rate (50-200) to enable the accurate measurement.

**iii) Zeta potential: (Craig et al., 1995)**

The zeta potential of SNEDDS were determined using Nano Zeta sizer (Horiba Instruments, Japan). Charge on emulsion droplets and their mean Zeta potential values (±SD) were obtained from the instruments.

**iv) Viscosity determination: (Patil P et al., 2007)**

The viscosity of SNEDDS formulation generally was very low. This was expected, because one of characteristics of SNEDDS formulation is lower viscosity. The viscosity of formulation (0.5 g) was determined without dilution using BROOKFIELD-DV-II+pro viscometer using spindle 00 UV adaptor at 25+_0.5 °C.
v) Refractive index: (Craig et al., 1995)

Refractive index of placebo formulations (without drugs), drug loaded formulation and one year old formulation was using Abbes type refractometer. When the refractive index values for formulation were compared with those of placebo and one year old formulation, it was found that there were no significant differences between the values, therefore it can be concluded that the SNEDDS formulation were not only thermodynamically stable but also chemically stable and remain isotropic; thus there were no interaction between SNEDDS excipients and drug.

vi) Conductivity determination:

A conductometer (LABINDIA pico+) was used in non-linear temperature compensation mode, according to EN 27888 conductivity was determined during heat between 45 & 90°C under magnetic stirring at an agitation of 250 rpm. This temperature ranges permit a steady state to be achieved, either as an emulsion o/w (high steady state) or as an emulsion w/o (low steady state) in different condition tested. The recording of conductivity relative to temperature permits the determination of phase inversion temperature. Conductivity values lower than 10 micro cm⁻¹ means that the continuous phase is oil, where as a highly steady state shows that water is the continuous phase.

vii) Polydispersity:

Polydispersity is the ratio of standard deviation to mean droplet size, so it indicates the uniformity of droplet size within the formulation. The higher the polydispersity, the lower the uniformity of the droplet size in the formulation.

viii). Entrapment efficiency: A weighed quantity of SNEDDS were added to 100mL of phosphate buffer of pH 7.4 The resulting mixture was keep for 24 hours at dark place. Then the solution was filtered through membrane filter of 0.45 µm pore size and 1mLof this solution was diluted to 10 mL using phosphate buffer of pH 7.4. After further suitable dilution, the samples were analyzed by HPLC for the drug content at 238 nm. The drug entrapment efficiency was determined using the relationship.
Experimental drug content x 100

Drug entrapment efficiency = \[
\frac{\text{Experimental drug content}}{\text{Theoretical drug content}} \times 100
\]

**ix) Transmission electron microscopy (TEM)**

The morphology and structure of the drug loaded oil droplets in the nano emulsion formulations were visualized with TEM analysis. It is also important to visualize any precipitation of the drug upon addition of the aqueous phase. Briefly, a drop of each sample was placed over the formvar coated grid. The samples were then negatively stained with phosphotungstic acid (1% w/v) solution for 10 min at room temperature. Excess liquid was blotted with a piece of Whatman filter paper. The samples were then observed with TEM (Hitachi, H-7500, Tokyo, Japan) operated at 80 kV.

**3.B.12: In vitro drug release studies**

Drug release tests on each batch of the SNEDDS were carried out using a USP I dissolution rate test apparatus at a stirring speed of 50 rpm and temperature of 37 ± 0.5°C. An amount of the SNEDDS equivalent to 10mg of drug was filled in a hard gelatin capsule (Size no.1) and was placed in the dissolution medium containing 900mL of phosphate buffer pH 7.4. A 5mL quantity of the dissolution medium was sampled at predetermined time intervals, and fresh dissolution medium was simultaneously used to replenish the dissolution medium on each occasion to keep the volume constant. The sample was filtered through filter disc and the filtrate was diluted with fresh dissolution medium if necessary. The samples were analyzed using RP-HPLC UV detector at 238nm.

**3.B.13: In vitro Bio-equivalence Studies:**

The Bio-equivalence studies were performed by comparing the rate and extent of drug release from the developed formulation and the commercial tablet formulation (Simvas 10mg, Micro Labs). The release of Simvastatin from the ME formulation and commercial tablet formulation was determined according to USP dissolution apparatus type-II. The procedure is same as the in vitro release studies; here the drug release from the commercial tablet is also determined.
3.B.14: In Vitro Intestinal Permeation Studies (Buch P et al., 2010):

The methods employed were modified from experimental procedures well described in the literature. Male Sprague-Dawley rats (250-300g) were killed by overdose with pentobarbitone administered by intravenous injection. To check the intra duodenal permeability, the duodenal part of the small intestine was isolated and taken for the in vitro diffusion study. Then this tissue was thoroughly washed with cold Ringer’s solution to remove the mucous and lumen contents. The SMEDDS sample was diluted with 1 mL of distilled water (outside mixing for 1 minute by vortex mixer), and for the tablet sample a suspension of tablet was made in distilled water. The resultant sample (1 mg/mL) was injected into the lumen of the duodenum using a syringe, and the 2 sides of the intestine were tightly closed. Then the tissue was placed in a chamber of organ bath with continuous aeration and a constant temperature of 37°C. The receiver compartment was filled with 30mL of phosphate-buffered saline (pH 5.5). At predetermined time intervals of 5min (up to 1 hour), 2 ml of the samples were withdrawn and the drug concentration was determined by HPLC at maximum wavelength 238nm. The percent diffusion of drug was calculated against time and plotted on a graph.

