CHAPTER-VI

A Synchronized UPLC Method for Quantitative and Qualitative Estimation of Biomolecules – Atosiban, Bivalirudin, Eptifibatide and Exenatide.
Introduction:

The core of innovation in pharmaceutical industry relies on the research of new high value added drugs to address unmet medical needs. However, the decreasing number of approved drugs, which is accompanied by increasing R&D expenditures, demands alternative approaches to increase R&D productivity. This situation contributes to a revival of interest in peptides as potential tools and drug candidates. With peptides as medicines for the future, there has been a rapid expansion in its use as drugs over the last decade, and this is likely to continue.

Peptides are natural or synthetic short polymers of amino acids linked by a peptide bond which is a chemical amide linkage, -NH-CO-, formed by the condensation of the amino group of one amino acid with the carboxyl group of another. Peptides regulate most physiological processes, acting at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors. They are already being used therapeutically in such diverse areas as neurology, endocrinology and haematology.

A brief discussion on peptides namely Atosiban, Bivalirudin, Eptifibatide and Exenatide concedes in the following.

ATOSIBAN:

Molecular Formula: C_{45}H_{71}N_{11}O_{14}S_{2}
Formula Weight: 994.19

Atosiban is a synthetic peptide which is a competitive antagonist of human oxytocin at receptor level in the uterus, and potentially also in the decidual and fetal membranes. Pharmacological inhibition of uterine contractility (tocolysis) to postpone delivery is the current mainstay of management of pre-term labour. Pre-term labour affects 25% of human pregnancy and leads to pre-term delivery. It is a cause of concern
as premature delivery of infants (before 37 weeks of gestation) leads to complications such as neurosensory deficits, respiratory distress syndrome, low body weight, subnormal height and possible neonatal death. Prevention of pre-term labour requires pharmacological inhibition of uterine contraction (tocolysis). Drugs used to suppress contractions such as magnesium sulfate, ritodrine, terbutaline, salbutamol (β agonists) suffer from adverse side effects. Atosiban has been developed as a new tocolytic therapy in the treatment of pre-term labour.

Pharmacokinetic studies in pregnant women showed a half-life of 18 min, clearance 42 l/hr and a distribution volume of 18 litres. In clinical trials atosiban reduced the number of premature deliveries over seven days compared with placebo and no fetal adverse effects were seen and it is used to delay pre-term birth between 24 and 33 weeks of gestational age for 48 hr. In pivotal studies conducted Atosiban when compared with the beta 2-agonists ritodrine, salbutamol and terbutaline may be less effective, but appears to be better tolerated.

BIVALIRUDIN:

![Bivalirudin molecule]

Molecular Formula: C_{99}H_{138}N_{24}O_{33}
Molecular Weight: 2180.29

Bivalirudin is a single chain of 20 amino-acid polypeptide based on the structure of hirudin. It is a direct thrombin inhibitor that binds reversibly to thrombin at both the active site and the substrate recognition site. Bivalirudin inhibits all thrombin-catalysed or -induced reactions, including fibrin formation, activation of coagulation factors V,
VIII, and XIII, activation of protein C and platelet aggregation. It is effective against fluid phase and clot bound thrombin and is alleged to inhibit protease-activated receptor (PAR) mediated activation of platelets.

Bivalirudin is an anticoagulant intended for parenteral use during invasive intravascular procedures such as percutaneous coronary intervention (PCI), including percutaneous transluminal coronary angioplasty (PTCA) procedures like angioplasty and balloon angioplasty and PTCA with stenting.

The preclinical and clinical pharmacokinetic and pharmacodynamic characteristics of bivalirudin have been studied in normal volunteers, patients with varying degrees of renal impairment, and in patients with acute coronary syndromes. The relationship between dose and plasma concentration of bivalirudin is direct and linear, and the therapeutic range of bivalirudin plasma levels is approximately 1–15 g/ml, depending on the dose used in specific applications [32]. The recommended dose is an intravenous bolus of 0.75 mg/kg followed immediately by an intravenous infusion at a rate of 1.75 mg/kg/h for at least the duration of the procedure. The infusion may be continued for up to 4 hours post-PCI as clinically warranted.

