Chapter – 2
2. DRUG AND EXCIPIENT PROFILES AND ANALYTICAL METHODS USED IN THE PRESENT INVESTIGATION

Simvastatin is cholesterol lowering agent and derived synthetically from a fermentation product of *Aspergillus terreus*. Simvastatin is administered as a prodrug, which is an inactive form. After oral administration simvastatin is hydrolyzed to the corresponding β-hydroxyacid form (Zhang *et al.*, 2010, Nirogi *et al.*, 2007 & Angelis 2004).

The objective of the work is to improve the oral bioavailability of simvastatin which has poor bioavailability due to poor dissolution and extensive hepatic first pass metabolism. Simavastatin drug belongs to BCS class II having low solubility and high permeability (Rao *et al.*, 2010 & TSRL Inc.). In this chapter physico-chemical, pharmacological and therapeutic profiles of simvastatin are described. Brief profiles of the important excipients used in the preparation of SLN and the analytical methods used for the estimation of simvastatin are also described.

2.1.1. **Chemical name:**

(1S,3R,7S,8S,8aR)-8-[(2R,4R)-4-hydroxy-6-oxotetrahydro-2H-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate.

2.1.2. **Therapeutic category:**

Anti hyperlipidemic

2.1.3. **Chemical Formula:**

C_{25}H_{38}O_{5}

2.1.4. **Molecular Weight:**

418.57

2.1.5. **Physical state and appearance:**

White to off-white, non hygroscopic, crystalline powder

2.1.6. **Solubility:**

It is practically insoluble in water but freely soluble in methanol, ethanol and chloroform; soluble in ether; sparingly soluble in acetonitrile and octanol. The water solubility was found to be 0.03 mg/mL (Serajuddin *et al.*, 1991).

2.1.7. **Melting point:**

135-138°C
2.1.8. Storage:

Simvastatin should be stored at room temperature preferably in the range of 20-25°C. It should be stored away from heat, moisture and light.

2.1.9. Mechanism of action:

Simvastatin is a prodrug and is hydrolyzed to its active β-hydroxy acid form, simvastatin acid, after administration. Simvastatin is a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early and rate limiting step in the biosynthetic pathway for cholesterol.

2.1.10. Half-life:

3 hrs

2.1.11. Bioavailability:

Less than 5%

2.1.12. Log P:

4.68

2.1.13. Pharmacokinetics: (Hwi-yoel Yun et al., 2005)

2.1.13.1. 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. However, because simvastatin undergoes extensive first pass metabolism, the availability of the drug in the systemic circulation is low. Peak plasma concentration occurs at 1.3-2.4 hrs after administration.

2.1.13.2. Distribution:

The protein binding of simvastatin and its active metabolite is more than 95%.

2.1.13.3. Metabolism:

Simvastatin undergoes extensive first-pass metabolism in the liver. It is rapidly hydrolyzed to the beta-hydroxyacid of simvastatin and its 6′-hydroxy, 6′-hydroxymethyl, and 6′-exomethylene derivatives.

2.1.13.4. Excretion:

Simvastatin is a substrate of CYP3A4. The major metabolites of simvastatin present in rat plasma are the β-hydroxy acid and four additional active metabolites. Following an oral dose of radioactive simvastatin to man, 13% of the radioactivity was excreted in the urine and 60% in the faeces within 96 hours. The amount recovered in the faeces represents absorbed medicinal product equivalents excreted in bile as well as unabsorbed medicinal product. Following an intravenous injection of the β-hydroxy acid metabolite, its half-life averaged 1.9 hr. An average of only 0.3% of the intravenous dose was excreted in urine as inhibitors.
2.1.14. Dosage and administration:

The usual dosage range is 5 to 40 mg/day. The recommended usual starting dose is 10 or 20 mg once a day in the evening. History of stroke or other cerebrovascular disease the recommended starting dose is 40 mg/day. Lipid determinations should be performed after 4 weeks of therapy and periodically thereafter.

