A New Stability-Indicating and Validated RP-HPLC Method for the Estimation of Liraglutide in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

A stability-indicating RP-HPLC method was developed and validated for the estimation of Liraglutide in bulk and pharmaceutical dosage forms. Shiseido C18 (250 mm x 4.6 mm I.D., 5 µm particle size) column was used as stationary phase with mobile phase consisting methanol+acetonitrile (80:20), phosphate buffer (pH 3.0 adjusted with ortho phosphoric acid) in the ratio of 75:25, v/v. The flow rate was maintained at 1.2 ml/min and effluents were monitored at 245 nm. The retention time was found to be 2.837 minutes. The forced degradation studies were performed as per ICH guidelines under acidic, alkali, oxidative, thermal, photostability and neutral conditions. The drug peak was well resolved from the peaks of degraded products. From the degradation studies it is evident that the drug showed instability under acidic, alkali, oxidative, thermal, photostability and neutral conditions. The linearity of the method was observed in the concentration range of 10-60 μg/ml with the number of theoretical plates & tailing factor being 5550 & 1.17 respectively with a correlation coefficient of 0.999. The percentage assay of Liraglutide was found to be 99.66%. The method was validated for its accuracy, precision and system suitability. The results obtained in the study were within the limits of ICH guidelines and hence this method can be used for the estimation of Liraglutide in bulk and pharmaceutical dosage forms.

Keywords: liraglutide, RP-HPLC, forced degradation, validation

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INTRODUCTION

Liraglutide is a long-acting glucagon-like peptide-1 receptor agonist, binding to the same receptors as does the endogenous metabolic hormone GLP-1 that stimulates insulin secretion [1-3]. It is an injectable drug developed by Novo Nordisk for the treatment of type 2 diabetes [4]. It is an acylated GLP-1 (Glucagon-Like Peptide-1) receptor agonist, which regulates intracellular cAMP resulting in the release of insulin given elevated blood glucose concentrations. Glucagon secretion can also be decreased in a glucose-dependent fashion by liraglutide [5, 6].

Literature survey revealed that very few RP-HPLC [6, 7] methods have been reported for the estimation of Liraglutide in bulk and pharmaceutical dosage forms. Moreover, no available method is found with forced degradation studies on this drug. Hence we made an attempt to develop and validate a new stability-indicating HPLC method as per ICH guidelines [8, 9] for the estimation of Liraglutide in bulk and pharmaceutical dosage forms.

MATERIALS AND METHODS

Instrumentation

To develop a high pressure liquid chromatographic method for quantitative estimation of Liraglutide using Shimadzu HPLC system, Shiseido C18 (250 mm x 4.6 mm I.D., 5 μm particle size) column was used. The instrument is equipped with manual injector and UV detector. A 20 μl rheodyne injector port was used for injecting the samples. Data was analyzed by using LC solutions software.
The working standard of Liraglutide was procured from Yarrow chemicals Pvt. Ltd., Mumbai, India. The market formulation Victoza® injection (Liraglutide 6 mg/mL) was procured from market. HPLC grade water, methanol and acetonitrile were purchased from E.Merck (India) Ltd., Mumbai, India. Potassium dihydrogen orthophosphate, triethylamine and orthophosphoric acid of AR grade were obtained from S.D. Fine Chemicals Ltd., Mumbai, India.

**Chromatographic conditions**

Methanol and acetonitrile (80:20): phosphate buffer (pH 3.0 adjusted with orthophosphoric acid) in the ratio of 75:25, v/v was found to be the most suitable mobile phase for ideal chromatographic separation of Liraglutide. The solvent mixture was filtered through a 0.45 μm membrane filter and sonicated before use. It was pumped through the column at a flow rate of 1.2 ml/min. Injection volume was 20 μl and the column was maintained at room temperature. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solution. The detection of the drug was monitored at 245 nm. The run time was set as 6 minutes.

**Preparation of 0.02 M phosphate buffer pH 3.0**

2.72 grams of potassium dihydrogen orthophosphate was weighed accurately, transferred into a 1000 ml beaker and dissolved in 500 ml of HPLC grade water. 1 ml of triethyl amine was added to the above solution. The solution was sonicated for 30 minutes, degassed and then made to total volume with water. The pH of the resulting solution was adjusted to 3.0 with dilute orthophosphoric acid and filtered through 0.45 μm membrane filter.