**EPTIFIBATIDE:**

![Eptifibatide](image)

Molecular formula: C_{35}H_{49}N_{11}O_{9}S_{2}

Molecular wt: 831.96

Disintegrins are naturally occurring polypeptides in viper snake venoms, which inhibit cell adhesion and platelet aggregation. The venom of the southeastern pigmy rattle-snake Sistrurus m barbouri contained a specific antagonist, which blocked only the binding of fibrinogen to \alpha IIbβ3. The purified protein was named barbourin.
The eptifibatide molecule is derived from the structure of barbourin. It is a glycoprotein IIb/IIIa inhibitor that blocks the final common pathway of platelet aggregation. Its major adverse effect is bleeding. The eptifibatide molecule contains a KGD (Lys-Gly-Asp) amino acid sequence, identical with that in the distal terminus of the \( \gamma \)-chains of fibrinogen \(^{[5-7]} \). The interaction between fibrinogen \( \gamma \)-chains and the GPIIb/IIIa receptor is through the binding site situated in a IIIa subunit. Eptifibatide tight fit into GPIIb/IIIa is fibrinogen binding pocket (a KGD binding motif) was proposed to be responsible for its ability to block integrin function with minimal receptor activation \(^{[5]} \).

Trials testing safety and efficacy of eptifibatide were conducted in the mid-1990s in healthy volunteers in different clinical settings \(^{[8-12]} \). Together they showed eptifibatide rapidly inhibited platelets reaching a maximum effect in 15 min, was able to maintain inhibition during infusion and inhibition was reversible with recovery of platelet activity in 2 - 4 hr. The amount of inhibition was dependent on the bolus dose. Boluses of 90 - 180 \( \mu g/kg \) and infusion doses of 0.75 -1.5 \( \mu g/kg/min \) produced inhibition of platelet aggregation of more than 75-80%. The failure of eptifibatide to show superiority compared with placebo in the Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis-II (IMPACT II) trial was explained by the discovery that eptifibatide affinity to GP is inversely related to the \( Ca^{++} \) concentration in the sample \(^{[13]} \). Citrate blood with a \( Ca^{++} \) concentration of 40 - 50 \( \mu mol/l \) falsely enhanced the platelet inhibitory activity of eptifibatide compared with noncitrate blood with a \( Ca^{++} \) of 1100 - 1200 \( \mu mol/l \) \(^{[14]} \).

Eptifibatide was approved for human use in 1998 for prevention of thrombotic complications during percutaneous coronary intervention \(^{[5]} \).
EXENATIDE:

Molecular Formula: C$_{184}$H$_{282}$N$_{50}$O$_{60}$S C$_2$H$_4$O$_2$

Molecular Weight: 4262.67

Exenatide is a synthetic version of exendin-4, a hormone found in the saliva of the Gila monster that was first isolated by Dr. John Eng in 1992 while working at the Veterans Administration Medical Center in the Bronx, New York.

Exenatide (synthetic exendin-4), an incretin mimetic, is a 39-amino acid peptide that has several gluco regulatory activities similar to mammalian incretin hormone glucagon-like peptide-1 (GLP-1) $^{[15]-[16]}$. Incretin mimetics increase glucose-dependent insulin secretion, suppress inappropriate glucagon secretion, delay gastric emptying (which reduces the rate of glucose absorption) and reduce appetite $^{[17]-[18]}$. It is given as a subcutaneous injection of 5 micrograms twice a day.

Exenatide is shown to have several beneficial glucoregulatory activites, including glucose-dependent insulinotropic, glucagonostatic, gastric slowing effects. It has also been reported that exenatide increases $\beta$-cell mass and enhances $\beta$-cell function $^{[19]-[21]}$.

Exenatide is a treatment option for people with type 2 diabetes mellitus who require:

(i) dual therapy but for whom a combination of metformin and a sulfonylurea is contraindicated or not tolerated.

