CHAPTER-III

Development and validation of a Stability-indicating Gradient RP-UHPLC method for the determination of impurities in Atorvastatin drug substance
Introduction:
Statins class of drugs that lower the level of cholesterol in the blood by reducing the production of cholesterol by the liver. It blocks the enzyme in the liver. Not only do they help to lower cholesterol levels, but studies have shown that fewer heart attacks, strokes and surgeries are less common when taking statins. Lipitor, Zocor, Mevacor, Pravachol and Crestor are the names of some brand name statin drugs.

History:
More than 100 years ago a German pathologist named Rudolf Virchow discovered that cholesterol was to be found in the artery walls of people who died from occlusive vascular diseases, like myocardial infarction. The cholesterol was found to be responsible for the thickening of the arterial walls and thus decreasing the radius in the arteries which leads in most cases to hypertension and increased risk of occlusive vascular diseases.

In the 1950s the Framingham heart study led by Dawber revealed the correlation between high blood cholesterol levels and coronary heart diseases. Following up from that study the researchers explored a novel way to lower blood cholesterol levels without modifying the diet and lifestyle of subjects suffering with elevated blood cholesterol levels. The primary goal was to inhibit the cholesterol biosynthesis in the body. Hence HMG-CoA reductase (HMGR) became a natural target. HMGR was found to be the rate-limiting enzyme in the cholesterol biosynthetic pathway. There is no build-up of potentially toxic precursors when HMGR is inhibited, because hydroxymethylglutarate is water soluble and there are alternative metabolic pathways for its breakdown.

In 1971, Akira Endo, a Japanese biochemist working for the pharmaceutical company Sankyo, began the search for a cholesterol-lowering drug. Research had already shown cholesterol is mostly manufactured by the body in the liver, using the enzyme HMG-CoA reductase. Endo and his team reasoned that certain microorganisms may produce inhibitors of the enzyme to defend themselves against other organisms, as mevalonate is a precursor of many substances required by organisms for the maintenance...
of their cell walls (ergosterol) or cytoskeleton (isoprenoids) \[^4\]. The first agent they identified was mevastatin (ML-236B), a molecule produced by the fungus *Penicillium citrinum*.

A British group isolated the same compound from *Penicillium brevicompactum*, named it compactin and published their report in 1976 \[^5\]. The British group mentions antifungal properties, with no mention of HMG-CoA reductase inhibition.

Mevastatin was never marketed, because of its adverse effects of tumors, muscle deterioration and sometimes death in laboratory dogs. P. Roy Vagelos, chief scientist and later CEO of Merck & Co, was interested and made several trips to Japan starting in 1975. By 1978, Merck had isolated Lovastatin (mevinolin, MK803) from the fungus *Aspergillus terreus*, first marketed in 1987 as Mevacor \[^3\].

Cholesterol researcher Daniel Steinberg writes that while the Coronary Primary Prevention Trial of 1984 demonstrated cholesterol lowering could significantly reduce the risk of heart attacks and angina, physicians, including cardiologists, remained largely unconvinced \[^6\].

To market statins effectively, Merck had to convince the public about the dangers of high cholesterol and doctors that statins were safe and would extend lives. As a result of public campaigns, people became familiar with their cholesterol numbers and the difference between "good" and "bad" cholesterol and rival pharmaceutical companies began producing their own statins, such as Pravastatin (Pravachol), manufactured by Sankyo and Bristol-Myers Squibb. In April 1994, the results of a Merck-sponsored study, the Scandinavian Simvastatin Survival study, were announced. Researchers tested Simvastatin, later sold by Merck as Zocor, on 4,444 patients with high cholesterol and heart disease. After five years, the study concluded the patients saw a 35% reduction in their cholesterol and their chances of dying of a heart attack were reduced by 42\% \[^3,\ 7\]. In 1995, Zocor and Mevacor both made Merck over US$1 billion \[^3\]. Endo was awarded the 2006 Japan Prize and the Lasker-DeBakey Clinical Medical Research Award in 2008.
Available forms:

The statins differ with respect to their ring structure and substituents. These differences in structure affect the pharmacological properties of the statins \(^8\), such as affinity for the active site of the HMGR.

- Rates of entry into hepatic and non-hepatic tissues.
- Availability in the systemic circulation for uptake into non-hepatic tissues.
- Routes and modes of metabolic transformation and elimination.

Statins have sometimes been grouped into two groups of statins according to their structure, like statins having substituted decalin-ring structure that resemble the first statin ever discovered, mevastatin has often been classified as type 1 like Simvastatin, Lovastatin and Pravastatin due to their structural relationship. Statins that are fully synthetic and have larger groups linked to the HMG-like (structure of HMG-CoA as below) moieties are often referred to as type 2 like Fluvastatin, Cerivastatin, Atorvastatin and Rosuvastatin \(^9\).

Another way of categorizing the statins into groups are: fermentation-derived and synthetic. They include, along with brand names, which may vary between countries \(^10\), (Table-1).
### Table 1: Statins categorized based on fermentation-derived and synthetic.

