2.1 Introduction

Atorvastatin (Atr) is a lipid lowering agent comes under the drug class known as statins, used for treatment of hyperlipidemia by lowering blood cholesterol levels. Atr is a BCS class II drug, used for the treatment of elevated total cholesterol, low density lipoprotein and triglycerides, and to elevate high density lipoprotein cholesterol. It also stabilizes plaque and prevents strokes through anti-inflammatory and other mechanisms. The mechanism of action of atorvastatin is selective inhibition of the rate limiting enzyme HMG-CoA reductase, which is involved in the biosynthesis of cholesterol (Law et al., 2003). Although, Atr was selected as a drug candidate because of synergistic properties with our synthesized material (Soy-Whey crosslink, sericin). It was taken as a model drug for present study. However any drug other than Atr, having a mode of action having synergies with antioxidant potential or a disease which is fallout of free radical response, can utilize the proposed system for its probable synergistic action.

2.2 Description of Atorvastatin (USP 2012, IP 2007)

Structural Formula

![Chemical structure of atorvastatin](image)

Fig. 2.1 Chemical structure of atorvastatin
Drug Profile and Preformulation Studies

Chemical Name: Calcium (βR,δR)-2-(p-fluorophenyl)-β,δ-dihydroxy-5-isopropyl-3-phenyl-4-(phenylcarbamoyl)pyrrole-1-heptanoic acid (1:2) trihydrate

Molecular Formula: \([\text{C}_{33}\text{H}_{35}\text{FN}_2\text{O}_5]_2 \text{Ca.3H}_2\text{O}\)

Category: Cardiovascular Agents

Physical Appearance: White to off white amorphous powder

Melting Point: 159.2-160.7 °C

Solubility: Freely soluble in methanol and soluble in dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF); insoluble in aqueous solution with pH less than 4.0. It is very slightly soluble in distilled water, Phosphate buffer (7.4) and acetonitrile; slightly soluble in ethanol. 20.4 ug/mL (pH 2.1), 1.23 mg/mL (pH 6.0)

Partition Coefficient: 6.36 (Octanol/Water)

Storage: Preserved it in well closed container, away from heat and damp places

2.2.1 Mechanism of Action

Atorvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co-A) reductase inhibitors, the rate-limiting enzyme in mevalonate synthesis, which is the precursor for cholesterol biosynthesis (Mekhail et al, 2012).

2.2.2 Biopharmaceutics and Pharmacokinetics

- Atorvastatin is rapidly absorbed after oral administration with maximum plasma concentrations achieved in 1 to 2 hours. The absolute bioavailability of atorvastatin (parent drug) is approximately 14% and the systemic availability of HMG-CoA reductase inhibitory activity is approximately 30%. The low
systemic bioavailability is due to presystemic clearance by hepatic first-pass metabolism.

✓ Mean volume of distribution of atorvastatin is approximately 381 L/m².
✓ Atorvastatin is ≥98% bound to plasma proteins.
✓ Mean plasma elimination half-life of atorvastatin in humans is approximately 14 hours.
✓ Less than 2% of a dose of atorvastatin is recovered in urine following oral administration. (Desager and Horsmans, 1996)

2.2.3 Contraindications

Cholesterol and other products of cholesterol biosynthesis are essential components for fetal development (including synthesis of steroids and cell membranes). Since HMG-CoA reductase inhibitors decrease cholesterol synthesis, they may cause fetal harm when administered to pregnant women. Therefore, HMG-CoA reductase inhibitors are contraindicated during pregnancy and in nursing mothers.

2.2.4 Adverse Effects

The adverse events may include constipation, flatulence, dyspepsia, and abdominal pain. Other possible side effects include myotoxic hepatotoxicity. Hepatitis and pancreatitis have been reported. Hypersensitivity reactions including anaphylaxis and angioedema have also occurred. Rarely, rhabdomyolysis with acute renal failure may develop (Golomb and Evans, 2008).

2.2.5 Drug Interactions

Aluminum hydroxide : The serum concentration of atorvastatin can be decreased.
Azithromycin : Increase the myopathic rhabdomyolysis activities of atorvastatin.
Digoxin : There is slight reduction in digoxin plasma level.
Colestipol : Plasma concentration of atorvastatin decreased.
2.2.6 Dose and Dosage Forms

The recommended starting dose of atorvastatin is 10 mg once daily. The dosage range is 10 to 80 mg once daily. Atorvastatin can be administered as a single dose at any time of the day, with or without food. Therapy should be individualized according to goal of therapy and response.