(ii) triple therapy for people who are already taking metformin and a sulfonylurea.

Exenatide when used in combination with metformin and/or a sulfonylurea reduces HbA1c levels. Two trials have assessed the efficacy of exenatide as dual therapy.
with either metformin or a sulfonylurea \[^{22-23}\]. Exenatide is associated with weight loss and thus may be a useful option for people who are overweight. However, whether weight loss with exenatide improves clinical outcomes or is maintained in the long-term, is currently unknown.

Common adverse effects with exenatide include gastrointestinal adverse effects (nausea, vomiting, diarrhoea, dyspepsia, gastro-oesophageal reflux disease and abdominal pain), headache, dizziness, jitteriness and injection-site reactions. Hypoglycaemia may also occur, mainly when exenatide is used with a sulfonylurea \[^{17}\]. Pancreatitis, allergic reactions and altered renal function have been reported rarely with exenatide \[^{17-26}\]. Precautions must be taken to avoid the use of exenatide in people with severe renal impairment or in pregnant women.

**Literature Survey:**

Biomolecules such as proteins and peptides represent a growing number of pharmaceutical drug entities. Drugs in this class are often more specialized than small molecules since they typically replace or augment an endogenous molecule known to change a disease or physiological state. Furthermore, since they are analogs to, synthetic versions of, or are endogenous compounds, they tend to be well-tolerated by the body. There are currently more than 40 peptide-based drugs on the market and more than 400 in late-stage clinical trials \[^{27}\]. With growing number of peptide and protein drugs getting approved for clinical trials, it is becoming important to have assay methods with improved sensitivity and selectivity. As scientists we may often be in a position where we have to choose a particular technique for measuring the protein concentration. Factors which may have to be considered are the amount of sample preparation required, their sensitivity and their speed. Other factors which may be important when selecting an appropriate technique are: the equipment available, ease of operation, the desired accuracy, and whether or not the technique is nondestructive. There are various methods used in analysis of peptides such as various UV-visible methods which require extensive sample preparation prior to analysis, electrophoretic methods, mass spectrophotometer, chromatographic methods and proteomic studies.
Peptide and protein identification from global proteomics studies of environmental samples is challenging because the standard approach used to identify peptides and proteins is difficult to effectively apply. The standard approach involves growing clonal cells in the laboratory, separating the proteins from other cellular components, digesting the proteins into smaller peptides (approximately 6-55 amino acids long), partially separating the peptides using liquid chromatography and then introducing each peptide into a mass spectrometer.

The classical proteomic quantification methods utilizing dyes, fluorophores or radioactivity have provided very good sensitivity, linearity and dynamic range, but they suffer from two important shortcomings: first, they require high-resolution protein separation typically provided by 2D gels, which limits their applicability to abundant and soluble proteins; and second, they do not reveal the identity of the underlying protein. Both of these problems are overcome by modern LC-MS/MS techniques. However, mass spectrometry is not inherently quantitative because proteolytic peptides exhibit a wide range of physicochemical properties such as size, charge, hydrophobicity, etc. which lead to large differences in mass spectrometric response.

Biologically active peptides used in therapy are most often produced using biosynthetic methods or by sequential chemical synthesis. Due to the starting biological material or multi-stage manufacturing methods, the formulations are a source of a number of issues related to appropriate quality assurance. The monitoring of any impurities is an important factor for the assurance of safety of use of such medicinal products. Considering their structure, similar to the active substance, the analysis of impurities requires specific and selective analytical methods.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) has become a widely used, well-established tool for the analysis and purification of biomolecules. The reason for the central role that RP-HPLC now plays in analyzing and purifying proteins and peptides is resolution. RP-HPLC is able to separate polypeptides of nearly identical sequences, not only for small peptides such as those obtained through trypsin digestion, but even for much larger proteins. Polypeptides which differ by a single
amino acid residue can often be separated by RP-HPLC. Intact proteins are analyzed by RP-HPLC to verify conformation and to determine degradation products. The scientific literature has many examples of RP-HPLC, RP-HPLC/CE methods developed to separate and determine peptides. Reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) are often used as complementary techniques for the separation of peptides. Hydrophilic interaction chromatography (HILIC) is another technique that is complementary to RP-HPLC and CE. HILIC is of general utility for polar analytes such as carbohydrates, nucleic acids, amino acids, and peptides. HILIC chromatography is related to normal phase chromatography since retention of solutes increases with increasing solute polarity and decreases with increasing mobile phase polarity.