2.1.15. Adverse effects:

Common side effects may include abdominal pain, diarrhoea, indigestion and a general feeling of weakness. Rare side effects include joint pain, memory loss and muscle cramps. Cholestatic hepatitis, hepatic cirrhosis, rhabdomyolysis (destruction of muscles and blockade of renal system) and myositis have been reported in patients receiving the drug chronically. Serious allergic reactions to simvastatin are rare. Signs of serious allergic reactions like rash, hoarsness itching/swelling, dizziness, difficulty in swallowing/breathing occurring during the therapy must be brought to the notice of the medical attention immediately.

2.1.16. Drug interactions:

The risk of myopathy and rhabdomyolysis is increased by high levels of statin activity in plasma. Simvastatin is metabolized by the cytochrome P450 isoform 3A4. Certain drugs which inhibit this metabolic pathway can raise the plasma levels of simvastatin and may increase the risk of myopathy. These include itraconazole, ketoconazole, posaconazole, voriconazole, the macrolide antibiotics
erythromycin and clarithromycin and the ketolide antibiotic telithromycin, HIV protease inhibitors, boceprevir, telaprevir the antidepressant nefazodone. Combination of these drugs with simvastatin is contraindicated. If short term treatment with strong CYP3A4 inhibitors is unavoidable, therapy with simvastatin must be suspended during the course of treatment.

2.1.17. Contraindications:

Simvastatin is contraindicated in active liver disease. It should be avoided during pregnancy due to potential birth defects. It disrupts the infant's lipid metabolism and hence breast feeding should be avoided during simvastatin therapy. High doses of simvastatin are also contraindicated with antihypertensive drug amlodipine and should not exceed a dosage greater than 20 mg/day when taken alongside amodipine.

Simultaneous administration of simvastatin with gemfibrozil, cyclosporine and danazol is contraindicated.

2.1.18. Available dosage forms:

Simvastatin is available in the form of tablets capsules and also in the form of oral suspension. The strengths of simvastatin available in Indian market are 5, 10, 20 and 40 mg in tablet forms from companies like Microlabs, Cipla, Emcure, Aristo Pharmaceuticals Ltd.
2.2. **Trimyristin** (Sigma Aldrich, T5141):

It is a saturated fat which is the triglyceride of myristic acid. Tramyristin is found naturally in many vegetable fats and oils.

![Trimyristin molecule](image)

2.2.1. **Chemical name:**

1,3-Di(tetradecanoyloxy)propan-2-yl tetradecanoate

2.2.2. **Chemical formula:**

\( \text{C}_{45}\text{H}_{86}\text{O}_6. \)

2.2.3. **Molecular weight:**

723.16 g mol\(^{-1}\)

2.2.4. **Specific Gravity/Density:**

1.080 g/cm\(^3\)

2.2.5. **Physical state appearance:**

Trimyristin is a white to yellowish-gray solid.

2.2.6. **Solubility:**

It is soluble in acetone, benzene, chloroform, ethanol (95%), ether, and aromatic and chlorinated solvents; practically insoluble in water.
2.2.7. Melting point:

56-57°C

2.2.8. Applications:

In pharmaceutical dosage forms trimyristin is used as emulsifying agent.

2.2.9. Storage:

It should be stored in a tightly closed container in a cool, dry place away from moisture and exposure to excessive heat and light should be avoided.

2.2.10. Safety:

Trimyristin is used in oral and topical pharmaceutical formulations and is generally regarded as nontoxic and nonirritant material at the levels employed as an excipient (Stenback & Shubik, 1974, Opdyke 1976 & Guillot et al., 1977). Trimyristin is often derived from vegetable sources and this must be done in accordance with the regulations for consumption.