3.C: In vivo methods

Fenofibrate was obtained as a gift sample from Dr. Reddys Laboratories Ltd (Hyderabad, India). Simvastatin was received as gift sample from Bright Labs, Hyderabad, India and all others were obtained from local market and donated by local labs. Cholesterol and triglycerides estimation kit is obtained by excel diagnostics, Hyderabad.

Animals

Male wistar rats (weighing 250 ± 30 g) were used for the study. They were kept in air-conditioned rooms (24°C-25°C) with constant humidity. The rats were caged and allowed water and food ad bitum before they were distributed by weight into experimental groups. The rats fasted overnight and were then intraperitoneally injected with 250 mg/kg Triton WR 1339 (Tyloxapol, Sigma chemical Co, St. Louis, MO) dissolved in 0.9 percent saline. Control groups of rats were given the vehicle (plain saline), and experimental groups were given plain
fenofibrate (9 mg/kg body weight) or the formulation (equivalent to 9 mg/kg fenofibrate). Without anesthesia and by restraining rats by hand, the oral dosing was performed by intubation using an 18-gauge feeding needle (the volume to be fed was 1.0 mL in all cases). To study the effect of formulation components on lipid lowering, 1 more group was included; this group was fed a placebo formulation. Blood samples were drawn at 0 hours, 24 hours, and 48 hours. Serum was separated by centrifugation at 10,000 rpm and used for biochemical analysis. Serum cholesterol and triglycerides were estimated in normal, control, and drug-treated groups by reported methods. Low-density lipoprotein (LDL) levels were estimated using the Friedewald formula. Statistical analysis of the collected data was performed using 1-way analysis of variance.

**Fig-4: Grouping of rats in cages**  
**Fig-5: Blood withdrawal from rats**

**Statistical analysis**  
The data obtained was subjected to one way analysis of variance (ANOVA) and the significance of difference between formulations was calculated by Benforni (compare selected pairs) with Instat Graphpad prism software (version 4.00; GraphPad Software, San Diego California). The level of statistical significance was considered to be significant when P<0.05.

**Experimental Design:** The obtained male healthy wistar albino rats were divided in to four groups, each group containing six animals.

**Group 1:** (normal control)

**Group 2:** (Triton treated )

**Group 3:** Triton treated + Plain drug
**Group 4:** Triton treated+formulation

**Parameters to be estimated**

1. Estimation of Total Cholesterol (Nader, R *et al.*, 2001)
2. Estimation of triglycerides (Bucolo G *et al.*, 1973)
3. Estimation of HDL cholesterol
4. Estimation of LDL cholesterol
5. Estimation of VLDL cholesterol.

**Estimation of Cholesterol Method: (CHOD - POD) (Nader, R *et al.* 2001)**

**Principle**

Enzymatic determination of total cholesterol according to the following reactions.

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids} \\
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol Oxidase}} 4\text{-Cholesterol-3-one} + \text{H}_2\text{O} \\
2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\text{H}_2\text{O}
\]

**Procedure**

Pipette in a clean dry test tube labelled as Blank (B) Standard(S), and Test (T)

<table>
<thead>
<tr>
<th>Enzyme Reagent</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Deionised water</td>
<td>0.01 ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>0.01 ml</td>
<td>-</td>
</tr>
<tr>
<td>Serum/Plasma</td>
<td>-</td>
<td>-</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

Mix and read the optical density (OD) at 500nm against blank after a 5 minute incubation (37°C). The final colour is stable for at least 1 hour.

**Calculation :**

\[
\text{Cholesterol Conc. in mg\%} = \frac{A \text{ of (T)}}{A \text{ of (S)}} \times 200 \text{ (Std. Conc)}
\]
**Estimation of HDL Cholesterol: (Bucolo G et al. 1973)**

**Step I: precipitation**

Serum 0.2 ml  
HDL PPT Reagent 0.3 ml  
Mix well and Stand at R.T. for 10 mins. Centrifuge at 3000 RPM for 10 mins.

**Step II: Colour Development**

Take 3 clean glass tubes labelled as Blank (B), Standard (S) and Test (T)

<table>
<thead>
<tr>
<th>Enzyme Reagent (ml)</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>hd1111</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant from</td>
<td>-</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Deionised water</td>
<td>0.01</td>
<td>0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubate for 5 mins. at 370C Read the optical Density at 500 nm against Blank

**Calculation:**

HDL Conc. = Abs of Test/ Abs of Standard x 50

**Triglycerides Estimation**

**Principle:**

Triglycerides are hydrolyzed by lipase to glycerol and free fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerokinase (GK) to Glycerol-3-Phosphate (G-3-P) which is oxidized by the enzyme Glycerol-3-Phosphate Oxidase (G-P-O) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-aminoantipyrine and ESPAS in the presence of the enzyme peroxidase (POD) to produce a Brown Colour Complex. The intensity of the color developed proportional to the triglycerides concentration.

**LDL**: Total cholesterol – HDL cholesterol – Triglycerides/ 5

**VLDL**: Triglycerides / 5

**Atherogenic index**: Total cholesterol – HDL Cholesterol / HDL Cholesterol.