Alan R. Oyler et al., designed a method that illustrated hydrophilic interaction chromatography (HILIC) on amine bonded-phase silica columns provides separations of peptides that are complementary to those obtained with reversed-phase HPLC and capillary electrophoresis. However the disadvantages were that though the analyses times were long, reduced times could be obtained at higher flow-rates with some sacrifice of resolution, high concentrations of salt were required to obtain acceptable peak shape and retention of analytes was very sensitive to the water content of the mobile phase.

Chen et al., have determined exenatide in human plasma using solid-phase extraction and HPLC analysis (C18 column and acetonitrile/water elution). But, the peak of exenatide was too time-consuming and "plumpness" to be separated from that of its main degradation ingredient on the base line.

LIU B in et al., designed a reverse-phase high performance liquid chromatography method to determine the levels of Exenatide in Poly (lactic-co-glycolic acid) microspheres. Separation was performed on a C4 column via a mobile phase consisting of ACN:KH2PO4 (0.02 mol/L, pH=2.5) gradient elution from 30:70 to 45:55 (volume ratio) in 30 min. Multi-diode array detection(DAD) was used to evaluate the spectral purity of exenatide. Though the method proved to be specific, accurate,
precise and robust with good resolution and peak symmetry, its drawback was the tedious preparation of exenatide loaded microspheres via the double emulsion method which may result in the drug degradation because of the existence of high-speed shearing force and sample preparation which required incubation at room temperature for 2 hr.

Malgorzata Jaworska et al., developed two-dimensional HPLC-CE technique was employed for the analysis of the impurities of octreotide, a cyclic octapeptide used in therapy. Because distinct separation mechanisms are used, the two-dimensional technique ensures higher separation efficiency and a more comprehensive impurity profile of the medicinal product than either of the techniques used separately.

Don Farthing et al., developed a high-performance liquid chromatographic (HPLC) method using solid-phase extraction, o-phthalaldehyde (OPA) derivatization and fluorescence detection for the determination of the direct thrombin inhibitor bivalirudin in human plasma and urine. A C18 bioanalytical column at a flow rate of 1 ml/min with an aqueous trifluoroacetic acid (0.1% TFA in deionized water, pH 2.2, v/v) mobile phase and methanol gradient was used. Though the method demonstrated precision, linearity from 3 to 20 g/ml bivalirudin in plasma, with a detection limit of 1 g/ml the absolute recovery for the plasma method at 5 and 15 g/ml (n = 3) was determined to be 85 and 89%, respectively. Another hitch was tiresome sample preparation process that involved OPA derivatization with each step using vacuum for sample processing.

Development of bioanalytical methods for the detection of small molecule pharmaceuticals in humans and animals is a challenging and time-consuming process. Regulatory guidelines require methods to be acceptable in terms of linearity, sensitivity, accuracy and precision, selectivity, stability and carryover. Although numerous HPLC methods with good selectivity, resolution, precision, linearity, sensitivity and accuracy were developed for the separation and quantification of peptides from matrix, a few shortcomings were observed due to tiresome sample preparation, long run time, high injection volume which in turn led to the requirement of more sample quantity, single method for estimation of a biomolecule and finally overall cost of analysis.
In order to save time and money a non tedious routine analysis method that can be used for estimation of biomolecules is always of prime importance. With due consideration of the limitations of HPLC methods the authors have developed a simple, synchronized, cost effective yet sensitive, precise, linear, accurate and robust method for the quantitative estimation of peptides. The analytical method discussed in this article utilizes a simple mobile phase composition at an extremely low flow rate for the simultaneous estimation of Atosiban, Bivalirudin, Eptifibatide and Exenatide in bulk and from commercial formulations. The method was validated in accordance with ICH guidelines and confirms that the analytical procedure employed is suitable for its intended purpose.