![Figure 2. Typical chromatogram of Liraglutide standard](image-url)
Preparation of mobile phase and diluent

The mobile phase was prepared by mixing 250 ml of 0.02M phosphate buffer pH 3.0 with 750 ml of mixture of methanol and acetonitrile (80:20). The solution was degassed in an ultrasonic water bath for 5 minutes and filtered through 0.45 µm filter under vacuum. Mobile phase was used as diluent.

Preparation of standard solution

10 mg of Liraglutide was accurately weighed, transferred to 10 ml volumetric flask and is dissolved in 7 ml of the diluent. Sonicated the solution for few minutes and dissolved the drug completely. Then it is filtered through 0.45 µm filter and the volume is made up to 10 ml with diluent to get a concentration of 1 mg/ml stock solution. Dilution was made to dilute concentration to 100 µg/mL of liraglutide. Further pipette 3 ml of the diluted solution into a 10 ml volumetric flask and diluted up to the mark with diluent to obtain required concentration of 30 µg/ml of Liraglutide.

Preparation of sample solution

One ml of formulation equivalent to 6 mg/mL of Liraglutide was transferred to 10 ml volumetric flask and is dissolved in 7 ml of the diluent. Sonicated the solution for few minutes and dissolved the drug completely. Then it is filtered through 0.45 µm filter and the volume is made up to 10 ml with diluent to get a concentration of 0.6 mg/ml stock solution. Dilution was made to dilute concentration to 60 µg/mL of liraglutide. Further pipette 5 ml of the above diluted solution into a 10 ml volumetric flask and diluted up to the mark with diluent to obtain required concentration of 30 µg/ml of Liraglutide.

Calibration plot

About 10 mg of Liraglutide was weighed accurately, transferred into a 10 ml volumetric flask and dissolved in 7 ml of a 75:25, v/v mixture of phosphate buffer pH 3.0 and methanol + acetonitrile (80:20). The solution was sonicated for 15 minutes and the volume made up to the mark with a further quantity of the diluent to get a 1000 µg/ml solution. From this, a working standard solution of the drug (100 µg/ml) was prepared by diluting with the above solution to 10 ml in a volumetric flask. Further dilutions ranging from 10-60 µg/ml were prepared from the solution in 10 ml volumetric flasks using the above diluent. The column was maintained at room temperature. The pump pressure was set at 300 psi. The run time was set at 6 minutes. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solution. Inject 20 µl of the standard, sample solutions six times into the chromatographic system at a flow rate of 1.2 ml/min and the corresponding chromatograms were obtained. From these chromatograms, the average area under the peak of each dilution was computed. The linearity curve constructed by plotting concentration of the drug against peak area was found to be linear in the concentration range of 10-60 µg/ml of the drug. The regression equation of this curve was
computed. This regression equation was later used to estimate the amount of Liraglutide in pharmaceutical dosage forms.

METHOD VALIDATION

Linearity

Several aliquots of standard solution of Liraglutide were taken in different 10 ml volumetric flasks and diluted up to the mark with diluent such that the final concentrations of Liraglutide were in the range of 10 to 60 µg/ml. The drug was eluted with UV detector at 245 nm, peak area was recorded for all the peaks.

Limit of Detection (LOD)

The limit of detection (LOD) is the smallest concentration that can be detected but not necessarily quantified as an exact value. LOD is calculated from the formula

\[ LOD = 3.3 \sigma / S \]

where, \( \sigma \) is the standard deviation of the response, \( S \) is the slope of calibration curve.

Limit of Quantitation (LOQ)

The limit of quantitation is the lowest amount of analyte in the sample that can be quantitatively determined with precision and accuracy. LOQ is calculated from formula

\[ LOQ = 10 \sigma / S \]

where, \( \sigma \) is the standard deviation of the response, \( S \) is the slope of calibration curve.
The precision was determined for Liraglutide in terms of interday precision and intraday precision.

Accuracy

The accuracy of the method was assessed by recovery study of Liraglutide in the dosage form at three concentration levels. A fixed amount of pre-analyzed sample was taken and standard drug was added at 50%, 100% and 150% levels. Each level was repeated three times. The content of Liraglutide was calculated.

System suitability

System suitability parameters like retention time, theoretical plates and tailing factor were calculated and compared with standard values.