2.2.7 Overdose

There is no specific treatment for atorvastatin overdosage. In the event of an overdose, the patient should be treated symptomatically, and supportive measures instituted as required. Due to extensive drug binding to plasma proteins, hemodialysis is not expected to significantly enhance atorvastatin clearance.

2.2.8 Precautions

Before instituting therapy with atorvastatin, an attempt should be made to control hypercholesterolemia with appropriate diet, exercise, and weight reduction in obese patients, and to treat other underlying medical problems (Martindale, 2009).

2.2.9 Clinical Uses

Atorvastatin is used for primary prevention in individuals with multiple risk factors for coronary heart disease (CHD) and as secondary prevention in individuals with CHD to reduce the risk of myocardial infarction, stroke, angina, and revascularization procedures.

It is recommended to reduce the risk of cardiovascular events in patients with acute coronary syndrome (ACS). May be used in the treatment of primary hypercholesterolemia and mixed dyslipidemia, homozygous familial hypercholesterolemia, primary dysbetalipoproteinemia, and/or hypertriglyceridemia as an adjunct to dietary therapy to decrease serum total and low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (apoB), and triglyceride concentrations, while increasing high-density lipoprotein cholesterol (HDL-C) levels (White C.M, 1999).
2.2.10 Marketed Products

<table>
<thead>
<tr>
<th>Brand product</th>
<th>Dosage form</th>
<th>Dose (mg per tablet)</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aztor</td>
<td>Tablet</td>
<td>5, 10, 20, 40</td>
<td>Sun Pharmaceutical Industries Ltd., India</td>
</tr>
<tr>
<td>Rotacor</td>
<td>Tablet</td>
<td>10, 20</td>
<td>Novartis, India</td>
</tr>
<tr>
<td>Caditor</td>
<td>Tablet</td>
<td>5, 10, 20</td>
<td>Cadila Pharmaceuticals Limited, India</td>
</tr>
<tr>
<td>Astin</td>
<td>Tablet</td>
<td>5, 10, 20, 40, 80</td>
<td>Micro Lab Ltd., India</td>
</tr>
<tr>
<td>Atorlip</td>
<td>Tablet</td>
<td>5, 10, 20, 40</td>
<td>Cipla Ltd., India</td>
</tr>
</tbody>
</table>

2.2.11 Analytical Techniques

Various methods have been reported in the literature for the identification and quantitative estimation of atorvastatin both in pharmaceutical formulations and biological fluids. These methods include spectrophotometric analysis and chromatographic methods.

**Spectrophotometric Method**

- **Ghanty et al, (2012)** reported UV spectrophotometric analysis of atorvastatin in 1:1 v/v methanol to water solution at 248 nm.

**Chromatographic Analysis**

- **Zarghi et al, (2005)** reported rapid and sensitive high-performance liquid chromatographic method for the determination of atorvastatin in plasma was developed in this study.


✓ Ertürk et al, (2003) described a simple high-performance liquid chromatographic (HPLC) method was developed for the analysis of atorvastatin (AT) and its impurities in bulk drug and tablets.

✓ Bullen et al, (1999) reported a liquid chromatographic/mass spectrometric method to quantitate atorvastatin (AT) and its active metabolites ortho-hydroxy (o-AT) and para-hydroxy (p-AT) atorvastatin in human, dog, and rat plasma was validated.

2.3 Excipients Profile

2.3.1 Soy Protein Isolate

Soy protein is abundant, renewable, inexpensive, biodegradable and expected to present a tailororable degradation profile varying with the crosslinking degree (Elzoghby et al, 2012). Isolated and purified soy proteins show interesting physicochemical and functional attributes in particular gel-forming, emulsifying and surfactant properties. Soy protein also holds promise as antioxidants, ACE inhibitors, anti-diabetics and in coronary heart disease (Gu et al, 2009). Purified soy protein is available in two form known as soy protein isolate and soy protein concentrate. Soy protein concentrates is a soy product containing less than 90% protein. Soy protein isolate is enriched form of soy protein with more than 90% protein and 18 diverse amino acids. The soy protein isolate generally shows a wide range of molecular weights (8–600 kDa). Dry soy protein isolate is a cream to light yellowish colored free flowing powder having characteristic odor of proteins.