Method Development and Optimization:

Initial trial experiments were conducted in order to select a suitable solvent system for accurate analysis of the drug substance. The suitability of the mobile phase and the flow rate was decided on the sensitivity of assay, resolution, use of readily available solvents and ease of preparation. These included Water-Orthophosphoric acid (0.005%v/v), Water-Acetonitrile-Orthophosphoric acid (50:50:0.005%v/v); Water-Trifluoroacetic acid (0.01%v/v), Water-Acetonitrile-Methanol-Trifluoroacetic acid (50:40:10:0.01%v/v). Variation in flow rate was between 0.7 mL/min to 0.5 mL/min.

To reduce the overall analysis time the gradient elution pattern was varied accordingly without compromising on resolution. The wavelength was set at 220nm. In order to obtain better separation and peak shape the column temperature was varied between 55°C and 60°C. The experimental work was conducted using a Water Acquity UPLC system consisting of a binary pump, a degasser, an autosampler, a column oven, a TUV detector and an Empower 2 software. Chromatographic conditions were optimized with Acquity UPLC® BEH Shield column (RP-18, 2.1 mm X 100 mm i.d, 1.7 μ particles). Column temperature was kept at 60°C. Mobile phase A was Water-Trifluoroacetic acid (0.01%v/v) and Mobile phase B was Water-Acetonitrile-Methanol-Trifluoroacetic acid (50:40:10:0.01%v/v). The mobile phase was filtered using 0.22 μ membrane filter and degassed using an ultrasonic water bath. Flow rate was adjusted to
0.5 mL/min. Injection volume was 1.0 μL. the wavelength was set at 220 nm. The gradient condition used is as per Table 1.

**Experimental:**

**Chemicals, reagents and standards:**

Lyophilized standard of Atosiban, Bivalirudin, Eptifibatide and Exenatide drug substance was obtained from Biocon Ltd (India). Trifluoro acetic acid AR grade from Rankem, gradient grade acetonitrile and methanol procured from E.Merck, India. Pure milli-Q water was collected from Millipore water purification unit.

**Preparations of solutions:**

Mobile phase A comprised of 0.01% trifluoro acetic acid in water and mobile phase B comprised of 0.01% trifluoro acetic acid in water, acetonitrile and methanol in the ratio of 50:40:10. The concentration of the test solution was 0.5 mg/mL in water (diluent) for all the four drug substances.

**Experimental condition:**

The chromatograph system comprised of Water Acquity UPLC system equipped with a TUV detector. The data was acquired and processed using Empower2 software. The samples were injected into an Acquity UPLC BEH Shield RP-18 1.7 μm, 2.1 X 100 mm column maintained at 60°C. The flow rate was 0.5 mL/min and the gradient program is as given in Table-1. The run time of the method was 8 min per injection and the elute was monitored at 220nm.

**Table-1: Gradient program**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>72</td>
<td>28</td>
</tr>
<tr>
<td>3.00</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>3.25</td>
<td>42</td>
<td>58</td>
</tr>
<tr>
<td>3.75</td>
<td>32</td>
<td>68</td>
</tr>
<tr>
<td>5.00</td>
<td>22</td>
<td>78</td>
</tr>
<tr>
<td>6.25</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>7.25</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>7.50</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>8.00</td>
<td>72</td>
<td>28</td>
</tr>
</tbody>
</table>
Forced Degradation:

Forced degradation study was conducted on drug substance in order to prove the stability indicating property and selectivity of the method. The drug substances were subjected to thermolytic and photolytic stress conditions. It is to be noted that scarcity of the material owing to its high cost restricted the author to conduct only solid state degradation study. Hence author could not perform liquid state degradation study.

Solid state degradation:

0.1 g of the drug substances (lyophilized peptide standards) were exposed to the condition as given in Table-2. After the elapse of the specified time the sample were prepared as per the method. Figure-1 illustrates the extent of degradation of all the four drugs when subjected to solid state stress.