Ruggedness and robustness

The ruggedness of the method was determined by carrying out the experiment on different instruments by different operators using different columns of similar types. The robustness of the method was determined by making slight changes in the chromatographic conditions such as flow rate and percent of composition of the mobile phase on the quantification of the drug substance and selectivity was studied.

Assay

20 µl of sample solution was injected and from the peak area of Liraglutide, amount of each drug in the sample were computed. The results were compared with the label claim of Liraglutide.

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Table 1. Optimized chromatographic conditions of Liraglutide

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>methanol+acetonitrile (80:20); phosphate buffer (pH 3.0 adjusted with ortho phosphoric acid) in the ratio of 75:25v/v</td>
</tr>
<tr>
<td>pH</td>
<td>3.0</td>
</tr>
<tr>
<td>Diluent</td>
<td>Mobile phase</td>
</tr>
<tr>
<td>Column</td>
<td>Shiseido C18 250 mm x 4.6 mm I.D., 5 µm particle size</td>
</tr>
<tr>
<td>Column temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Wave length</td>
<td>245 nm</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µl</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.2 ml/min</td>
</tr>
<tr>
<td>Run time</td>
<td>6 min</td>
</tr>
<tr>
<td>Retention time</td>
<td>2.837 min</td>
</tr>
</tbody>
</table>

Precision

The precision was determined for Liraglutide in terms of interday precision and intraday precision.
Table 2. Linearity results of Liraglutide

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Mean peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>504868</td>
</tr>
<tr>
<td>20</td>
<td>1041170</td>
</tr>
<tr>
<td>30</td>
<td>1533393</td>
</tr>
<tr>
<td>40</td>
<td>2038076</td>
</tr>
<tr>
<td>50</td>
<td>2599689</td>
</tr>
<tr>
<td>60</td>
<td>3118794</td>
</tr>
</tbody>
</table>

Table 3. Intraday Precision for Liraglutide

<table>
<thead>
<tr>
<th>Day</th>
<th>Peak area (50%)</th>
<th>%RSD</th>
<th>Peak area (100%)</th>
<th>%RSD</th>
<th>Peak area (150%)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>778182</td>
<td>0.96</td>
<td>1529932</td>
<td>0.72</td>
<td>2543513</td>
<td>1.76</td>
</tr>
<tr>
<td>2</td>
<td>763899</td>
<td>1.10</td>
<td>1552040</td>
<td>0.98</td>
<td>2605414</td>
<td>1.70</td>
</tr>
<tr>
<td>3</td>
<td>774771</td>
<td>0.96</td>
<td>1544341</td>
<td>0.98</td>
<td>2517635</td>
<td>1.70</td>
</tr>
</tbody>
</table>

Table 4. Interday precision for Liraglutide

<table>
<thead>
<tr>
<th>Day</th>
<th>Peak area (50%)</th>
<th>%RSD</th>
<th>Peak area (100%)</th>
<th>%RSD</th>
<th>Peak area (150%)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>774589</td>
<td>1.10</td>
<td>1526689</td>
<td>0.98</td>
<td>2543515</td>
<td>1.70</td>
</tr>
<tr>
<td>2</td>
<td>758964</td>
<td>0.96</td>
<td>1556941</td>
<td>0.98</td>
<td>2635418</td>
<td>1.70</td>
</tr>
<tr>
<td>3</td>
<td>774871</td>
<td>1.10</td>
<td>1543342</td>
<td>0.98</td>
<td>2585431</td>
<td>1.70</td>
</tr>
</tbody>
</table>

Table 5. Recovery studies of Liraglutide

<table>
<thead>
<tr>
<th>% Concentration level</th>
<th>Standard conc. (μg/ml)</th>
<th>Conc. added (μg/ml)</th>
<th>Conc. found (μg/ml)</th>
<th>% Recovery</th>
<th>% Mean recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 %</td>
<td>30</td>
<td>15</td>
<td>44.68</td>
<td>99.28%</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>30</td>
<td>30</td>
<td>59.29</td>
<td>98.82%</td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td>30</td>
<td>45</td>
<td>75.21</td>
<td>100.28%</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. System suitability parameters of Liraglutide

<table>
<thead>
<tr>
<th>System suitability</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (μg/ml)</td>
<td>10-60</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
</tr>
<tr>
<td>Theoretical plates (N)</td>
<td>5550</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.17</td>
</tr>
<tr>
<td>LOD (μg/ml)</td>
<td>0.64</td>
</tr>
<tr>
<td>LOQ (μg/ml)</td>
<td>1.94</td>
</tr>
</tbody>
</table>
DEGRADATION STUDIES

Acid degradation studies

To 1 ml of stock solution of liraglutide, 1 ml of 0.1 M HCl, 0.5 M HCl, 1M HCl & 2M HCl was added and refluxed for 30 minutes at 60°C. The resultant solution was diluted to obtain 30 µg/ml solution and 20 µl solution were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali degradation studies

To 1 ml of stock solution of liraglutide, 1 ml of 0.1 M NaOH, 0.5 M NaOH, 1M NaOH & 2M NaOH was added and refluxed for 30 minutes at 60°C. The resultant solution was diluted to obtain 30 µg/ml solution and 20 µl solution were injected into the system and the chromatograms were recorded to assess the stability of sample.

Oxidative degradation studies

To 1 ml of stock solution of Liraglutide, 1 ml of 1%, 3%, 5% and 10% hydrogen peroxide (H₂O₂) was added and refluxed for 30 minutes at 60°C. The resultant solution was diluted to obtain 30 µg/ml solution and 20 µl solution were injected into the system and the chromatograms were recorded to assess the stability of sample.

Thermal degradation studies

The standard drug Liraglutide solution was exposed to UV light by keeping the beaker in UV chamber for 7 days or 200 Watt hours/m² in photo stability chamber. The resultant solution was diluted to obtain 30 µg/ml solution and 20 µl solution were injected into the system and the chromatograms were recorded to assess the stability of sample.
Neutral degradation studies

To 1 ml of stock solution of Liraglutide, 1 ml of water was added and refluxed for 6 hours at 60°C. The resultant solution was diluted to obtain 30 µg/ml solution and 20 µl solution were injected into the system and the chromatograms were recorded to assess the stability of sample.

The percent of drug degraded in the presence of acidic, alkali, oxidative, thermal, photostability and neutral conditions were studied. The amount of drug recovered or degraded is calculated by comparing the area of the standard with that of the area of the degraded sample.

Figure 4. Chromatograms of Liraglutide showing degraded peaks under acidic conditions
RESULTS AND DISCUSSION

The stability-indicating RP-HPLC procedure was optimized with a view to develop an accurate method in injection dosage form using Shieshiedo C18 column (250 x 4.6 mm, 5 µm) in isocratic mode with mobile phase composition of 0.02M phosphate buffer pH 3.0 adjusted with orthophosphoric acid and methanol+ acetonitrile(80:20) in the ratio of 25:75, v/v. The use of phosphate buffer and methanol+ acetonitrile (80:20) in the ratio of 25:75, v/v resulted in peak with good shape and resolution. The flow rate was 1.2 ml/min and the drug component was measured with UV detector at 245 nm. The results of optimized chromatographic conditions were shown in Table 1. The quantification was linear in the concentration range of 10 to 60 µg/ml for Liraglutide with a correlation coefficient of 0.999. The regression equation of the linearity plot of concentration of Liraglutide over its peak area was found to be
y = 51939x - 10173, where x is the concentration of Liraglutide (μg/ml) and y is the corresponding peak area. The results show that an excellent correlation exists between peak area and concentration of drug within the concentration range indicated. The linearity results were shown in Table 2 and the linearity curve was shown in Figure 3.

The limit of detection and limit of quantification for Liraglutide were found to be 0.640 μg/ml and 1.940 μg/ml respectively, which indicate the sensitivity of the method. The % RSD for interday precision and intraday precision for Liraglutide were found to be 1.29% and 1.17% respectively (limit %RSD<2.0%) and hence the method is precise. The precision data of Liraglutide were furnished in Table 3 & 4. The mean recovery of the drug Liraglutide was 99.46% and the high percentage of recovery of Liraglutide indicates that the proposed method is highly accurate. The results of recovery studies of Liraglutide were shown in Table 5. The

Figure 6. Chromatograms of Liraglutide showing degraded peaks under oxidative conditions.
retention time for the drug Liraglutide was 2.837 minutes. The number of theoretical plates calculated was 5550, tailing factor was 1.17, which indicates efficient performance of the column and the summary of system suitability parameters and validation parameters were shown in Table 6. Typical chromatograms for the drug Liraglutide in standard was given in Figure 3. The robustness studies indicated that no considerable effect on the determination of the drug. Therefore the test method is robust for the quantification of the drug. Robustness data was given in Table 7.

Validated method was applied for the determination of Liraglutide in commercial formulations. The % assay of Liraglutide was found to be 99.66%. No interfering peaks were found in the chromatogram of the formulation within the run time indicating that excipients
used in tablet formulation did not interfere with the estimation of the drug Liraglutide by the proposed HPLC method. The assay results are shown in Table 8. A study on degradation of the drug Liraglutide in various stress conditions was conducted and the degradation of the drug was found to be appreciable. Moreover, though the drug showed degradation the degraded peaks were not noticed in the chromatograms. The probable reason may be due to the lack of chromophore in the degraded component which may be identified with further research using LC-MS.

**Table 9.** Degradation results of Liraglutide

<table>
<thead>
<tr>
<th>Degradation parameter</th>
<th>Peak area of sample</th>
<th>Peak area of standard</th>
<th>% Recovery</th>
<th>% Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M HCl</td>
<td>153255.70</td>
<td>1535628</td>
<td>99.80</td>
<td>0.20</td>
</tr>
<tr>
<td>0.5 M HCl</td>
<td>146806.00</td>
<td>1535628</td>
<td>95.60</td>
<td>4.40</td>
</tr>
<tr>
<td>1 M HCl</td>
<td>118243.40</td>
<td>1535628</td>
<td>77.00</td>
<td>23.00</td>
</tr>
<tr>
<td>2 M HCl</td>
<td>114650.00</td>
<td>1535628</td>
<td>74.64</td>
<td>25.34</td>
</tr>
<tr>
<td>0.1 M NaOH</td>
<td>149693.00</td>
<td>1535628</td>
<td>97.48</td>
<td>2.52</td>
</tr>
<tr>
<td>0.5 M NaOH</td>
<td>146283.90</td>
<td>1535628</td>
<td>95.26</td>
<td>4.74</td>
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<tr>
<td>1 M NaOH</td>
<td>142045.60</td>
<td>1535628</td>
<td>92.50</td>
<td>7.50</td>
</tr>
<tr>
<td>2 M NaOH</td>
<td>141154.90</td>
<td>1535628</td>
<td>91.92</td>
<td>8.08</td>
</tr>
<tr>
<td>1 % H₂O₂</td>
<td>152549.30</td>
<td>1535628</td>
<td>99.34</td>
<td>0.66</td>
</tr>
<tr>
<td>3 % H₂O₂</td>
<td>152349.70</td>
<td>1535628</td>
<td>99.21</td>
<td>0.79</td>
</tr>
<tr>
<td>5 % H₂O₂</td>
<td>147834.90</td>
<td>1535628</td>
<td>96.27</td>
<td>3.73</td>
</tr>
<tr>
<td>10 % H₂O₂</td>
<td>92137.68</td>
<td>1535628</td>
<td>60.00</td>
<td>40.00</td>
</tr>
<tr>
<td>UV</td>
<td>150368.70</td>
<td>1535628</td>
<td>97.92</td>
<td>2.08</td>
</tr>
<tr>
<td>Thermal</td>
<td>149263.00</td>
<td>1535628</td>
<td>97.20</td>
<td>2.80</td>
</tr>
<tr>
<td>Neutral</td>
<td>152887.10</td>
<td>1535628</td>
<td>99.56</td>
<td>0.44</td>
</tr>
</tbody>
</table>

**Figure 8.** Plot showing degradation pattern of Liraglutide in different conditions
Typical chromatograms for Liraglutide in various degradation conditions were shown from Figure 4 to Figure 7. The degradation studies results are furnished in Table 9.

CONCLUSION

A simple, selective and sensitive stability-indicating RP-HPLC method with UV detection for Liraglutide was developed and validated. The proposed study showed acceptable accuracy, precision and wide linear concentration range. The results of analysis proved that the method is suitable for the determination of Liraglutide in bulk and parenteral dosage forms without any interference from the degradation products and it is recommended for routine quality control analysis of the Liraglutide in pharmaceutical dosage forms.

REFERENCES