2.2.11. Regulatory status:

GRAS listed and USP/NF, EP, BP compliance. Included in the FDA Inactive Ingredients Database (orals, injectables and rectals)

2.3. Tripalmitin (Sigma Aldrich, T5888):

Tripalmitin is a triglyceride derived from the fatty acid of palmitic acid. Tripalmitin is found in many natural oils and fats.
2.3.1. **Chemical name:**

1, 2, 3-Propanetriyl trihexadecanoate

2.3.2. **Chemical formula:**

$C_{51}H_{98}O_{6}$

2.3.3. **Molecular weight:**

807.29 g mol$^{-1}$

2.3.4. **Specific Gravity/Density:**

0.886 g/cm$^3$

2.3.5. **Physical state appearance:**

Tripalmitin is a white powder.

2.3.6. **Solubility:**

It is practically insoluble in ethanol and in water and freely soluble in ether, benzene and chloroform.

2.3.7. **Melting point:**

63 - 64°C

2.3.8. **Applications:**

In pharmaceutical dosage forms tripalmitin is used as a surfactant and emulsifying agent.
2.3.9. **Storage:**

The bulk material should be stored in a well-closed container in a cool, dry place away from heat and light.

2.3.10. **Safety:**

Tripalmitin is used in oral and topical pharmaceutical formulations and is generally regarded as nontoxic and nonirritant material (Frosch & Kligman, 1976, Opdyke & Letizia, 1982) at the levels employed as an excipient.

2.3.11. **Regulatory status:**

GRAS listed and USP/NF, EP, BP compliance. Included in the FDA Inactive Ingredients Database (orals, injectables and rectals).

2.4. **Tristearin** (Sigma Aldrich, T2501200):

Tristearin or glyceryl tristearate is a triglyceride derived from three units of stearic acid. Most triglycerides are derived from at least two and more commonly three different fatty acids. Stearin is obtained from animal fats created as a byproduct of processing beef. It can also be found in tropical plants such as palm.

![Triglyceride Structure](image)

2.4.1. **Chemical name:**

1, 3-Di (octadecanoyloxy) propan-2-yl octadecanoate
2.4.2. Chemical formula:

$$C_{57}H_{110}O_6$$

2.4.3. Molecular weight:

891.48 g mol\(^{-1}\)

2.4.4. Specific Gravity/Density:

0.862 g/cm\(^3\)

2.4.5. Physical state appearance:

Tristearin is a white powder

2.4.6. Solubility:

It is practically insoluble in water and freely soluble in ether, ethanol and chloroform.

2.4.7. Melting point:

68°C

2.4.8. Applications:

In pharmaceutical dosage forms tristearin is used as nonionic emulsifier, stabilizer, emollient, and plasticizer in a variety of food, pharmaceutical, and cosmetic applications. It is used as a hardening agent in the manufacture of candles and soap.

2.4.9. Storage:

The bulk material should be stored in a well-closed container in a cool, dry place away from heat and light.
2.4.10. Safety:

Tristearin is used in oral and topical pharmaceutical formulations and is generally regarded as nontoxic and nonirritant at the levels employed as an excipient.

2.4.11. Regulatory status:

GRAS listed and USP/NF, EP, BP compliance. Included in the FDA Inactive Ingredients Database (orals, injectables and rectals)

2.5. Soya lecithin (Raymond et al., 2006):

Soya lecithin contains three kinds of phospholipids; Phosphatidylcholine (PC), Phosphatidylethanolamine (PE) and Phosphatidylinositol (PI). Soya Lecithin is an alliance of naturally-occurring phospholipids, which are extracted during the processing of soybean oil.

2.5.1. Synonyms:

Phosphatidylcholine.

2.5.2. Chemical name:

(2R)-2,3-di(tetradecanoyloxy)propyl]2-(trimethylazaniumyl) ethyl phosphate.
2.5.3. Chemical Formula:

\[ C_{36}H_{72}NO_{8}P \]

2.5.4. Molecular Weight:

677.93 gmol\(^{-1}\)

2.5.5. Viscosity:

80 - 120 Poise

2.5.6. Physical state appearance:

Lecithins vary greatly in their physical form, from viscous semi liquids to powders, depending upon the free fatty acid content. They may also vary in color from brown to light yellow, depending upon whether they are bleached or unbleached or on the degree of purity.