Several components associated with soy protein have been implicated in lowering cholesterol: trypsin inhibitors, saponins, isoflavons. Small amounts of the heat-stable
Bowman–Birk inhibitor (trypsin inhibitors) may exert a hypo-cholesteremic effect by increasing the secretion of cholecystokinin. Saponins may contribute to cholesterol lowering by increasing bile excretion. Soy protein containing isoflavones are known to lower cholesterol significantly and also to inhibit formation of atherosclerotic lesions in primates. On the other hand, whey proteins work by hampering the absorption of cholesterol by way of reduced micellar cholesterol solubility in intestine (Arliss et al, 2002).

Soy protein isolate has been emerging as a favourable protein based biomaterial due to its low cytotoxicity, therapeutic properties and its ability to form nanoparticulate system for delivery of drug. In present work soy protein isolate is selected as biomaterial for the crosslinking with whey protein concentrate.

2.3.2 Whey Protein Concentrate

Whey protein is a valuable by-product of the dairy industry (Gunasekaran et al, 2007) Whey protein mainly consists of the globular proteins β-lactoglobulin (45%) and α-lactalbumin (20%) with minor fractions of serum albumin (5%), immunoglobulins (10%) and proteose-peptone (20%). Both α-lactalbumin and β-lactoglobulin possess molar masses <20 kDa. Whey protein concentrate (WPC) containing 70-85% of pure protein. WPC have good nutritional and functional properties and can entrap hydrophobic compounds successfully. Furthermore it can be digested in digestive system easily and releases its contents (Zemel & Ha, 2003).

Whey protein has also been reported to hold cholesterol reducing activity, which could be favorable for cardio vascular diseases. WPC consists of protein content of <80% and is rich in hydrophilic groups such as –OH, –CONH, –CONH₂, –COOH, and –SO₃H (Winkler et al, 2015). WPC is more ideal material as it can be able to crosslinked with SPI using amine-amine crosslinker. It will also show synergistic cholesterol reducing activity with SPI and hypolipidemic drug.
2.3.3 Sericin

Sericin is a natural occurring unique protein extracted from silk cocoons during the degumming process. Sericin is mainly comprised of serine and aspartic acid with strong polar side chains containing hydroxyl, carboxyl, or amino groups, which makes it water soluble. The other major amino acids present are glycine, histidine, glutamic acid, aspartic acid, threonine, and tyrosine. Bioactivities such as antioxidant, anticancer, anti-tyrosinase, anticoagulant, and wound healing and antihyperlipidemic activity have been attributed to sericin over the few past decades. The remarkable mechanical properties, versatile processing in an aqueous environment, biocompatibility, and controlled degradation suggest that silks (both native as well as recombinant) are attractive biomaterials for controlled and sustained release, stabilization and delivery of bioactive molecules (Numata et al, 2010).

Another important attribute of sericin is their process ability into different material format, such as films, hydrogels, nanofibers, nanoparticles, and three-dimensional porous scaffold. (Lamboni et al, 2015). Nanoparticles prepared from sericin could improve the targeted sustained delivery, bioavailability, and biostability of drugs. It was hypothesized that the encapsulation capabilities and cholesterol reducing properties of sericin may enhance the biological properties of hypolipidemic drug incorporated in NPs.

2.2.3.1 Extraction of Sericin

Silkworm cocoons (Bombyx mori) which were produced in a controlled environment cut into small pieces and extraction was carried out using a high temperature and pressure degumming technique (Zhang et al, 2004). Briefly, the 25 g of cocoons were washed with distilled water to remove debris and autoclaved (Indfos 110PB, India) at 120 °C for 60 min after mixing with 375 ml water. Autoclaved sample were then filtered through a membrane to get fibroin free sericin rich protein solution and concentrated at low temperature until the desired concentration of sericin [approximately 7% (w/v)]. The concentration of sericin in solution was measured by Bradford method. After that, the concentrated sericin solution was frozen and lyophilized (Christ Alpha 1–4 lyophilizator, Osterode, Germany) to get powdered sericin (Aramwit et al, 2010).
2.2.3.2 Characterization of Extracted Sericin

Fourier Transform Infrared Spectroscopy (FTIR)
The extracted sericin was characterized by using FTIR using Shimadzu FTIR spectrophotometer. The sericin sample was grounded into pestle mortar and was sealed into a KBr pellet (thickness less than 0.5 mm) by a hydraulic press prior to measurement at ambient temperature. The measurements were carried out in the spectral regions of 4000–400 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
SDS-PAGE (Genetix Biotech Asia, Pvt Ltd, India) was performed to determine the molecular weight of sericin according to previously described method with some modifications. Sample solution was prepared and loaded onto 12% polyacrylamide gel and 5% gradient gel, polypeptide bands were stained by 0.25% Coomasie Brilliant Blue R-250 staining, protein ladder from 30 to 250 kDa (Himedia, Mumbai) was used to determine molecular weight (Zhang et al, 2004).

Bradford Assay
Sericin concentration was determined by Bradford assay at 595 nm using a UV-VIS spectrophotometer. Briefly, the protein samples were mixed with Bradford reagent and incubated for 5 min. The absorbance was measured at 595 nm and the amount of sericin present was compared with a bovine serum albumin (BSA) standard curve.

Preparation of Bradford Reagent
Bradford reagent was prepared by mixing 100 mg of Coomasie Brilliant Blue R-250 staining dye in 50 ml of methanol and 100 ml of 80% phosphoric acid. The solution was filtered and then volume was made up to 1000 ml.

Calibration Curve of BSA by Bradford Method
The accurately weighed 10 mg of BSA was dissolved in required volume of distilled water in clean and dry 10 ml volumetric flask. The volume was made up to 10 ml with
distilled water. Proper aliquots were prepared so as to obtain the range of 10-100 μg/ml and the volume of each aliquot was made upto 0.6 ml with distilled water. Bradford reagent (2.4 ml) was added to each aliquot and allowed to stand for 5 min. The absorbance of each concentration was determined spectrophotometrically at $\lambda_{max}$ 595 nm (Labtronics, LT 2910, India) against mixture of distilled water (0.6 ml) and Bradford reagent (2.4 ml) used as a blank. The calibration curve was prepared between absorbance and concentration which was subsequently linearly regressed. The sericin solution was treated with Bradford reagent with similar details as mentioned above and the concentration of protein was calculated.

2.2.3.3 Results

Extraction of Sericin

Sericin was successfully extracted from silkworm cocoons (Bombyx mori) using a high temperature and pressure degumming technique. The extracted sericin was further characterized by SDS-PAGE, IR and Bradford assay.

Fourier Transform Infrared Spectroscopy (FTIR)

The characteristic peak of sericin is shown in Fig. 2.2. As shown in Table 2.1, in case of sericin, the absorption band at 1650 cm$^{-1}$ is assigned to (amide I), 1524 cm$^{-1}$ is assigned to (amide II), 1240 cm$^{-1}$ is assigned to (amide III) and 543 cm$^{-1}$ is assigned to (amide V) (Zhang et al, 2012).

<table>
<thead>
<tr>
<th>IR Absorption Band (cm$^{-1}$)</th>
<th>Assignment of Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1650</td>
<td>amine I</td>
</tr>
<tr>
<td>1524</td>
<td>amide II</td>
</tr>
<tr>
<td>1240</td>
<td>amide III</td>
</tr>
<tr>
<td>543</td>
<td>amide V</td>
</tr>
</tbody>
</table>

Table 2.1: Interpretation of FT-IR spectra of sericin
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The extracted sericin was characterized by broad bands which indicate the wide range of molecular weight from 30 to 250 kDa as shown in Fig. 2.3. The observation revealed that sericin was a mixture of different molecular weight polypeptide chains.

Fig. 2.2: FTIR Spectra of sericin

Fig.2.3: SDS PAGE of sericin, MW: molecular weight (kDa), (1) Marker, (2) sericin from Bombyx mori
**Calibration Curve of BSA by Bradford Method**

The calibration curve of the BSA was prepared using UV-absorption method at $\lambda_{\text{max}}$ 595 nm in distilled water. A straight line was obtained in a concentration range of 10 to 100 $\mu$g/ml with $R^2$ value of 0.993 (Table 2.2 and Fig. 2.4). The observation revealed that Beer Lambert’s law is followed in UV spectroscopy.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance</th>
<th>Statistical Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.135</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.228</td>
<td>$R^2 = 0.9930$</td>
</tr>
<tr>
<td>40</td>
<td>0.255</td>
<td>Equation of line $Y = 0.005x + 0.041$</td>
</tr>
<tr>
<td>50</td>
<td>0.338</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.598</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2: Calibration curve of BSA by Bradford method at $\lambda_{\text{max}}$ 595 nm**

![Figure 2.4: Calibration curve of BSA by Bradford method at $\lambda_{\text{max}}$ 595 nm](image)
2.3.4 Genipin

Biodegradable polymers such as sericin, soy protein and whey proteins needs to be crosslinked in order to improve their physical properties for application in drug delivery. Glutaraldehyde and epoxy compounds are synthetic chemicals used for crosslinking of protein based polymers. The most common synthetic chemicals used as crosslinking reagents are glutaraldehyde, formaldehyde and dialdehyde starch. However, most of these crosslinking agents are synthetic chemicals which are toxic. On the other hand genipin (Gn) is advantageous in terms of being natural and a non toxic crosslinker with efficient property for the crosslinking of amino groups (Fig 2.5). Gn is obtained from fruits of Gardenia jasminoides. Gn itself is colorless but it reacts spontaneously with amino acids to form blue pigments. The properties of genipin are recorded in Table 2.3 (Djerassi et al, 1960).

The crosslinking mechanism of genipin involves a nucleophilic attack of the primary amine on the C3 carbon of genipin. This causes an opening of the dihydropyran ring. An attack on the resulting aldehyde group by the secondary amine group then follows (Yao et al, 2004).

The proposed reaction mechanism with genipin involves the reaction of genipin with molecules that contain a primary amine group protein. The final step in the formation of the crosslinking material is believed to be dimerization produced by radical reactions. This indicates that genipin can be used to form intramolecular and intermolecular crosslinks that have a heterocyclic structure with materials that contain a primary amine group (Chang et al, 2003).

![Fig. 2.5: Structure of genipin](image)
Table 2.3: Properties of genipin

<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>Cyclopenta(c) pyran-4-carboxylic acid, 1,4a-alpha,5,7a-alpha-tetrahydro-1-hydroxy-7-(hydroxymethyl)-, methyl ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>$\text{C}<em>{11}\text{H}</em>{14}\text{O}_5$</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>226.227</td>
</tr>
<tr>
<td>Melting Point</td>
<td>120-121 °C</td>
</tr>
<tr>
<td>Physical Form</td>
<td>Crystalline Solid</td>
</tr>
</tbody>
</table>

2.4 Preformulation Studies of Drug

A successful formulation process has four stages: preformulation, stabilization of the active substance in bulk form, formulation in the designated dosage forms (drug delivery), and fill and finish of aseptic manufacturing activities. Preformulation stage of the formulation development is defined as the science which deals with physicochemical profiling of candidate drug. Data obtained from preformulation studies provide important information for formulation design.

Every drug has inherent chemical and physical properties which have to be considered before development of pharmaceutical formulation. Accordingly, the present chapter involves the preformulation studies of the selected drug atorvastatin. Partition coefficient, solubility, melting point, UV, IR spectroscopy etc. were carried out for the development of suitable drug delivery system(s).

2.4.1 Physical Parameters

Atorvastatin was obtained as a gift sample from M/s Ipca Laboratories, Ltd., Mumbai, India. The visual observation method was used to determine the physical properties of drug. A small amount of the drug sample was placed on a glass slide. The physical appearance, color and crystalline nature of the drug were observed.
2.4.2 Melting Point
Melting point range of Atr was determined by using melting point apparatus (Superfit, Mumbai, India) after filling the drug powder in capillary tubes (heat sealed at one end).

2.4.3 Partition Coefficient
The partition coefficient is defined as the ratio of unionized drug distributed between the organic and aqueous phase at equilibrium (Niazi et al, 2006). The partition coefficient directly influences the permeability of drug through various membranes. The partition coefficient of the drug was measured in n-octanol: PBS (pH 7.4). Briefly, 10 mg of drug was taken in 10 ml of n-octanol: PBS (pH 7.4) (1:1 v/v) mixture. The vials were placed on shaker and shaken for 24 hr. Separating funnel was used to separate both the phases and the drug concentration in aqueous phase was determined spectrophotometrically at $\lambda_{\text{max}}$ 241 nm (Labtronics, LT 2910 UV Visible spectrophotometer, India).

2.4.4 Solubility Studies
Solubility is defined as the extent to which the solute dissolves at a given temperature. The solubility of Atr was determined in various aqueous and non-aqueous solvents by phase solubility analysis. 10 mg of drug sample was taken in 10 ml of each solvent at room temperature in screw capped test tubes and shaken for 24 hr in shaker.

2.4.5 UV Visible Spectroscopy
The UV scanning of Atr in the range of 200-400 nm in methanol was carried out using UV Visible spectrophotometer (Labtronics, LT 2910, India). The drug was dissolved in methanol and the $\lambda_{\text{max}}$ of drug sample as per official standards was determined.

2.4.6 Infrared Spectral Analysis
IR spectroscopy of Atr was carried out by potassium bromide (KBr) pellets technique using IR spectroscope (Shimadzu, 8400S, Japan). The measurements were taken in the spectral regions of 4000–400 cm$^{-1}$. Various peaks in IR spectrum were recorded and interpreted for the presence of different chemical groups.
2.4.7 Preparation of Calibration Curves

2.4.7.1 Calibration Curve of Atr in Methanol by UV Method

Accurately weighed 10 mg of Atr was dissolved in required volume of methanol in clean and dry 100 ml volumetric flask. The volume was made up to 100 ml with methanol. Proper aliquots were prepared so as to obtain the range of 2-20 μg/ml. The absorbance of each concentration was determined spectrophotometrically at $\lambda_{max}$ 246 nm (Labtronics, LT 2910, India) against methanol used as a blank. The calibration curve was prepared between absorbance and concentration which was subsequently linearly regressed. The each data point of standard curve was an average of three recorded observations (Prajapati et al, 2011).

2.4.7.2 Calibration Curve of Atr in PBS (pH 7.4) by UV Method

The phosphate buffer was prepared according to the official method (IP 2007). As per procedure, 50.0 ml of 0.2 M potassium dihydrogen phosphate was placed in a 200 ml dry volumetric flask and the specified volume (39.1 ml) of 0.2 M sodium hydroxide added. The pH was adjusted to 7.4 and volume was made up by addition of water. Accurately weighed 10 mg of Atr was dissolved in minimum required volume of methanol in clean and dry 100 ml volumetric flask. The volume was made up to 100 ml with PBS (pH 7.4): methanol (8:2 v/v). Proper aliquots were prepared so as to obtain the range of 2-20 μg/ml. the absorbance of each concentration was determined spectrophotometrically at $\lambda_{max}$ 241 nm (Labtronics, LT 2910, India) against PBS (pH 7.4): methanol (8:2 v/v) used as a blank. The calibration curve was prepared by plotting a graph between absorbance and concentration which was subsequently linearly regressed. Each data point of standard curve was an average of three recorded observations.

2.4.7.3 Calibration curve of Atr in PBS (pH 7.4) by using HPLC

Calibration curve of Atr was prepared in plasma using HPLC as method reported elsewhere with slight modification (Anwar et al, 2011). A 50 μl volume of methanol containing known concentration of Atr (0.05-1 μg/ml) were added to the plasma (50 μl).
To the above solution 50 μl of internal standard (simvastatin: 5 μg/50 μl) was added and the mixture was then vortexed for few seconds. The above mixture was centrifuged for 20 min at 10,000 rpm (Spinwin MC-01, Tarsons), and the supernatant was collected and evaporated. The residue was dissolved in 0.2 ml of mobile phase and centrifuged for 10 min at 5000 rpm, filtered through 0.22 μm membrane filter and injected in HPLC system.

HPLC system (Waters 2489, with UV-Visible Detector) consisting of reverse phase column (Spherosorb C$_{18}$, 250 X 4.6 mm, 5μ) and the column effluent was monitored by UV detector at 246 nm. The mobile phase consisted of sodium dihydrogen phosphate (0.05 M, pH 5.1) : methanol (30 : 70 v/v). The mobile phase was filtered through a 0.22 μm membrane filter and degassed by ultrasonication under vacuum before use. The flow rate was 1.0 ml/min, and the effluent was monitored by UV absorption at 246 nm. The calibration curve was prepared by plotting peak area ratio verses Atr concentration in plasma.

2.5 Results

2.5.1 Physical Parameters
Atr was obtained as white to off-white crystalline, odorless powder (Table 2.4). The physical appearance, color and crystalline nature of the drug were found as reported in official literature.

2.5.2 Melting Point
The melting point of Atr was determined and found to be 160 ±1°C as shown in Table 2.4.

2.5.3 Partition Coefficient
The partition coefficient (Log P) of Atr was found 6.14 in octanol: PBS (pH 7.4) which indicated the hydrophobic nature of the drug (Table 2.4).
Table 2.4: Physical evaluation and identification of Atr

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical appearance</td>
<td>A white to off-white, crystalline powder</td>
</tr>
<tr>
<td>Melting point</td>
<td>160±1°C</td>
</tr>
<tr>
<td>UV scanning (200-400 nm) in methanol</td>
<td>$\lambda_{\text{max}}$ 246 nm</td>
</tr>
<tr>
<td>Partition coefficient [n-octanol/PBS (pH 7.4)]</td>
<td>6.14</td>
</tr>
</tbody>
</table>

2.5.4 Solubility Studies

Atr was found to be freely soluble in methanol; soluble in dimethyl formamide and dimethylsulfoxide; slightly soluble in PBS (pH 7.4) and distilled water (Table 2.5).

Table 2.5: Solubility profiles of Atr at room temperature

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>+++</td>
</tr>
<tr>
<td>Dimethyl formamide</td>
<td>++</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>++</td>
</tr>
<tr>
<td>PBS pH 7.4</td>
<td>+</td>
</tr>
<tr>
<td>Distilled water</td>
<td>+</td>
</tr>
</tbody>
</table>

+++Freely soluble; ++ Soluble; + Slightly soluble; - Practically insoluble

2.5.5 UV Visible Spectroscopy

The absorption maxima ($\lambda_{\text{max}}$) of the drug were recorded while scanning the drug dissolved in methanol, within a range of 200-400 nm using double beam spectrophotometer. The value of $\lambda_{\text{max}}$ was found to be 246 nm that is in accordance with the reports published in the literature (Fig. 2.6).

2.5.6 Infrared Spectral Analysis

The IR spectrum of Atr showed characteristic peak of which confirmed the presence of different groups (Fig. 2.8). The peaks at 1106 cm$^{-1}$ confirmed the presence of -OH group (Table 2.6).
Fig. 2.6: UV Scan of Atr

Table 2.6: Infrared spectrum data of Atr

<table>
<thead>
<tr>
<th>IR Absorption Band (cm(^{-1}))</th>
<th>Assignment of Peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>3365</td>
<td>N-H stretching</td>
</tr>
<tr>
<td>3059</td>
<td>C-HO stretching alcoholic group</td>
</tr>
<tr>
<td>2967</td>
<td>C-H stretching</td>
</tr>
<tr>
<td>1651</td>
<td>C=O stretching</td>
</tr>
<tr>
<td>1315</td>
<td>C-N stretching</td>
</tr>
<tr>
<td>1106</td>
<td>O-H bending</td>
</tr>
<tr>
<td>747, 694</td>
<td>C-F bending</td>
</tr>
</tbody>
</table>
Fig. 2.7: Infrared Spectra of Atr (reference)

Fig. 2.8: Infrared Spectra of Atr (sample)
2.5.7 Calibration Curve of Atr in Methanol by UV Method

The calibration curve of the Atr was prepared using UV-absorption method at $\lambda_{\text{max}}$ 246 nm in methanol (Fig. 2.9). A straight line was obtained in a concentration range of 2 to 22 $\mu$g/ml with $R^2$ value of 0.998 (Table 2.7). This result reveals that Beer Lambert’s law is followed in the used concentration range (2 to 22 $\mu$g/ml) in UV spectroscopy.