Table-2: Solid state degradation-% purity

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>ATOSIBAN</th>
<th>BIVALIRUDIN</th>
<th>EPTIFIBATIDE</th>
<th>EXENATIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-8°C</td>
<td>97.5</td>
<td>97.3</td>
<td>98.6</td>
<td>90.2</td>
</tr>
<tr>
<td>25°C- 60%</td>
<td>97.2</td>
<td>97.0</td>
<td>98.7</td>
<td>81.9</td>
</tr>
<tr>
<td>40°C-75%</td>
<td>94.3</td>
<td>97.2</td>
<td>98.6</td>
<td>79.6</td>
</tr>
<tr>
<td>60°C</td>
<td>86.7</td>
<td>95.7</td>
<td>98.4</td>
<td>81.8</td>
</tr>
<tr>
<td>Photolytic</td>
<td>92.7</td>
<td>96.2</td>
<td>96.2</td>
<td>76.2</td>
</tr>
</tbody>
</table>
Figure-1: A trend chart of the % purity of all the four drugs when subjected to solid state stress.

Table-3: Chromatograms of all the four drug substance at different stress conditions

Atosiban Control Sample: A sample was kept in its intended packing for 72 hr (2-8°C).
Atosiban At Accelerated conditions 25°C/60% RH for 72 hr

Atosiban At Accelerated Conditions i.e. at high temperature and humidity (as per ICH): 40°C/75% RH for 72 hr
Atosiban Under Elevated temperature (Heat): 60°C for 24 hr

Atosiban: Photolysis – Exposure to UV-visible light: As per ICH Q1B Guideline, photolytic conditions of fluorescent light (1.2x10^6 LUX hrs), UV light for a total exposure of 200 W-hr/m^2.
Bivalirudin Control Sample: A sample was kept in its intended packing for 72 hr (2-8°C).

Bivalirudin At Accelerated conditions 25°C/60% RH for 72 hr
Bivalirudin At Accelerated Conditions i.e. at high temperature and humidity (as per ICH): 40°C/75% RH for 72 hr

Bivalirudin Under Elevated temperature (Heat): 60°C for 24 hr
Bivalirudin Photolysis – Exposure to UV-visible light: As per ICH Q1B Guideline, photolytic conditions of fluorescent light (1.2x10^6 LUX hrs), UV light for a total exposure of 200 W·hr/m^2.

Eptifibatide Control Sample: A sample was kept in its intended packing for 72 hr (2-8°C)
Eptifibatide At Accelerated conditions 25°C/60% RH for 72hr

Eptifibatide At Accelerated Conditions i.e. at high temperature and humidity (as per ICH): 40°C/75% RH for 72 hr
Eptifibatide Under Elevated temperature (Heat): 60°C for 24 hr

Eptifibatide Exposure to UV-visible light: As per ICH Q1B Guideline, photolytic conditions of fluorescent light (1.2x10^6 LUX hours), UV light for a total exposure of 200 W·hr/m².
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Exenatide Control Sample: A sample was kept in its intended packing for 72 hr (2-8°C).

Exenatide At Accelerated conditions 25°C/60% RH for 72 hr
Exenatide At Accelerated Conditions i.e. at high temperature and humidity (as per ICH): 40°C/75%RH for 72 hr

Exenatide Under Elevated temperature (Heat): 60°C for 24 hr
Exenatide Exposure to UV-visible light: As per ICH Q1B Guideline, photolytic conditions of fluorescent light (1.2x10^6 LUX hr), UV light for a total exposure of 200 W·hr/m².

Method Validation:

Specificity (Selectivity):

Specificity was demonstrated by injecting the individual drug substance (lyophilized peptide standard). From the data it was evident that there was no interference from the blank at the retention time of four drug substances and also no interference was observed from each drug substance at the retention time of the rest proving the method to be selective. Figure-2 illustrates the retention time and the separation of the four drugs.
Figure-2: Chromatogram of all the four drugs- EPT, BIV, ATO and EXE.