<table>
<thead>
<tr>
<th>Statin</th>
<th>Structure</th>
<th>Brand name</th>
<th>Derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td><img src="structure_atorvastatin.png" alt="Structure of Atorvastatin" /></td>
<td>Lipitor, Torvast</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Cerivastatin</td>
<td><img src="structure_cerivastatin.png" alt="Structure of Cerivastatin" /></td>
<td>Lipobay, Baycol. (Withdrawn from the market in August, 2001 due to risk of serious Rhabdomyolysis)</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td><img src="structure_fluvastatin.png" alt="Structure of Fluvastatin" /></td>
<td>Lescol, Lescol XL</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Mevastatin</td>
<td><img src="structure_mevastatin.png" alt="Structure of Mevastatin" /></td>
<td>Compactin</td>
<td>Naturally occurring compound. Found in red yeast rice.</td>
</tr>
<tr>
<td>Statin</td>
<td>Structure</td>
<td>Brand name</td>
<td>Derivation</td>
</tr>
<tr>
<td>---------------------</td>
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<td>---------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td><img src="image" alt="Structure" /></td>
<td>Livalo, Pitava</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Pravastatin</td>
<td><img src="image" alt="Structure" /></td>
<td>Pravachol, Selektine, Lipostat</td>
<td>Fermentation-derived. (A fermentation product of bacterium <em>Nocardia autotrophica</em>).</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td><img src="image" alt="Structure" /></td>
<td>Crestor</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Simvastatin</td>
<td><img src="image" alt="Structure" /></td>
<td>Zocor, Lipex</td>
<td>Fermentation-derived. (Simvastatin is a synthetic derivative of a fermentation product of <em>Aspergillus terreus</em>.)</td>
</tr>
<tr>
<td>Simvastatin+Ezetimibe</td>
<td>--</td>
<td>Vytorin</td>
<td>Combination therapy</td>
</tr>
<tr>
<td>Lovastatin+Niacin</td>
<td>--</td>
<td>Advicor</td>
<td>Combination therapy</td>
</tr>
<tr>
<td>extended-release</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atorvastatin+Amlodipine</td>
<td>--</td>
<td>Caduet</td>
<td>Combination therapy - Cholesterol+Blood Pressure</td>
</tr>
<tr>
<td>Besylate</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin+Niacin</td>
<td>--</td>
<td>Simcor</td>
<td>Combination therapy</td>
</tr>
<tr>
<td>extended-release</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LDL-lowering potency varies between agents. Cerivastatin is the most potent, (withdrawn from the market in August, 2001 due to risk of serious rhabdomyolysis) followed by (in order of decreasing potency), rosuvastatin, Atorvastatin, Simvastatin, Lovastatin, Pravastatin and Fluvastatin \(^{[11]}\). The relative potency of pitavastatin has not yet been fully established.
Some types of statins are naturally occurring and can be found in such foods as oyster mushrooms and red yeast rice. Randomized controlled trials found them to be effective, but the quality of the trials was low. Most of the block-buster branded statins has become generic by 2012, including Atorvastatin, the largest-selling branded drug.

Table-2: Statin equivalent dosages

<table>
<thead>
<tr>
<th>% LDL reduction (approx.)</th>
<th>Atorvastatin</th>
<th>Fluvastatin</th>
<th>Lovastatin</th>
<th>Pravastatin</th>
<th>Rosuvastatin</th>
<th>Simvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-20%</td>
<td>--</td>
<td>20 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>--</td>
<td>5 mg</td>
</tr>
<tr>
<td>20-30%</td>
<td>--</td>
<td>40 mg</td>
<td>20 mg</td>
<td>20 mg</td>
<td>--</td>
<td>10 mg</td>
</tr>
<tr>
<td>30-40%</td>
<td>10 mg</td>
<td>80 mg</td>
<td>40 mg</td>
<td>40 mg</td>
<td>5 mg</td>
<td>20 mg</td>
</tr>
<tr>
<td>40-45%</td>
<td>20 mg</td>
<td>--</td>
<td>80 mg</td>
<td>80 mg</td>
<td>5-10 mg</td>
<td>40 mg</td>
</tr>
<tr>
<td>46-50%</td>
<td>40 mg</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>10-20 mg</td>
<td>80 mg*</td>
</tr>
<tr>
<td>50-55%</td>
<td>80 mg</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>20 mg</td>
<td>--</td>
</tr>
<tr>
<td>56-60%</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>40 mg</td>
<td>--</td>
</tr>
</tbody>
</table>

* 80-mg dose no longer recommended due to increased risk of rhadomyolysis.

Table-3: Starting dose.

<table>
<thead>
<tr>
<th>Starting dose</th>
<th>10–20 mg</th>
<th>20 mg</th>
<th>10–20 mg</th>
<th>40 mg</th>
<th>10 mg; 5 mg if hypothyroid, &gt;65 yo, Asian</th>
<th>20 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>If higher LDL reduction goal</td>
<td>40 mg if &gt;45%</td>
<td>40 mg if &gt;25%</td>
<td>20 mg if &gt;20%</td>
<td>--</td>
<td>20 mg if LDL &gt;190 mg/dL (4.87 mmol/L)</td>
<td>40 mg if &gt;45%</td>
</tr>
<tr>
<td>Optimal timing</td>
<td>Anytime</td>
<td>Evening</td>
<td>With evening meals</td>
<td>Anytime</td>
<td>Anytime</td>
<td>Evening</td>
</tr>
</tbody>
</table>
Medical uses:

Clinical practice guidelines generally recommend people to try "lifestyle modification", including a cholesterol-lowering diet and physical exercise, before statins use is considered.[13, 14]

Primary prevention:

There is debate over whether or not statins are effective in those with high cholesterol, but no other health problems.[15] One review did not find a mortality benefit in those at high risk but without prior cardiovascular disease.[15] Other reviews concluded there is a mortality and morbidity benefit[16, 17], but there were concerns regarding the quality of the evidence.[18] With respect to quality of life, evidence of improvement is limited when statins are used for primary prevention.[18] No studies as of 2010 show improved clinical outcomes in children with high cholesterol though statins decrease cholesterol levels.[19]

Secondary prevention:

Statins are effective in decreasing mortality in people with pre-existing Cardiovascular disease (CVD). They are also currently advocated for use in patients at high risk of developing heart disease.[20] On average, statins can lower LDL cholesterol by 1.8 mmol/l (70 mg/dl), which translates into a 60% decrease in the number of cardiac events (heart attack, sudden cardiac death) and a 17% reduced risk of stroke after long-term treatment.[21] They have less effect than the fibrates or niacin in reducing triglycerides and raising HDL-cholesterol ("good cholesterol").

Mechanism of action:

The HMG-CoA reductase pathway, which is blocked by statins via inhibiting the rate limiting enzyme HMG-CoA reductase. Statins act by competitively inhibiting HMG-CoA reductase, the first committed enzyme of the HMG-CoA reductase pathway. Because statins are similar to HMG-CoA on a molecular level, they take the place of HMG-CoA in the enzyme and reduce the rate by which it is able to produce mevalonate,
the next molecule in the cascade that eventually produces cholesterol, as well as a number of other compounds. This ultimately reduces cholesterol via several mechanisms.

**Inhibiting cholesterol synthesis:**

By inhibiting HMG-CoA reductase, statins block the pathway for synthesizing cholesterol in the liver. This is significant because most circulating cholesterol comes from internal manufacture rather than the diet. When the liver can no longer produce cholesterol, levels of cholesterol in the blood will fall. Cholesterol synthesis appears to occur mostly at night \[^{22}\]\, so statins with short half-lives are usually taken at night to maximize their effect. Studies have shown greater LDL and total cholesterol reductions in the short-acting Simvastatin taken at night rather than the morning \[^{23, 24}\]\, but have shown no difference in the long-acting Atorvastatin \[^{25}\].

**Adverse effects:**

The most common adverse side effects are raised liver enzymes and muscle problems. In randomized clinical trials, reported adverse effects are low; but they are "higher in studies of real world use" and more varied \[^{26}\]. In randomized trials, statins increased the risk of an adverse effect by 39% compared to placebo (odds ratios 1.4); two-thirds of these were myalgia or raised liver enzymes, with serious adverse effects similar to placebo \[^{27}\]. However, reliance on clinical trials can be misleading indications of real-world adverse effects – for example, the statin cerivastatin was withdrawn from the market in 2001 due to cases of rhabdomyolysis (muscle breakdown), although rhabdomyolysis did not occur in a meta-analysis of cerivastatin clinical trials \[^{26}\]. Other possible adverse effects include cognitive loss, neuropathy, pancreatic and hepatic dysfunction and sexual dysfunction \[^{26}\].

Some patients on statin therapy report myalgias \[^{28}\], muscle cramps \[^{28}\] or, less frequently, gastrointestinal or other symptoms. Multiple other side effects occur rarely; typically also at similar rates with only placebo in the large statin safety/efficacy trials. Two randomized clinical trials found cognitive issues, while two did not; recurrence upon reintroduction suggests these are causally related to statins in some individuals \[^{29}\]. A Danish case-control study published in 2002 suggested a relationship between long-term statin use and increased risk of nerve damage or polyneuropathy, \[^{30}\] but suggested this
side effect is "rare, but it does occur" \[^{31}\]; other researchers have pointed to studies of the effectiveness of statins in trials involving 50,000 people which have not shown nerve damage as a significant side effect \[^{32}\].

Rare reactions include myositis and myopathy, with the potential for rhabdomyolysis (the pathological breakdown of skeletal muscle) leading to acute renal failure \[^{33, 34}\].

Graham et al. (2004) reviewed records of over 250,000 patients treated from 1998–2001 with the statin drugs Atorvastatin, Cerivastatin, Fluvastatin, Lovastatin, Pravastatin and Simvastatin \[^{15}\]. The incidence of rhabdomyolysis was 0.44 per 10,000 patients treated with statins other than Cerivastatin. However, the risk was over 10-fold greater if Cerivastatin was used, or if the standard statins (Atorvastatin, Fluvastatin, Lovastatin, Pravastatin, or Simvastatin) were combined with fibrate (Fenofibrate or Gemfibrozil) treatment. Cerivastatin was withdrawn by its manufacturer in 2001. Statins may slightly increase the risk of diabetes \[^{36}\], with higher doses appearing to have a larger effect \[^{37}\].

Although there have been concerns that statins might increase cancer, several meta-analyses have found no relationship to cancer, the largest of which as of 2006 included nearly 87,000 participants \[^{38}\]. In 2007, though, a meta-analysis of 23 statin treatment arms with 309,506 person-years of follow-up found the risk of cancer was significantly associated with lower achieved LDL-C levels; the authors said this requires further investigation \[^{39}\].

**Drug interactions:**

Combining any statin with a fibrate or niacin, another category of lipid-lowering drugs, increases the risks for rhabdomyolysis to almost 6.0 per 10,000 person-years \[^{35}\]. Most physicians have now abandoned routine monitoring of liver enzymes and creatine kinase, although they still consider this prudent in those on high-dose statins or in those on statin/fibrate combinations and mandatory in the case of muscle cramps or of deterioration in renal function.

Consumption of grapefruit or grapefruit juice inhibits the metabolism of certain statins. Bitter oranges may have a similar effect \[^{40}\]. Furanocoumarins in grapefruit juice
(i.e. bergamottin and dihydroxybergamottin) inhibit the cytochrome P450 enzyme CYP3A4, which is involved in the metabolism of most statins (however, it is a major inhibitor of only Lovastatin, Simvastatin and to a lesser degree, Atorvastatin) and some other medications \[^{[41]}\] (flavonoids (i.e. naringin) were thought to be responsible). This increases the levels of the statin, increasing the risk of dose-related adverse effects (including myopathy/rhabdomyolysis). The absolute proscription of grape fruit juice consumption for users of some statins is controversial \[^{[42]}\].

The FDA notified healthcare professionals of updates to the prescribing information concerning interactions between protease inhibitors and certain statin drugs. Protease inhibitors and statins taken together may raise the blood levels of statins and increase the risk for muscle injury (myopathy). The most serious form of myopathy, rhabdomyolysis, can damage the kidneys and lead to kidney failure, which can be fatal. \[^{[43]}\].

After the literature survey regarding the efficacy and adverse effects of statin drugs, author considered Atorvastatin for study. Due to the change in lifestyle of people i.e., diet and physical activity, present generation needs these kinds of drugs. Hence a proper estimation of impurity in drug substance is very important, as the presence of impurities in drug substance may alter the pharmacological activity which in turn leads to adverse effects. Author developed and validated a highly sensitive method of analysis to have a control over impurity levels. A highly sensitive, specific, precise and accurate method development and validation is taken as a task in this study so that the method can be used routinely for its intended purpose.

**About Atorvastatin:**

Atorvastatin (ATV) is a member of the drug class known as statins, used for lowering blood cholesterol. It also stabilizes plaque and prevents strokes through anti-inflammatory and other mechanisms. ATV is a synthetic hydroxy methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor that has been demonstrated to be efficacious in reducing both cholesterol and triglyceride.
ATV calcium is a synthetic lipid-lowering agent. ATV is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase an enzyme found in liver tissue that plays a key role in production of cholesterol in the body. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. Inhibition of the enzyme decreases de novo cholesterol synthesis, increasing expression of low-density lipoprotein receptors (LDL receptors) on hepatocytes. This increases LDL uptake by the hepatocytes, decreasing the amount of LDL-cholesterol in the blood. Like other statins, Atorvastatin also reduces blood levels of triglycerides and slightly increases levels of HDL-cholesterol. In clinical trials, drugs that block cholesterol uptake like ezetimibe combine with and complement those that block biosynthesis like Atorvastatin or Simvastatin in lowering cholesterol or targeting levels of LDL \[^{[44-46]}\].

Atorvastatin calcium is an organic acid with a pKa of 4.46 and is a white to off-white crystalline powder that is insoluble in aqueous solutions of pH 4 and below. It is very slightly soluble in distilled water, pH 7.4 phosphate buffer and acetonitrile; slightly soluble in ethanol; and freely soluble in methanol \[^{[47]}\]. Numerous methods for the determination of ATV and impurities were found during our literature survey which included HPLC and spectrophotometric methods \[^{[47-54]}\].

M. Sudheer et.al, developed a method for the quantification of Atorvastatin and Fenofibrate in tablets using HPLC with DAD detector. The developed method consisting the mobile phase ACN: Buffer (1.36 gm of K$_2$HPO$_4$ was added in 1000 ml of water and pH adjusted to 3.0 (±0.1) with OPA) in the ratio 70 : 30 with isocratic programming, Inertsil ODS C18 column (250 mm×4.6 mm, 5 μm) column as stationary phase with a flow rate of 1.5 mL/minute. Proposed method was found to be linear in the concentration range of 10 to 30 μg/mL for Atorvastatin Calcium and 200 to 600 μg/mL for Fenofibrate respectively \[^{[47]}\].

Saravanamuthukumar, M et.al, in their work describes a HPLC method for simultaneous estimation of Atorvastatin calcium and Ubidecarenone as in the bulk drug and in tablet dosage forms. The separation was achieved using a PEERLESS C8 reverse
phase column (250 x 4.6 mm, 5 μ, L7 pack) at ambient temperature with an isocratic mixture of methanol and acetonitrile in the ratio of 80:20 %V/V at a flow rate of 1.5mL/minute and detection at 290nm. The retention times for Atrovastatin calcium and Ubidecarenone were 1.692 and 10.709 minutes respectively.\(^{[48]}\)

Kadav, A.A et.al, has developed and validated the simultaneous determination of Atorvastatin, Fenofibrate and their impurities in tablets. The chromatographic separation was performed on Acquity UPLC\(^{\text{TM}}\) BEH C18 column (1.7 μm, 2.1 mm×100 mm) using gradient elution of acetonitrile and ammonium acetate buffer (pH 4.7; 0.01 M) at flow rate of 0.5 ml/min. UV detection was performed at 247 nm. Total run time was 3 min within which main compounds and six other known and major unknown impurities were separated.\(^{[49]}\)

Sidika Ertu”rk et.al, has developed a high-performance liquid chromatographic (HPLC) method for the analysis of Atorvastatin (ATV) and its impurities in bulk drug and tablets, using a Luna C18 column with acetonitrile/ammonium acetate buffer pH 4-tetrahydrofuran (THF) as mobile phase. Samples were eluted gradiently with the mobile phase at flow rate 1.0 ml/min and detected at 248 nm.\(^{[50]}\)

A simple, sensitive and accurate spectrophotometric method has been developed by Hasan, M et.al for the determination of Atrovastatin calcium in raw material and tablets. The λ max of the Atrovastatin was found to be 248 nm.\(^{[51]}\)

A high-performance liquid chromatographic (HPLC) method of analysis of Atorvastatin Calcium in pharmaceutical dosage form was developed and validated by Zaheer, Z et al. The chromatographic conditions comprised of a reversed-phase C18 column (250 x 4.6 mm), 5 μ with a mobile phase consisting of a mixture of methanol: acetonitrile: phosphate buffer solution in the ratio (45:45:10). Flow rate was 1 mL / min. Detection was carried out at 246 nm. The retention time of Atorvastatin was 6.98 min.\(^{[52]}\)

A high-performance liquid chromatographic (HPLC) method of analysis of Atorvastatin Calcium in pharmaceutical drug substance having chromatographic conditions comprising of a reversed-phase C8 column (250 x 4.6 mm), 5 μ with a mobile phase...
consisting of mobile phase A a mixture of acetonitrile, stabilizer-free tetrahydrofuran and buffer in the ratio (21:12:67) and mobile phase B a mixture of acetonitrile, stabilizer-free tetrahydrofuran and buffer in the ratio (61:12:27). Flow rate was 1.5 mL/min with the gradient elution of T0/100:0, T40/100:0, T70/20:80, T85/0:100, T100/0:100, T105/100:0, T115/100:0. Detection was carried out at 244 nm with injection volume of 20μL. The system suitability criteria being the resolution between Atorvastatin and impurity B not less than 1.5. This is an official USP and Ph.Eur method of analysis.

From recent past the method development for the determination of impurities has been one of the critical and tedious task in pharmaceutical industry, this is even more tedious if more number of impurities are to be resolved. Moreover, ICH Guidance Q3A (R2) emphasises the importance of controlling impurities in drug substances.

Taking this into consideration ATV should be monitored together with their degradation compounds (Table-4), preferably in single chromatographic run. Ultra high performance liquid chromatography (UHPLC) is a recent technique in liquid chromatography, which enables significant reductions in separation time and solvent consumption. Literature indicates that UHPLC system allows about tenfold decrease in analysis time as compared to the conventional HPLC system using 5 μm particle size analytical columns and about three fold decrease in analysis time in comparison with 3 μm particle size analytical columns without compromise on overall separation. The purpose of this study was to develop a stability indicating method for the simultaneous determination of ATV and its impurities. UHPLC technique was chosen because of its above mentioned advantages. The proposed method was able to separate the impurities listed in table 4 and as well as other unknown degradation products within 17 min. The method was validated according to ICH Q2 (R1) guideline and also extended to separation of degradation products formed under various stress conditions such as acid hydrolysis, base hydrolysis and oxidative degradation. Photostability is performed according to ICH Q1B guideline.

However there are no UHPLC methods reported in the literature for the quantification of Atorvastatin and its related impurities. Hence the objective of the work is
to develop an economic, time efficient, RP-UHPLC method and demonstrate its stabilityindicating capabilities by forced degradation followed with method validation of the developed method for the accurate quantification of impurities of Atorastatin in bulk drug samples.

Structures of Atorvastatin and its impurities under study:

**TABLE-4: Atorvastatin and its impurities considered in this study.**[^53,^54]

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Structure</th>
<th>Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td><img src="image" alt="Structure" /></td>
<td>((3R, \ 5R)-7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1H-pyrrol-1-yl]-3, 5-dihydroxyheptanoate trihydrate)</td>
</tr>
<tr>
<td>Impurity H/ Lactone</td>
<td><img src="image" alt="Structure" /></td>
<td>((4R, 6R)-6-[2-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1H-pyrrol-1-yl]ethyl]-4-hydroxytetrahydro-2H-pyran-2-one.)</td>
</tr>
<tr>
<td>3-Oxo Atorvastatin</td>
<td><img src="image" alt="Structure" /></td>
<td>((8R)-2-(4-Fluorophenyl)-\delta-hydroxy-5-(1-methylethyl)-\beta-oxo-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic Acid.)</td>
</tr>
<tr>
<td>Impurity A/ Desfluoro</td>
<td><img src="image" alt="Structure" /></td>
<td>((3S,5R)-7-(3-(phenylcarbamoyl)-2-isopropyl-4.5-diphenyl-1H-pyrrol-1-yl)-3,5-dihydroxyheptanoic acid.)</td>
</tr>
<tr>
<td>Impurity B/ Diastereomer</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>C_{35}H_{35}FN_{15}O_{5} Mol Wt: 558.64</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Impurity C/ Fluoro</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>C_{36}H_{37}FN_{15}O_{5} NaO_{5} Mol Wt: 598.61</td>
</tr>
<tr>
<td>Impurity D</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>C_{36}H_{37}FN_{15}O_{5} Mol Wt: 598.61</td>
</tr>
<tr>
<td>Impurity F/ amide</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>C_{48}H_{48}FN_{15}O_{5} Mol Wt: 798</td>
</tr>
<tr>
<td>Impurity G/ O-methyl</td>
<td><img src="image" alt="Molecular Structure" /></td>
<td>C_{48}H_{48}FN_{15}O_{5} Mol Wt: 594.65</td>
</tr>
</tbody>
</table>

(3S,5R)-7-(3-(phenylcarbamoyl)-5-(4-fluorophenyl)-2-isopropyl-4-phenyl-1Hpyrrol-1-yl)-3,5-dihydroxyheptanoic acid.

(3R,5R)-7-[3-(Phenylcarbamoyl)-4,5-bis(4-fluorophenyl)-2-isopropyl-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid, sodium salt
(" Difluoro Atorvastatin Sodium Salt")

3-(4-Fluorobenzoyl)-2-isobutyryl-3-phenyl-oxirane-2-carboxylic acidphenylamide.
(" Atorvastatin Epoxide")

Atorvastatin Amide Acid Sodium Salt"; "Atorvastatin Diamino Impurity Sodium Salt.

(3R,5R)-7-(3-(phenylcarbamoyl)-2-isopropyl-4,5-diphenyl-1H-pyrrol-1-yl)-5-hydroxy-3-methoxyheptanoic acid
Method development included forced degradation to ensure that the method is stability indicative.

EXPERIMENTAL:

Materials and Reagents:

The investigated sample of ATV and its impurities were received from synthetic laboratory of Biocon Ltd, Bangalore, India. Acetonitrile of HPLC grade was procured from Merck (Darmstadt, Germany), ammonium acetate was procured from Merck (Mumbai, India), ExcelaR grade glacial acetic acid was supplied by Qualigens fine chemicals (Mumbai, India) and tetrahydrofuran (THF) from Rankem (RFCL Ltd. India). Water obtained from Millipore system (Millipore Inc., USA). The filters with pore size of 0.2 µm (Waters, Milford, USA) were used for the filtration of mobile phase.
Preparation of Solutions:

Buffer solution was prepared by adjusting the pH of 0.01M ammonium acetate solution to 5.0 using glacial acetic acid. Mobile phase A, comprised of acetonitrile, buffer and tetrahydrofuran in the ratio of 21:67:12 (v/v/v) and mobile phase B comprised of acetonitrile, buffer and tetrahydrofuran in the ratio of 61:27:12 (v/v/v). A mixture of acetonitrile and water in the ratio of 40:60 (v/v) was used as the diluent for the preparation of solutions. The buffer preparation, mobile phase preparation and diluent were found stable with respect to pH and visual clarity for 48 hr.

Test sample solution concentration of 1mg/ml was prepared for the determination of related substances. Stock solution of ATV standard and all the impurities of 100 ppm were prepared and were further diluted adequately to study the validation attributes. The resolution solution was prepared at 1ppm by diluting the above stock solution.

RESULTS AND DISCUSSION:

Method Development and Optimization:

Primarily, chromatographic system analyses were performed on Agilent HPLC 1200, consisting of a Quaternary pump and variable wavelength detector. Instrument equipped with a Rheodyn injection system with a 100 µl loop.

Conventional HPLC[^53,^54]: Column: Zorbax SB C8 (250 x 4.6 mm, 5 µm)

Injection volume: 20 µL; Run time: 115 min

Gradient program: T0/100:0, T40/100:0, T70/20:80, T85/0:100, T100/0:100, T105/100:0, T115/100:0
Flow rate: 1.5 mL/min; Wavelength: 244 nm

A theoretical approach for the method migration from conventional HPLC to UHPLC was calculated using the Method Translator and Cost Saving Calculator. The mobile phase was filtered and degassed using 0.22 µ filter when used for the UHPLC systems. Chromatographic system analyses were performed on Agilent UHPLC 1290, consisting of a binary pump and diode array detector. Instrument equipped with a Rheodyn injection system with a 10 µl loop.

**UHPLC Method Trial-1:** Column: Zorbax SB C18 (50 x 2.1 mm, 1.8 µm)
Injection volume: 1.0 µL; Run time: 25 min.
Gradient program: T₀/100:0, T₈/100:0, T₁₄/20:80, T₁₇/0:100, T₂₀/0:100, T₂₁/100:0, T₂₅/100:0
Flow rate: 0.31mL/min; Wavelength: 244 nm.

When resolution mixture was injected with this method we observed peak tailing, poor resolution and merging of peaks. This method was a simple conversion from the method translator which was not satisfactory. Hence the method needed to be optimized further to achieve the good resolution.

The UHPLC trial-1 method was modified to achieve better separation and further reduction of run time from 25 min to 17 min. Resolution mixture chromatograph under this chromatographic condition was satisfactory with the better resolution, but the disadvantage was with the working pressure which was about 1000 bar and which was
not compatible with more injections continuously. Also there was no proper resolution between the 2\textsuperscript{nd} and 3\textsuperscript{rd} peak and also there was interference from the gradient.

**UHPLC Method Trial-2:** Column: Zorbax SB C18 (100 x 2.1 mm, 1.8 µm)
Injection volume: 1.0 µL; Run time: 17 min.
Gradient program: T\textsubscript{0}/100:0, T\textsubscript{5}/20:80, T\textsubscript{16}/100:0, T\textsubscript{17}/100:0
Flow rate: 0.4 mL/min; Wavelength: 244 nm.

Pressure about 1000 bar

In order to optimize the method with this flow rate, we opted for a poroshell column which allows high flow rate with less pressure but not compromising on the resolution.

The poroshell technology offers an alternative for very high resolution analyses, because of its superficially porous column technology \cite{59} which have particles with a solid core and a superficially porous shell. These particles consist of a 1.7 µm solid core with a 0.5 µm porous silica shell. In total, the particle size is about 2.7 µm \cite{60}.
The 2.7 μm superficially porous particles provide 40–50 % lower back pressure and 80–90 % of the efficiency of a sub-2 μm totally porous particle. The superficially porous particles have a narrower particle size distribution than a totally porous particle. This results in a more homogeneous column and reduces diffusion in the column. At the same time the small particle and the porous shell allow for lower resistance to mass transfer. The result is higher flow rates without loss of efficiency.

For a symmetrical peak we use the following equation to calculate the plate number (N): 

\[ N = 5.54 \left( \frac{RT}{W} \right)^2 \]

Where, RT is the retention time and W the peak width at half height.

Porous shell columns represent a real alternative to sub-2 μm columns. The lower back pressure allows flow rates of 1 mL/min for a 4.6 mm x 150 mm, 2.7 μm column. In general, for this column 35000 plates are achievable or more than 235000 plates/meter. The final concluded method showed good resolution and baseline stabilization with the working pressure less than 500 bar.
UHPLC Method Trial-3: Column: Zorbax SB C18 poroshell 120 (100 x 2.1mm, 2.7μm)
Injection volume: 1.0 μL; Run time: 17 min.
Gradient program: T_0/100:0, T_15/20:80, T_16/100:0, T_17/100:0
Flow rate: 0.5 mL/min; Wavelength: 244 nm.

Pressure less than 500 bar.

Instrument and Operating Conditions:
Agilent UHPLC 1290 (Agilent technologies, USA) equipped with a binary pump, diode array detector, Rheodyn injection system with a 10 μl loop. Detection was accomplished with Diode array detector. Zorbax SB C18 (100 mm x 2.1 mm, 2.7 μm) column maintained at 35°C was used for the separation. The flow rate and injection volume was 0.5 mL/min and 1.0 μL respectively. The analysis was carried out under gradient program such as time (min)/A: B (v/v); T_0/100:0, T_15/20:80, T_16/100:0 and T_17/100:0. The data acquired at 244 nm for 17 min was processed using chemstation and chemstore software systems.
Forced Degradation Study:

Forced degradation study was conducted on bulk drug substance in order to prove the stability-indicating property and selectivity of the established method. Forced degradation of Atorvastatin was carried out under acidic/basic hydrolysis, oxidative, thermolytic and photolytic stress conditions.

Solid state degradation:

About 1.0 g of the sample was taken and exposed to the conditions as explained with chromatograms below. After the specified time the samples were prepared as per the method. The analysis was carried out with the final concluded method by UHPLC.

Solid State degradation – Control Sample: A sample was kept in its intended packing for 72 hr.
At high humidity conditions: 25°C/90% RH for 72 hr.

At Accelerated Conditions i.e. at high temperature and humidity (as per ICH): 40°C/75%RH for 72 hr.
Chapter III

Under Elevated temperature (Heat): 80°C for 24 hr.

Photolysis – Exposure to UV-visible light: As per ICH Q1B Guideline, photolytic conditions of fluorescent light (1.2 x106 LUX hours), UV light for a total exposure of 200 W·hr/m².
Liquid State Degradation:

The sample was dissolved with a minimum quantity (about 5 to 10 ml) of the diluent as per the method. 5 ml of the stated degradation vehicle is added as indicated under the following chromatograms. After the specified time all the samples were made up to 50 ml with diluent. The analysis was carried out with the final concluded method by UHPLC. Results of both solid and liquid state degradation were compiled and presented in table 5.

Acidic degradation: The sample is subjected to 0.1 N HCl at room temperature for 6 hr.

Alkaline degradation: The sample is subjected to 1.0 N NaOH at room temperature for 6 hr.
**Oxidative degradation:** The sample is subjected to 3% Hydrogen peroxide at room temperature for 6 hr.

**TABLE-5:** Impurity profile when subjected to stress

| Impurity Profile under Solid and Liquid State | Impurity A | Impurity B | Impurity C | Impurity D | Impurity E | Impurity F | Impurity G | Impurity H | Alprazolam diol | Alprazolam methyl ester | Total impurities |
|---------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|----------------|----------------|----------------|
| Ambient for 72hrs                           | 0.071     | 0.034     | 0.098     | 0.056     | 0.058     | 0.026     | 0.053     | 0.027     | 0.030     | 0.089                       | 0.762                      |
| 25°C/60%RH for 72hrs                        | 0.069     | 0.042     | 0.097     | 0.073     | 0.139     | 0.043     | 0.057     | 0.056     | 0.029     | 0.095                       | 0.824                      |
| 25°C/90%RH for 72hrs                        | 0.068     | 0.034     | 0.098     | 0.057     | 0.023     | 0.028     | 0.062     | 0.037     | 0.029     | 0.062                       | 0.799                      |
| 40°C/75%RH for 72hrs                        | 0.068     | 0.038     | 0.098     | 0.056     | 0.019     | 0.029     | 0.074     | 0.038     | 0.028     | 0.086                       | 0.841                      |
| 80°C for 24hrs                              | 0.066     | 0.049     | 0.096     | 0.053     | 0.022     | 0.005     | 0.198     | 0.065     | 0.029     | 0.177                       | 1.388                      |
| Photo degradation                           | 0.053     | 0.073     | 0.074     | 0.055     | 0.074     | 0.007     | 0.098     | 0.028     | 0.033     | 0.171                       | 1.094                      |
| 0.1N HCl for 6 hours                         | 0.036     | 0.021     | 0.047     | 0.034     | 0.059     | 0.014     | 0.004     | 48.618    | 0.030     | 0.284                       | 49.419                     |
| 1N NaOH for 6 hours                          | 0.061     | 0.047     | 0.095     | 0.057     | 0.032     | 0.085     | 0.050     | 0.017     | 0.0       | 0.007                       | 0.657                      |
| 3% H2O2 for 6 hours                          | 0.067     | 5.761     | 0.105     | 0.041     | 0.036     | 0.010     | 0.137     | 0.052     | 0.057     | 0.588                       | 9.763                      |
METHOD VALIDATION:

Specificity (Selectivity):
Specificity was demonstrated by injecting the individual impurities and the ATV separately, peak purity for individual impurities was found to be greater than the purity threshold and finally specificity mixture was injected. There were no interfering peaks from the diluent and system blank at the retention time of ATV and its impurities.

Linearity:
The linearity was established from LOQ to 10 ppm (LOQ, 2, 4, 8 and 10 ppm) for ATV and its impurities. The linear regression data for all the components tested are presented in Table-6.

Limit of Detection and Quantization (LOD and LOQ):
The LOD and LOQ for all the impurities and ATV were determined based on the signal to noise method. The LOQ for impurities and ATV ranged from 0.14 ppm to 0.48 ppm, the LOD values ranged from 0.05 ppm to 0.16 ppm. The LOQ concentrations were further verified for precision by injecting six individual preparations of ATV and its impurities. The RSD of LOQ precision was in the range of 2.58-9.26%. The results are depicted in Table-6.

Precision:
System precision was evaluated by repeated injections (n=6) of the specificity mixture. The RSD of ATV and its impurities were found to be 1.89 %. Precision of the method was studied under method and intermediate precision. Replicate (n=6) sample was prepared and analyzed, the content of impurities were calculated and the RSD for all the impurities were in the range of 0.29% - 1.58%. Similar to the method precision, intermediate precision was carried out by different analyst, different day using different instrument. The percentage relative standard deviation of the results was found to be 0.38%-1.69%. The results of method precision and intermediate precision were compared, the overall RSD (n=12) for percentage of impurities was found to be 0.38%-7.82%. The results are reported in the Table-6.
Accuracy (Recovery):

Accuracy of the method was evaluated for all the impurities of ATV by spiking the impurities to the ATV drug substance from LOQ to 10 ppm (LOQ, 4 ppm and 10 ppm). Each level was analyzed in triplicate preparations and duplicate injections. The samples were analyzed by the proposed method and the amount of impurities recovered after making corrections for the amount already present were calculated. Table-6 provides the % recovery at each selected level. The recovery of all the impurities was found to be within the predefined acceptance criteria of 80.0-120.0%.

Solution Stability:

Sample solutions were prepared and analyzed at different time intervals up to 24 hours to determine the stability of sample solution. The sample was found to be stable for 24 hr without increase in the impurities level.

Mobile Phase Solution Stability:

The resolution between impurity B and ATV was monitored in the resolution solution for a time period of 24 hr at regular intervals and the resolution was within the acceptance criteria (NLT 1.2). The mobile phase was found to be stable for 24 hr.

Robustness:

The robustness of the developed method was evaluated by deliberate changes in the chromatographic conditions and monitoring the resolution between the closely eluting peak pair, that is, impurity B and ATV. The flow rate of the mobile phase was 0.5 mL/min. To study the effect of flow rate on the resolution, the flow rate was altered by 0.05 mL/min, that is, 0.45 mL/min and 0.55 mL/min. The effect of column temperature on the resolution was studied at 33°C and 37°C instead of 35°C. All the other chromatographic conditions were held constant. In all the deliberate variations in the chromatographic conditions the resolution between impurity B and ATV was greater than 1.2. Very minor changes in the resolution were observed in all the robustness conditions demonstrating the robustness of the developed method.
TABLE-6: Validation data results of related substances of Atorvastatin

<table>
<thead>
<tr>
<th>Validation data results of related substances of Atorvastatin</th>
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</thead>
<tbody>
<tr>
<td>Impurity</td>
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<tr>
<td>Precision; Mean % content</td>
</tr>
<tr>
<td>Method precision (n=6)</td>
</tr>
<tr>
<td>Intermediate precision (n=6)</td>
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<tr>
<td>Overall results (n=12)</td>
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<tr>
<td>Limit of detection (LOD)</td>
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<tr>
<td>In ppm</td>
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<tr>
<td>Limit of quantization (LOQ)</td>
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<tr>
<td>In ppm</td>
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<tr>
<td>Correlation coefficient</td>
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<td>Accuracy: mean recovery; LOQ to 10ppm</td>
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<tr>
<td>LOQ</td>
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<tr>
<td>4ppm</td>
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<tr>
<td>10ppm</td>
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CONCLUSION:

A simple, economic, selective and time efficient stability indicating reverse phase ultra high performance liquid chromatography (UHPLC) method is reported for the purpose of determination of degradation products and impurities in Atorvastatin (ATV). The reported method was developed on a Agilent poroshell 120 Zorbax SB C-18, 2.1 mm x 100 mm 2.7 μm particle size column with chromatographic conditions like, mobile phase comprising of acetonitrile, buffer and tetrahydrofuran, a flow rate of 0.5 ml/min, gradient programmed for 17 min and UV detector set at 244 nm, resulted in equal or better chromatographic separation when compared with conventional HPLC method. The poroshell column was selected because of its porous technology which helps in reducing 40-50% pressure compared to 1.8 μ particle column. Forced degradation study supports the fact that the optimized and validated method is stability indicative and can be used in the routine analysis of ATV drug substance.
This chapter presents a rapid, simple, precise, robust, accurate and stability indicative gradient UHPLC method that separates the degradation products and impurities of ATV with good resolution. The optimized method was validated to ensure the compliance in accordance with ICH guidelines for its intended purpose. The sensitivity, time and solvent saving characteristic of this method concludes that method can be used for routine testing and stability analysis of ATV drug substance.
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