2.5.8 Calibration Curve of Atr in PBS (pH 7.4) by UV Method

The calibration curve of the Atr was prepared using UV-absorption method at $\lambda_{\text{max}}$ 241 nm in PBS (pH 7.4) (Fig. 2.10). A straight line was obtained in all the cases in a concentration range of 2 to 22 $\mu$g/ml with $R^2$ value of 0.997 (Table 2.8). This result reveals that Beer Lambert’s law is followed in the used concentration range (2 to 22 $\mu$g/ml) in UV spectroscopy.

<table>
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<th>Concentration (µg/ml)</th>
<th>Absorbance</th>
<th>Statistical Parameters</th>
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Table 2.7: Calibration curve of Atr in methanol at $\lambda_{\text{max}}$ 246 nm

- **Correlation Coefficient**: $R^2=0.9980$
- **Equation of line**: $Y=0.042x$
Fig. 2.9: Calibration curve of Atr in methanol at $\lambda_{\text{max}}$ 246 nm

Table 2.8: Calibration curve of Atr in PBS (pH 7.4) at $\lambda_{\text{max}}$ 241 nm

<table>
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**Correlation Coefficient**

$R^2 = 0.9970$

**Equation of line**

$Y = 0.040x$
2.5.9 **Calibration curve of Atr in PBS (pH 7.4) by using HPLC**

The validated HPLC analysis presented well resolved peak of Atr from the standard sample. The standard curve was drawn by plotting the peak area of Atr versus drug concentration in plasma. The chromatograms were recorded at 246 nm with the retention time of 4.6 for Atr and 6.7 for simvastatin. **Fig 2.11** displays the chromatograms of Atr and simvastatin (Internal standard; IS) in plasma. The calibration graph was linear in concentration range of 0.05-1.0 µg/ml with correlation coefficient of 0.995 (**Fig. 2.12 and Table 2.9**). It was concluded that HPLC assay method has been successfully used in further studies of Atr after oral administration of Atr loaded sericin nanoparticles in mice model.

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**Fig. 2.10: Calibration curve of Atr in PBS (pH 7.4) at λ<sub>max</sub> 241 nm**

**Fig. 2.11: HPLC chromatogram of Atr in plasma and simvastatin (IS) in plasma**
Table 2.9: Calibration curve of Atr in plasma

<table>
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<tr>
<th>Concentration (μg/ml)</th>
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- **Correlation Coefficient** $R^2 = 0.995$
- **Equation of line** $Y = 99940x$

Fig. 2.12: Calibration curve of Atr in plasma using HPLC

2.5.10 Discussion

A preformulation study helps in understanding the physico-chemical properties of the drug molecule. It provides the foundation for development of a robust dosage form. Efforts spent on preformulation provide cost savings in the long run, by reducing
challenges during formulation development. It is the investigation of the physical and the chemical properties of the drug substance alone and when combined with formulating excipients to get information useful to the formulator in developing stable and bioavailable dosage form.

It is necessary to detect the purity or homogeneity of the drug substance, as impurities may have undesirable effects. The atorvastatin was standardized as per the specifications given in the Monograph in IP 2007. The organoleptic properties of drug were found as reported in official literature. The melting point and partition coefficient (Log P) of Atr was found to be 160±1° and 6.14 in octanol: PBS (pH 7.4), respectively.

This result of calibration curve in methanol reveals that Beer Lambert’s law is followed in the concentration range (2-22 µg/ml) in UV spectroscopy. A straight line was obtained in calibration curve of Atr in PBS (pH 7.4) in a concentration range of 2-22 µg/ml with R² value of 0.997 (Y=0.040x). Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the solution. The equation obtained from calibration curve was used to determine the concentration of drug in unknown sample.

High-performance liquid chromatography (HPLC) is a versatile analytical technology widely used for the analysis of pharmaceuticals, biomolecules, polymers, and many organic and ionic compounds. The chromatograms were recorded at 246 nm with the retention time of 4.6 for Atr and 6.7 for simvastatin. The calibration curve of Atr in PBS (pH 7.4) was linear in concentration range of 0.05-1.0 µg/ml with correlation coefficient of 0.995. It was concluded that HPLC assay method has been successfully used in analysis of drug in physiological fluids (plasma, serum) after oral administration of Atr loaded sericin nanoparticles in mice model.
REFERENCES

Drug Profile and Preformulation Studies

- Niazi S.K. Handbook of preformulation-Chemical, Biological and Botanical Drugs, Taylor & Francis, 2006, 105-106.