Precision:

The precision of the system was established by replicate (n=6) injections of standard mixture consisting of all the four drug substance and the percent relative standard deviation (%RSD) was found to be 1.47, 1.65, 0.99 and 0.70 respectively for Atosiban, Bivalirudin, Eptifibatide and Exenatide.

Replicate (n=6) samples (liquid drug substance with assay NLT 8.00 mg/mL) were prepared as per the methodology and the %RSD obtained for assay was found to be 0.67, 1.97, 0.29 and 1.63 respectively for Atosiban, Bivalirudin, Eptifibatide and Exenatide (refer Table 4).
Table-4: Precision results for all the four drugs

<table>
<thead>
<tr>
<th>Replicates</th>
<th>ATOSIBAN mg/mL</th>
<th>BIVALIRUDIN mg/mL</th>
<th>EPTIFIBATIDE mg/mL</th>
<th>EXENATIDE mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.36</td>
<td>8.87</td>
<td>8.80</td>
<td>9.13</td>
</tr>
<tr>
<td>2</td>
<td>8.46</td>
<td>8.96</td>
<td>8.81</td>
<td>9.34</td>
</tr>
<tr>
<td>3</td>
<td>8.47</td>
<td>9.10</td>
<td>8.83</td>
<td>9.39</td>
</tr>
<tr>
<td>4</td>
<td>8.41</td>
<td>9.29</td>
<td>8.85</td>
<td>9.35</td>
</tr>
<tr>
<td>5</td>
<td>8.49</td>
<td>9.30</td>
<td>8.81</td>
<td>9.54</td>
</tr>
<tr>
<td>6</td>
<td>8.52</td>
<td>9.22</td>
<td>8.78</td>
<td>9.53</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>8.45</strong></td>
<td><strong>9.12</strong></td>
<td><strong>8.81</strong></td>
<td><strong>9.38</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td><strong>0.06</strong></td>
<td>0.18</td>
<td>0.03</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>%RSD</strong></td>
<td><strong>0.67</strong></td>
<td><strong>1.97</strong></td>
<td><strong>0.29</strong></td>
<td><strong>1.63</strong></td>
</tr>
</tbody>
</table>

Table-5: Precision chromatograms

[Atosiban Sample chromatogram]

Atosiban- Sample chromatogram
Exenatide- Sample chromatogram

Linearity:

The range of reliable quantification was set at 0.3-1000 μg/mL, for each drug injected as a combination solution for simultaneous estimation in a single run. The standard curve had reliable reproducibility (n=3) for all the four analyte across the calibration range. The linear regression data for the calibration plot has good linear relationship with coefficient of correlation, $r^2>0.999$ for all the four drugs over a wide range. Table-6 shows the linearity parameters for calibration curve of the four drugs.

Table-6: Linearity parameters for calibration curve of the four drugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration range (μg/mL)</th>
<th>Slope</th>
<th>Intercept</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atosiban</td>
<td>4 to 1000</td>
<td>1784.5</td>
<td>18360</td>
<td>0.9997</td>
</tr>
<tr>
<td>Bivalirudin</td>
<td>0.2 to 1000</td>
<td>1136.3</td>
<td>11767</td>
<td>0.9992</td>
</tr>
<tr>
<td>Eptifibatide</td>
<td>0.4 to 1000</td>
<td>4213.7</td>
<td>17496</td>
<td>0.9999</td>
</tr>
<tr>
<td>Exenatide</td>
<td>0.125 to 1000</td>
<td>1165.7</td>
<td>-2468.6</td>
<td>0.9998</td>
</tr>
</tbody>
</table>
Table 7: Linearity graphs of the four drugs

Detection (LOD) and Quantification (LOQ) Limits:

The limit of detection was found to be 1.2, 0.06, 0.12 and 0.04 µg/mL for Atosiban, Bivalirudin, Eptifibatide and Exenatide respectively. The drug peaks could be detected without any baseline noise disturbances (>3 times) at these concentrations. The limit of quantification was 4, 0.2, 0.4 and 0.125 µg/mL. The analyte’s response at these concentration level were > 10 times the baseline noise. The precision and accuracy at these concentration levels for the four drugs were within the acceptable range. This
indicated that the method can be used for simultaneous detection and quantification of these four drugs over a wide concentration range.