2.5.7. Solubility:

Lecithins are soluble in aliphatic and aromatic hydrocarbons, halogenated hydrocarbons, mineral oil, and fatty acids. They are practically insoluble in cold vegetable and animal oils, polar solvents, and water.

2.5.8. Applications:

Soya Lecithin is used as a natural emulsifying or stabilizing agent in several food and pharmaceutical applications.

2.5.9. Storage:

All lecithin grades should be stored in well-closed containers protected from light and oxidation. Lecithins decompose at extreme
pH. They are also hygroscopic and subject to microbial degradation. Observe normal precautions appropriate to the circumstances and quantity of material handled.

2.5.10. Safety:

Soya lecithin is used in oral and topical pharmaceutical formulations and is generally regarded as nontoxic and nonirritant at the levels employed as an excipient.

2.5.11. Regulatory Status:

It is GRAS listed and included in the FDA Inactive Ingredients Database (inhalations; IM and IV injections; otic preparations; oral capsules, suspensions and tablets; rectal, topical, and vaginal preparations).

2.6. Poloxamer 188 (Sigma Aldrich, 15759):

Poloxamers are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly (propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly (ethylene oxide)).

![Poloxamer 188 molecular structure](attachment:image.png)
2.6.1. Synonym:

Lutrol, Monolan, Pluronic, poloxalkol, poloxamera, polyethylene–propylene glycol copolymer, polyoxyethylene–polyoxypropylene copolymer, Supronic, Synperonic

2.6.2. Chemical Name:

2-methyloxirane; oxirane

2.6.3. Chemical Formula:

(C₃-H₆OC₂H₄O)

2.6.4. Molecular weight:

102.1 g mol⁻¹

2.6.5. Melting point:

52°C

2.6.6. Physical state and appearance:

Poloxamers generally occur as white, waxy, free-flowing prilled granules, or as cast solids. They are practically odorless and tasteless.

2.6.7. Solubility:

Soluble in water, ethanol; partially soluble in toluene. Insoluble in kerosene and ethylene glycol.

2.6.8. Applications:

It is used as emulsifier, solubilizer, suspension stabilizer, thickener and gellant in liquid oral, topical and parenteral pharmaceuticals. It is used in solid oral dosage forms as tablet
lubricant, plasticizer, dispersant, wetting agent, solubilizer, bioavailability enhancer, absorption enhancer for low solubility drugs. It is also used as a solubilizer for actives and essential oils in pharmaceuticals and cosmetics.

2.6.9. Storage:

Poloxamers are stable materials. Aqueous solutions are stable in the presence of acids, alkalis, and metal ions. However, aqueous solutions support mold growth. The bulk material should be stored in a well closed container in a cool, dry place.

2.6.10. Safety:

Poloxamers are used in a variety of oral, parenteral, and topical pharmaceutical formulations, and are generally regarded as nontoxic and nonirritant materials.

2.6.11. Regulatory Status:

It is a GRAS listed and USP/NF, BP, EP, FDA 21CFR 172.808, 173.340, 175.105, 176.180, 176.200, 176.210, 177.1200, 177.1210 compliance. It is included in the FDA Inactive Ingredients Database (inhalations, oral capsules, powders, suspensions, syrups and tablets; topical and vaginal preparations).

2.6.12. Trade names:

The nonproprietary name ‘Poloxamer’ is followed by a number, the first two digits of which, when multiplied by 100, corresponds to the approximate average molecular weight of the polyoxy propylene
portion of the copolymer and the third digit, when multiplied by 10, corresponds to the percentage by weight of the polyoxy ethylene portion.

Similarly, with many of the trade names used for poloxamer, e.g., Pluronic F68 (BASF Corp.), the first digit arbitrarily represents the molecular weight of the polyoxy propylene portion and the second digit represents the weight percent of the oxyethylene portion. The letters ‘L’ and ‘F’, stand for the physical form of the Poloxamer: liquid, flakes and shown in Table 2.1.

<table>
<thead>
<tr>
<th>Nonproprietary name</th>
<th>BASF</th>
<th>CRODA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poloxamer 124</td>
<td>Pluronic L44NF</td>
<td>Synperonic PE/L44</td>
</tr>
<tr>
<td>Poloxamer 188</td>
<td>Pluronic F68NF</td>
<td>Synperonic PE/F68</td>
</tr>
<tr>
<td>Poloxamer 237</td>
<td>Pluronic F87NF</td>
<td>Synperonic PE/F87</td>
</tr>
<tr>
<td>Poloxamer 338</td>
<td>Pluronic F108NF</td>
<td>Synperonic PE/F108</td>
</tr>
<tr>
<td>Poloxamer 407</td>
<td>Pluronic F127NF</td>
<td>Synperonic PE/F127</td>
</tr>
</tbody>
</table>

2.7. Analytical method for the in vitro estimation of simvastatin:

In vitro estimation of simvastatin was done by using HPLC method in the present investigation by using the method developed by Zhang Z, et al., 2010.

2.7.1. Instrument details:

The HPLC system (Model Waters 2496 LC 10 AT) with auto sampler and UV detector was used and the system was equipped with Winchrome software. Samples were chromatographed on a C-18
Symmetry (25 cm x 4.6 mm) column. The column temperature was maintained at 30°C and a flow rate of 1 mL/min was used with isocratic mode.

2.7.2. Preparation of phosphate buffer:

2.72 grams of sodium di hydrogen phosphate was accurately weighed and transferred into a 1000 mL volumetric flask, dissolved and diluted to 1000 mL with HPLC grade water. pH was adjusted to 4.0 with ortho phosphoric acid.

2.7.3. Preparation of mobile phase:

Mobile phase was prepared by mixing 400 mL (40%) phosphate buffer and 600 mL (60%) of acetonitrile. The air bubbles in the mobile phase were removed by sonication using ultrasonic water bath for 5 min and filtered through 0.45 µm filter under vacuum.

2.7.4. Preparation of stock solution and standard solutions

Primary stock solution of simvastatin was prepared by dissolving 25 mg of simvastatin in 50 mL of methanol to obtain the concentration of 500 µg/mL. Secondary stock solutions were prepared by dilution of above solution with methanol to yield concentrations of 20 µg/mL. The secondary stock solution of simvastatin was subsequently diluted with pH 6.8 phosphate buffer to obtain a series of working standard solutions containing 0.2, 0.5, 1, 2, 4, 6, 8 and 10 µg/mL. All standard solutions were filtered through 0.45 µm
membrane filter and 20 µL was injected into HPLC column and measured at 238 nm.

2.7.5. Results and discussion

The concentration and peak area values of the solutions are shown in Table 2.1 and the calibration curve in Fig. 2.2. The present analytical method obeyed Beer’s law in the concentration range of 0.2-10 µg/mL and suitable for the estimation of simvastatin. The correlation coefficient (r) was found to be 0.9999 in the calibration curve indicating a positive correlation between the concentration of simvastatin and the corresponding peak area values.

2.7.6. Precision and accuracy

The intra-day (within-run) and inter-day (between-run) precision and accuracy of the present HPLC method were estimated by subjecting the simvastatin standard solutions (0.5, 4 and 8 µg/mL). Intra-day precision and accuracy were determined by assaying the drug in triplicate for each concentration within one day, whereas inter-day precision and accuracy were determined by assaying the samples in triplicate for each concentration for three consecutive days. Percentage of coefficient of variation (% CV) was determined for intra-day and inter-day precision and percentage of relative error (% RE) was determined for accuracy.

\[
% \text{ CV} = \frac{s.d.}{M} \times 100
\]  

-Eq. 2.1
M is the mean of the experimentally determined concentrations and s.d. is the standard deviation

\[
\% \text{ RE} = \left( \frac{\text{measured concentration} - \text{target concentration}}{\text{target concentration}} \right) \times 100 \quad \text{-Eq. 2.2}
\]

The acceptable precision and accuracy were \% CV < 15 and \% RE < 15. The results are shown in Table 2.2.

The method demonstrated excellent chromatographic specificity with no interference from mobile phase or dissolution medium at the retention time of simvastatin. Representative chromatograms of simvastatin in the pH 6.8 phosphate buffer are shown in Fig. 2.1.
Each of the samples was injected three times and the same retention times were observed in all the cases with a variation of ±0.05 min. The column pressure varied from 132 to 138 Kgf/cm². The peak area was reproducible as indicated by low coefficient of variation (<0.107%) shown in Table 2.2.

Table 2.2: Concentration vs. peak area values for the estimation of simvastatin (n=3)

<table>
<thead>
<tr>
<th>Simvastatin concentration in µg/mL</th>
<th>Peak area</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>74016</td>
<td>0.103</td>
</tr>
<tr>
<td>0.5</td>
<td>151772</td>
<td>0.045</td>
</tr>
<tr>
<td>1.0</td>
<td>306209</td>
<td>0.031</td>
</tr>
<tr>
<td>2.0</td>
<td>614496</td>
<td>0.062</td>
</tr>
<tr>
<td>4.0</td>
<td>1234770</td>
<td>0.068</td>
</tr>
<tr>
<td>6.0</td>
<td>1851902</td>
<td>0.091</td>
</tr>
<tr>
<td>8.0</td>
<td>2442171</td>
<td>0.073</td>
</tr>
<tr>
<td>10.0</td>
<td>3032448</td>
<td>0.086</td>
</tr>
</tbody>
</table>
The calibration curves for simvastatin showed good linearity with correlation coefficient greater than 0.99 in the concentration range of 0.2-10 µg/mL. This indicated a good correlation between peak area and drug concentration. The regression equations presented in Fig. 2.2 were used for calculation of the amount of simvastatin in estimations. The limit of detection (LOD) was found to be 10-23 ng/mL (obtained from LC Solution software).

![Fig. 2.2: Calibration curves for the in vitro estimation of simvastatin](image)

The results of the precision and accuracy determinations are shown in Table 2.3. The maximum % CV and % RE values were found to be 1.40 and 2.00 respectively (0.5 µg/mL in pH 6.8 phosphate buffer) intra-day precision and accuracy studies. Similarly, for the inter-day precision and accuracy studies the maximum % CV and % RE values were 3.11 and 4.50 respectively for 4 µg/mL in pH 6.8 phosphate buffer. However, these values were less than the prescribed
limits of 15%. Hence the results indicated good precision and accuracy over the concentration ranges selected.

**Table 2.3: Inter-day and Intra-day precision and accuracy of simvastatin**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Simvastatin concentration (µg/mL)</th>
<th>% CV</th>
<th>% RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>Measured (Mean, n=3)</td>
<td>Intra-day</td>
<td>Inter-day</td>
</tr>
<tr>
<td>pH 6.8 phosphate buffer</td>
<td>0.5</td>
<td>0.51</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4.05</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>8.28</td>
<td>8.19</td>
</tr>
</tbody>
</table>

The solution remained stable in pH 6.8 phosphate buffer dissolution media tested for the time period specified and no degradation products were observed in any chromatogram. The results of the study indicated that the present HPLC method was simple, sensitive, precise and accurate and hence used for the estimation of simvastatin.

**2.8. HPLC method for the estimation of simvastatin in rat plasma samples:**

Carlucci *et al.* reported the simultaneous estimation of simvastatin along with its active metabolite (Carlucci *et al.*, 1992). However, this method involved the extraction process in two steps. The method was modified and used in the present investigation. The rat plasma samples were processed by protein precipitation method instead of liquid-liquid extraction method in order to avoid the use of
organic solvents and protein free plasma samples were directly injected into the HPLC column.

The instrument details, preparation of phosphate buffer and preparation of mobile phase were followed as mentioned above in the \textit{in vitro} estimation of simvastatin.

\textbf{2.8.1. Preparation of stock solution and standard solutions:}

Primary stock solution of simvastatin was prepared by dissolving 25 mg of simvastatin in 50 mL of methanol to obtain the concentration of 500 µg/mL. The primary stock solution of simvastatin was subsequently diluted with methanol to obtain a series of working standard solutions containing 0.2, 0.5, 5, 10, 20, 50, 100 and 200 µg/mL. All standard solutions were filtered through 0.45 µm membrane filter and 20 µL of the sample was injected into HPLC column and measured at 238 nm.

\textbf{2.8.2. Chromatographic conditions:}

The chromatographic system consisted of Waters 2496 LC 10 AT solvent delivery pump equipped with a 20 µL loop and Rheodyne sample injector and SPD 10 AVP Dual wavelength UV-visible detector. The column used for analysis was a C-18 (25 cm × 4.6 mm). The column temperature was maintained at 30°C and a flow rate of 1 mL/min was used with isocratic mode. Analysis was carried out using ultraviolet detector at wavelength of 238 nm. Sensitivity selected was 0.001 absorbance unit full scale (AUFS). The data was recorded and calculated using Winchrome software.
2.8.3. Sample preparation:

Extraction of the drug from the plasma was carried out by precipitation method. An aliquot of 250 µL of drug free plasma spiked with 10 µL of different working standards of simvastatin. The spiked samples were vortexed for 5 min. Methanol was added (double the volume of plasma) as a precipitating agent and vortexed for 5 min and then centrifuged for 15 min at 5000 rpm. The supernatant solution was separated and filtered through 0.45 µm filter and 20 µL of the solution was injected into HPLC column.

2.8.4. Method validation:

The validation parameters like specificity, linearity, sensitivity, accuracy, precision and recovery in rat plasma, were done according to the US-FDA guidance for industry, bioanalytical method development and validation (USFDA Bioanalytical method Dev. - CDER, 2001 & ICHQ2B., 1996). Selectivity was studied by comparing the chromatograms of six different batches of plasma sample obtained from six albino rats of either sex approved by the ethical committee (ICMR- Ethical Guidelines, 2000). Simvastatin containing plasma samples are spiked and calibration curves were prepared by assaying standard plasma samples of simvastatin, ranging from 2-2000 ng/mL. The linearity of method was determined by plotting the peak area of simvastatin versus the nominal concentration (x) of simvastatin, respectively. The calibration curves were constructed by the method of least squares linear regression.
The intra-and inter-day precision and accuracy of the present HPLC method were estimated by subjecting the three simvastatin QC samples viz. low quality control (LQC), medium quality control (MQC) and high quality control (HQC) to HPLC analysis for five different times on five different days. The concentrations chosen for LQC, MQC and HQC are 10, 250 and 1500 ng/mL. Precision was calculated by using the percent relative standard deviation (%RSD or %CV = 100 s.d./M where, M is the mean of the experimentally determined concentrations and s.d. is the standard deviation of M). Accuracy is defined as the percent relative error (%RE) and was calculated using the following formula

\[ \%\text{RE} = \frac{100(E-T)}{T} \]

where E is the experimentally determined concentration and T is the theoretical concentration.

2.9. Results and discussion:

2.9.1. Calibration of standards:

Working standards of 0.2, 0.5, 5, 10, 20, 50, 100 and 200 μg/mL were prepared from the working stock standard solution. An aliquot of 10 μL of each working standard was spiked to 100 μL of plasma separately to get the concentrations of 2, 5, 50, 100, 200, 500, 1000 and 2000 ng/mL and followed the sample (extraction) procedure mentioned above. The baseline was monitored for 20 min and then 20 μL of these solutions were injected for linearity. Then the standard graph was plotted by taking concentration on x-axis and ratio of peak area of drug on y-axis. The amount of drug present in the sample was
calculated through the standard calibration curve. The present method linearity range 2-2000 ng/mL, it will cover all the strengths of simvastatin in plasma. This calibration curve was used for the determination of concentration of drug in plasma in kinetic study or *in vivo* study.

\[ y = 167.47x + 5054.6 \]

Where, \( y \) is the peak area at 238 nm and \( x \) is the concentration of simvastatin in ng/mL. The \( r \) is square root of \( R^2 \) (Coefficient of determination) \( r \) value was found to be 0.9998 indicating a positive correlation between the concentration of simvastatin and the corresponding peak area values.

**2.9.2. Linearity:**

The calibration curves were obtained at eight concentration levels of simvastatin (2–2000 ng/mL) in methanol. The peak area values were determined at 238 nm in triplicate and the data obtained is shown in Table 2.4. The calibration curve was plotted between concentration of simvastatin and corresponding peak area (shown in Fig. 2.3)
Table 2.4: Concentration vs. peak area values for the estimation of simvastatin in plasma (n=6)

<table>
<thead>
<tr>
<th>Plasma concentration in ng/mL</th>
<th>Peak area</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5385</td>
<td>1.83</td>
</tr>
<tr>
<td>5</td>
<td>5887</td>
<td>1.68</td>
</tr>
<tr>
<td>50</td>
<td>12954</td>
<td>0.73</td>
</tr>
<tr>
<td>100</td>
<td>22154</td>
<td>0.46</td>
</tr>
<tr>
<td>200</td>
<td>41021</td>
<td>0.26</td>
</tr>
<tr>
<td>500</td>
<td>84201</td>
<td>0.11</td>
</tr>
<tr>
<td>1000</td>
<td>175241</td>
<td>0.05</td>
</tr>
<tr>
<td>2000</td>
<td>339541</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Fig. 2.3: Calibration curve for the estimation of simvastatin in plasma

2.9.3. Selectivity:

The selectivity of the present method was established by checking the chromatogram of plasma spiked with drug (simvastatin) with chromatograms of blank rat plasma (without spiking with
simvastatin and IS). There was no interference found at the retention time of the drug peak (simvastatin) shown in Fig. 2.4

2.9.4. Sensitivity:

The LLOQ of this method was verified by injecting the 2 ng/mL. The percent accuracy of LLOQ was 98.64% and precision denoted by % RSD was 4.73%.
2.9.5. Precision and accuracy, recovery:

The coefficients of variation for the intra and inter-day precision were <2%. The intra and inter-day accuracies were 97.36-104.15% for simvastatin. The low levels of coefficients of variation (Table 2.5), indicate the method is accurate and precise.

<table>
<thead>
<tr>
<th></th>
<th>Standard (ng/mL)</th>
<th>Accuracy means±s.d.</th>
<th>%RSD</th>
<th>%RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=5)</td>
<td>LQC 10</td>
<td>102.15</td>
<td>1.68</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>MQC 250</td>
<td>101.43</td>
<td>1.95</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>HQC 1500</td>
<td>104.15</td>
<td>1.85</td>
<td>1.3</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=5)</td>
<td>LQC 10</td>
<td>97.36</td>
<td>2.09</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>MQC 250</td>
<td>101.32</td>
<td>1.77</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>HQC 1500</td>
<td>102.80</td>
<td>1.56</td>
<td>0.5</td>
</tr>
</tbody>
</table>

2.9.6. Robustness:

Robustness of the method was done by changing slight variation in the parameters like mobile phase composition, flow rate and wavelength. Present method didn’t show any significant change when the critical parameters were modified. The tailing factor for the drugs was always less than 2.0 and the components were well separated under all the changes carried out (i.e. mobile phase composition, flow rate and pH of buffer). Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying the experiment at room temperature indicate the method found to be robust.
2.9.7. Ruggedness:

Ruggedness was studied along with precision and accuracy of batches where the effect of the column, and analyst change was observed. The observed value for analyst variation and results obtained for precision and accuracy were within the acceptance criteria (i.e. there are no changes in the retention time, recovery and precision of the drug) according to ICH guidelines.

The results of this study indicated that the method was sensitive, precise and accurate. Hence this method is used for the estimation of simvastatin in rat plasma samples during the in vivo studies.