Blank chromatogram

Table- 8: LOQ /LOD chromatograms of the four drugs.

Atosiban-LOQ chromatogram

Atosiban-LOD chromatogram
Accuracy:
The accuracy of the method was studied at three different concentrations, LOQ, 100 μg/mL and 1000 μg/mL for all the four drugs. The percent recovery for all the four drugs at three levels were found is between 80% and 120%. Further to confirm the accuracy 100 μg/mL of each drug substance (API) were spiked to the individual drug products and the recovery was well within 80% and 120%.

Robustness:
The robustness of the method was evaluated for instrumental parameters such as change in flow rate (±0.05 mL/min), change in wavelength (±1 nm) and change in column temperature (± 2°C). Table-9 shows the content of all the four drugs for the robustness conditions said above and percent cumulative RSD for each drug with method precision. Figure-3 demonstrates the variation in the resolution between ATO and BIV under different robustness conditions.
Figure-3: A trend chart for the resolution between BIV and ATO at different robustness conditions.

Table-9: Results of Robustness study

<table>
<thead>
<tr>
<th>Robustness parameter</th>
<th>Flow rate in mL/min</th>
<th>Wavelength in nm</th>
<th>Column Temperature in °C</th>
<th>Method precision</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.55 (FH)</td>
<td>0.45 (FL)</td>
<td>221 (NME)</td>
<td>219 (NML)</td>
<td>62.0 (TH)</td>
</tr>
<tr>
<td>Peptide</td>
<td>Content in mg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atosiban</td>
<td>8.57</td>
<td>8.59</td>
<td>8.47</td>
<td>8.53</td>
<td>8.48</td>
</tr>
<tr>
<td>Eptifibatide</td>
<td>8.97</td>
<td>9.04</td>
<td>9.02</td>
<td>9.00</td>
<td>9.05</td>
</tr>
</tbody>
</table>
Figure 4: Trend chart for the Assay of ATO, BIV, EPT and EXE for different robustness conditions
Results and Discussion:

A validated method in compliance with ICH guidelines is reported for simultaneous determination of Atosiban (ATO), Bivalirudin (BIV), Eptifibatide (EPT) and Exenatide (EXE) using ultra performance liquid chromatography (UPLC). A bioanalytical BEH Shield RP-18 1.7 μm, 2.1 X 100 mm column maintained at 60°C and a flow rate of 0.5 mL/min with an aqueous trifluoro acetic acid 0.01% in water as mobile phase A and 0.01% in a mixture of water, acetonitrile and methanol was used. The elute was monitored at 220 nm.

The method was validated for selectivity, precision, accuracy, robustness, limit of quantization and detection in accordance to FDA and ICH guidelines. ATO (4.135 min), BIV (3.901 min), EPT (1.915 min) and EXE (5.471 min) separated with good resolution in a single chromatographic run of 8.0 min. The resolution between closely eluting peaks ATO and BIV was 6.7. Linearity relationship ($r^2 > 0.999$) was observed between the peak area and concentration for all the four compounds within the range (LOQ- 1000 μg/mL). Accuracy was between the pre set criteria of 80 to 120%. LOQ values were 4, 0.2, 0.4 and 0.125 μg/mL respectively for ATO, BIV, EPT and EXE. The method was robust for the minor changes in flow rate, column temperature and wavelength.

Conclusion:

A single, validated UPLC method for the synchronized analysis of peptide molecules namely Atosiban, Bivalirudin, Eptifibatide and Exenatide is reported. The results reported suggest the utility of the method for qualitative and quantitative analysis of the four individual peptide molecule or for the simultaneous quantitative estimation of all the four drug substance.

Simple, cost effective, time saving, non tedious sample preparation being the main attributes of this method makes it conducive and feasible for routine and stability analysis of all the four drug substance.
REFERENCES:


