Chapter 2

New RP HPLC method development, validation and forced degradation study for simultaneous analysis of Dapagliflozin and Saxagliptin in pharmaceutical dosage form
2.1 Introduction

Dapagliflozin (DGFZ) belongs to gliflozin class is indicated for the treatment of diabetes mellitus type 2, and functions to improve glycemic control in adults when combined with diet and exercise. The drug was approved by US FDA in 2014 [1-4]. The mechanism of drug action is by inhibiting subtype 2 of the sodium-glucose transport proteins (SGLT2) which are responsible for at least 90% of the glucose reabsorption in the kidney. Blocking of glucose reabsorption in the kidney, results in the elimination of blood glucose through the urine [5-7]. The drug is available as tablet form along with other drugs for oral administration. Side effects of the drug include rapid weight loss and tiredness, dehydration. It is also associated with hypotensive reactions, diabetic ketoacidosis [8] and may raise the risk of genital thrush and urinary tract infections.

Saxagliptin (SGPT) belongs to dipeptidyl peptidase-4 (DPP-4) inhibitor approved by FDA in 2009. It is an orally active hypoglycemic used in the treatment of type 2 diabetes mellitus in combination with other agents or as monotherapy [9, 10]. Mechanism of action of the drug is by affecting the action of natural hormones in the body called incretins [11-13]. The drug is available as tablet dosage form and it is more effective when administered as combined dosage form than as monotherapy. Major side effects of the drug include upper respiratory tract infection, urinary tract infection, and headache [14].
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2.2 Literature review

Various qualitative and quantitate analysis have been reported with DGFZ and SGPT individually, combined and drugs with other combinational drugs. Among them reversed phase High performance liquid chromatography methods have been studied and listed.


First derivative method UV spectroscopic method has been described by Jani B.R et al (2015) for the estimation of Dapagliflozin and Metformin hydrochloride in synthetic mixture [25]. UV spectroscopic methods have been described for Dapagliflozin with other drugs by Jani B.R et al (2015) for simultaneous estimation of Dapagliflozin and Metformin hydrochloride in synthetic mixture [26].
HPLC method for Saxagliptin analysis individually has been described by Saiful Islam Md et al (2016) for bulk and tablet dosage form [27], Pawanjeet.J.C et al (2014) for its forced degradation impurities in bulk drug and pharmaceutical dosage form [28].

2.3 Materials and Methods

2.3.1 Instrumentation:

<table>
<thead>
<tr>
<th>S No</th>
<th>Instrument</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPLC Instrument</td>
<td>PEAK chromatographic system</td>
</tr>
<tr>
<td>2</td>
<td>HPLC-Pump</td>
<td>LC-P7000 pump</td>
</tr>
<tr>
<td>3</td>
<td>Inject port</td>
<td>Rheodyne manual injector with 20μl fixed volume loop</td>
</tr>
<tr>
<td>4</td>
<td>UV detector</td>
<td>Variable wavelength programmable UV7000 detector</td>
</tr>
<tr>
<td>5</td>
<td>Chromatographic Software</td>
<td>PEAK Chromatographic Software version 1.06</td>
</tr>
<tr>
<td>6</td>
<td>UV-Visible spectrophotometer</td>
<td>Tech comp UV2301</td>
</tr>
<tr>
<td>7</td>
<td>Softer</td>
<td>Hitachi version 2.0</td>
</tr>
<tr>
<td>8</td>
<td>Cuvette</td>
<td>10mm path length Quartz (MCQ-4/104) Cuvettes</td>
</tr>
</tbody>
</table>

Table 2.1: List of instruments

2.3.2 Chemicals and Reagents:

Working standard drug DGFZ with purity of 99.15 % was purchased from Hikal Ltd, Mumbai and SGPT purity of 99.02 % was obtained from Jubilant Life Sciences Ltd., Amroha. Solvents used for analysis like methanol, water and acetonitrile were HPLC grade and were purchased from Merck chemicals, Mumbai, India. 0.45µm membrane filter papers were purchased from Merck Millipore, Mumbai, India. Laboratory reagent grade ammonium dihydrogen phosphate (NH₄H₂PO₄) and ortho phosphoric acid were purchased from Fisher scientific, Mumbai, India.

2.3.3 Preparation of solutions:

2.3.3.i. Preparation of standard stock solutions:

50mg of each standard drug DGFZ and SGPT were weighed accurately and were dissolved in 50ml of methanol. Standard solution having a concentration of 1000μg/ml of DGFZ and SGPT were obtained separately. From this stock solution concentration range of 2.5 μg/ml – 40μg/ml of DGFZ and 1.25 μg/ml – 20μg/ml of SGPT obtained. Equal volume of same concentration level was mixed separately to get calibration dilution having known concentration of DGFZ and SGPT. The resultant solutions were analyzed in the developed method for construction of calibration curve.
2.3.3.ii. Preparation of bulk drug solution:

The bulk drug sample obtained from fresh batch was used for the bulk drug analysis of DGFZ and SGPT in the developed method. Accurately weighed 50mg of DGFZ and SGPT were dissolved in 50ml methanol separately to get bulk drug sample at 1000μg/ml concentration of DGFZ and SGPT. This solution was further diluted up to DGFZ at 10μg/ml and SGPT at 5μg/ml concentration. Equal volumes of these two solutions were mixed separately to get bulk drug sample having 10μg/ml DGFZ and 5μg/ml of SGPT.

2.3.3.iii. Preparation of formulation solution:

The formulation sample prepared by AstraZeneca UK Limited with brand name Qtern having 5 mg of SGPT and 10 mg DGFZ was used for the formulation analysis. Ten tablets of Qtern were powdered uniformly. An amount of powder equivalent to 25mg of was weighed DGFZ was weighed accurately and was dissolved in 25ml methanol. The content was mixed well and was filtered through 0.45μ nylon membrane filter paper. This sample stock solution was diluted get a solution having 10μg/ml of DGFZ. As per the label claim of both drugs, the solution contains 5μg/ml of SGPT. This sample solution was used for the formulation analysis of DGFZ and SGPT.

2.3.4 Method development:

Systematic experimental design was used for the optimization of several operating condition such as stationary phase, mobile phase compassion, mobile phase pH, mobile phase flow rate, detector wavelength for improving the chromatographic separation performance. The optimization steps are given below

Step 1: Selection of detector wavelength:

The UV absorption wavelength of working standard drug solution at a concentration of 10μg/ml of DGFZ and 10μg/ml of SGPT was measured in UV region separately. The iso-absorption wavelength of DGFZ and SGPT was observed from the overlay scanning spectra. The iso-absorption wavelength observed was used for the simultaneous analysis of DGFZ and SGPT.
Step 2: Selection of flow rate and elution:
The method development was performed in isocratic elution mode and the flow rate of the mobile phase was selected at 1.0 ml/min and based on the results observed the flow rate of the mobile phase was changed and the flow rate that produce best results with high resolution was considered as optimized flow rate.

Step 3: Selection of stationary phase:
The initial method development trails was performed on C18 column of different configurations including 100mm ×4.6mm×3.5µ id, 250mm ×4.6mm×3.5µ id and ambient column temperature was selected for method development.

Step 4: Selection of mobile phase:
Standard drug solution having 10µg/ml concentrations of both DGFZ and SGPT was used for method development. The optimization of mobile phase was initiated with methanol as organic modifier and different strengths of phosphate buffer as pH modifier. Systematic trails were performed with different mobile phase compositions and different strengths of pH modifiers. In each trail studied, peak shape, response and system suitable conditions were verified. The conditions that give symmetric peaks with high response, acceptable system suitability were considered for further validation.

2.3.5 Method validation:
The applicability of the developed method for the separation and quantification of DGFZ and SGPT was studied by method validation steps as per ICH guidelines

2.3.5.1 System suitability:
Standard drug solution in the calibration range was analyzed triplicates in the optimized method conditions. The system suitability parameters like resolution, theoretical plates and tailing factor were studied for DGFZ and SGPT. Number of theoretical plates of >2500, <2 tailing factor and >2 resolution factor was considered as acceptable system suitability.
2.3.5.ii Linearity and range:
Standard drug solution at a concentration range of $2.5 \mu g/ml - 40\mu g/ml$ of DGFZ and $1.25 \mu g/ml - 20\mu g/ml$ of SGPT obtained was prepared and the solution was analysed in triplicates in the developed method. The linear calibration curve of DGFZ and SGPT was constructed using peak response versus concentration prepared. From the resultant calibration curve, regression equation and correlation coefficient were observed.

2.3.5.iii Accuracy:
The accuracy of the method developed for the analysis of DGFZ and SGPT was carried by recovery technique. Recovery test was conducted at the levels of 50%, 100%, 200% levels for DGFZ and SGPT. The percentage of recovery and the %RSD of each recovery level were calculated.

2.3.5.iv Precision:
The repeatability and reproducibility of the developed method was confirmed by precision studies. Standard drug solution at a concentration of $10\mu g/ml$ of DGFZ and $5\mu g/ml$ of SGPT was prepared and was analyzed six times in same day for intraday precision and six times in three different days for intraday precision in the developed method conditions. The % RSD of the peak area responses was calculated and the % RSD is less than 2 was considered as precise.

2.3.5.v Ruggedness:
Ruggedness study was conducted by determining the variations of the experimental results on change in the analyst. Standard drug solution at a concentration of $10\mu g/ml$ of DGFZ and $5\mu g/ml$ of SGPT was prepared by three different analysts and the solutions were analyzed six times in the developed method conditions. The %RSD of peak area response was used for calculating %RSD and the %RSD is <2 was considered as rugged.

2.3.5.vi Robustness:
The reliability of the developed analytical method was determined by robustness study. Robustness was carried by small variations in the developed method and the percentage of
change in the results was calculated by comparing the standard calibration results. In robustness study, ±15% change in mobile phase ratio, ± 0.2 change in pH of buffer solution and ± 3 nm changes in detector wavelength was studied. Percentage of change was calculated by comparing the standard values. Percentage of change of ±2 was accepted as the developed method was robust.

2.3.5.vii Sensitivity:
Sensitivity of the developed method was confirmed by determining limit of detection (LOD) and limit of quantification (LOQ) of DGFZ and SGPT. Signal vs noise ration method was adopted for the determination of LOD and LOQ.

\[
\text{LOD} = 3.3 \times \frac{N}{S}.
\]

\[
\text{LOQ} = 10 \times \frac{N}{S}
\]

Where,
N = standard deviation of the peak areas of the drug and impurities.
S = Slope of the corresponding calibration curve.

2.3.5.viii Solution stability studies:
The stability period of the prepared standard drug solution was determined by analyzing the 100% standard solution at different time intervals. The 100% standard solution at a concentration of 10μg/ml of DGFZ and 5μg/ml of SGPT was prepared and was analyzed after 6, 12, 18, 24, 30 and 36 hours. All insignificant changes in the resultant chromatograms were observed. The percentage of stability and stability period was calculated by comparing the results with standard calibration curve results.

2.3.5.ix Forced degradation studies:
Various forced degradation studies like oxidation, acid, alkali, thermal heat photo stability studies were carried for both the standard drugs. Oxidation degradation was studied with 20% hydrogen peroxide, acidic degradation was studied with 2N hydrochloric acid, alkali degradation was studied with 2 N NaOH, thermal degradation was studied by exposing standard drugs at 105°C for 6 hours in hot air oven and Photo stability studies was carried by keeping the drugs in UV chamber for seven days at 200 Watt h/m². After the expose of both drugs in different stress conditions, both the standard drugs were diluted up to 100%
solution concentration and were analyzed in the standard method conditions. The number of degradation products formed was observed from the resultant chromatograms and the percentage of degradation was calculated by comparing the results with 100% calibration results.

2.3.5. Sample analysis:
The sample solutions prepared from bulk drug and formulation was analyzed in the developed method and the percentage of assay was calculated using standard calibration curve.

2.4 Results:
2.4.1 Method Development:
Liquid chromatographic method development for the separation and quantification of DGFZ and SGPT was initiated by selecting the suitable UV absorption wavelength. UV detector wavelength was confirmed by scanning both the standard drugs in UV region.

The overlay scanning spectra of both the drugs DGFZ and SGPT gives the iso-absorption wavelength. The iso-absorption wavelength of DGFZ and SGPT was found to be 280 nm. Hence 280 nm was selected as suitable UV detector wavelength for further development study. The overlay UV absorption spectrum of DGFZ and SGPT was given in figure 2.2.
Several mobile phase trails were performed for the separation and simultaneous quantification of DGFZ and SGPT using HPLC. Trails were optimized based on the separation and system suitability acceptance of both DGFZ and SGPT. Phosphate buffer was used as the mobile phase and the pH of the mobile phase was adjusted with ortho phosphoric acid. Different pH range of mobile phase from 4.0 to 7.0 was studied. At very less acidic pH of 6.8 was found to be produce best results than acidic range. Different configurations of stationary phase were studied for the separation of DGFZ and SGPT. The optimized separation was achieved on Intersil ODS C18 column (250 mm × 4.6 mm × 5 μ). The flow rate of mobile phase was studied in the range of 0.5-1.5ml/min for the separation of DGFZ and SGPT. Finally expected peak shape, resolution between DGFZ and SGPT and acceptable system suitability was achieved using Intersil ODS C18 column (250 mm × 4.6 mm × 5 μ) as stationary phase, ammonium dihydrogen phosphate buffer (pH 6.8) and methanol in a ratio of 65:35 v/v as mobile phase at a flow rate of 1.5ml/min and UV detection was at a wavelength of 280nm. The development trail chromatograms were given in figure 2.3.
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HPLC Report

[Graph]

HPLC Report

[Graph]
The results of systematic trials in the method development steps for the separation and quantification of DGFZ and SGPT confirms that, the molecules can be separate on Intersil ODS C18 column (250 mm × 4.6 mm i.d., and particle size 5μm, maintained at ambient temperature). The mobile phase optimized as ammonium dihydrogen phosphate buffer at pH of 6.8 and methanol in the ratio of 65:35 (v/v) at a flow rate of 1.5ml/min. UV detection was carried at a wavelength of 280 nm. In these conditions, the retention time of DGFZ and SGPT were found to be 4.6 min and 6.7 min respectively. The developed method further studied for validation.

2.4.2 Method Validation:

2.4.2.1 System suitability:
Standard sample solution at 100% concentration of DGFZ and SGPT was analyzed in the optimized conditions. A very high resolution factor of 8.31 was observed for DGFZ and SGPT represents that the method was found to be highly resolved. The number of theoretical plates were found to be 2374, 6123 and tailing factor was found to be 0.02, 1.16 for DGFZ and SGPT respectively. Hence the method obeys system suitable acceptance.
limits. Table 2.2 shows the System suitability results observed for DGFZ and SGPT in the developed method.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter</th>
<th>DGFZ</th>
<th>SGPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Retention time</td>
<td>4.6min</td>
<td>6.7min</td>
</tr>
<tr>
<td>2</td>
<td>Resolution</td>
<td>----</td>
<td>8.31</td>
</tr>
<tr>
<td>3</td>
<td>Theoretical plates</td>
<td>2374</td>
<td>6123</td>
</tr>
<tr>
<td>4</td>
<td>Tailing factor</td>
<td>0.02</td>
<td>1.16</td>
</tr>
</tbody>
</table>

*Table 2.2: System suitability results*

HPLC Report

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Retain.T</th>
<th>Height</th>
<th>Area</th>
<th>Conc</th>
<th>Tail Factor</th>
<th>Theo_Plate</th>
<th>Res</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DGFZ</td>
<td>4.672</td>
<td>74204</td>
<td>912022.4</td>
<td>100.000</td>
<td>1.22</td>
<td>2880</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Sum: 74204 912022.4 100.0000
Figure 2.4: System suitability (individual and 100% mixed solution) chromatograms of DGFZ and SGPT
2.4.2.ii Linearity and range:

The calibration curve standard dilutions prepared in the concentration range of 2.5 – 40µg/ml of DGFZ and 1.25 – 20µg/ml of SGPT were analysed in the optimized conditions and the calibration curve was plotted using peak area response vs concentration of the standard solution. Linear calibration curve was found to be $Y = 37678.69x + 609348.4$ ($r^2 = 0.9992$), $y = 26255.1x + 415025$ ($r^2 = 0.9990$) for DGFZ and SGPT respectively. Standard calibration curve results were given in table 2.3 and calibration curves were given in figure 2.5 for DGFZ and SGPT.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Percentage of Concentration</th>
<th>DGFZ</th>
<th>SGPT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Concentration (µg/ml)</td>
<td>Peak Area</td>
</tr>
<tr>
<td>1</td>
<td>25%</td>
<td>2.5</td>
<td>670011</td>
</tr>
<tr>
<td>2</td>
<td>50%</td>
<td>5.0</td>
<td>808182</td>
</tr>
<tr>
<td>3</td>
<td>100%</td>
<td>10</td>
<td>1008292</td>
</tr>
<tr>
<td>4</td>
<td>200%</td>
<td>20</td>
<td>1374293</td>
</tr>
<tr>
<td>5</td>
<td>400%</td>
<td>40</td>
<td>2106062</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Slope</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Intercept</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Linearity calibration curve results

![Calibration curves of DGFZ and SGPT](image-url)

**Figure 2.5: Calibration curves of DGFZ and SGPT**
2.4.2.iii Accuracy:
The accuracy study results (table 2.4) at 50%, 100%, and 200% levels of calibration curve concentrations of DGFZ and SGPT confirms that the method was found to be accurate. The percentage of recovery was calculated for both DGFZ and SGPT in each concentration level and the average percentage of recovery was found to be 99.66%, 100.87%, 100.21% for DGFZ and 99.79%, 100.07% and 100.44% for SGPT in 50%, 100% and 150% spiked levels respectively. The percentage of recovery was found to be within the acceptable range of 98%-102% for DGFZ and SGPT hence the developed method was found to be accurate and precise.

![Table 2.4: Recovery results](chart.png)

2.4.2.iv Precision:
The % RSD of repeatability study was found to be 0.759, 0.583 and 1.292, 0.915 for DGFZ and SGPT in Intraday and Interday Precision respectively. The % RSD was found to be less than for both the drugs studied in the developed method. This confirms that the change in the analytical duration does not influence the results and similar results were observed for both DGFZ and SGPT. Hence the method developed for the analysis of DGFZ and SGPT was found to be precise.
### Table 2.5: Precision results

<table>
<thead>
<tr>
<th>S. No</th>
<th>Peak area</th>
<th>Intraday Precision</th>
<th>Interday Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DGFZ at 20μg/ml</td>
<td>SGPT at 10μg/ml</td>
<td>DGFZ at 20μg/ml</td>
</tr>
<tr>
<td>1</td>
<td>995731</td>
<td>553501</td>
<td>991325</td>
</tr>
<tr>
<td>2</td>
<td>998322</td>
<td>551839</td>
<td>1020672</td>
</tr>
<tr>
<td>3</td>
<td>1008369</td>
<td>557830</td>
<td>996804</td>
</tr>
<tr>
<td>4</td>
<td>1010634</td>
<td>557359</td>
<td>992875</td>
</tr>
<tr>
<td>5</td>
<td>1014100</td>
<td>560927</td>
<td>1004969</td>
</tr>
<tr>
<td>6</td>
<td>1012090</td>
<td>556493</td>
<td>1019076</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.759</td>
<td>0.583</td>
<td>1.292</td>
</tr>
</tbody>
</table>

#### 2.4.2. v Ruggedness:

Standard stock solution containing DGFZ at 20μg/ml and SGPT at 10μg/ml was analyzed by two different analysts and the % RSD of six replicate analyses was calculated. The % RSD was found to be 0.880 and 1.745 for DGFZ and SGPT respectively. The % RSD was found to be very less for both DGFZ and SGPT and hence the method was found to be rugged. Results were given in table 2.6

### Table 2.6: Ruggedness results

<table>
<thead>
<tr>
<th>S. No</th>
<th>Peak area</th>
<th>DGFZ at 20μg/ml</th>
<th>SGPT at 10μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>992651</td>
<td>542577</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>999359</td>
<td>563535</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>998286</td>
<td>550007</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1014129</td>
<td>550240</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1000895</td>
<td>564304</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1013941</td>
<td>565792</td>
<td></td>
</tr>
<tr>
<td>% RSD</td>
<td>0.880</td>
<td>1.745</td>
<td></td>
</tr>
</tbody>
</table>
2.4.2.\textit{vi Robustness:}

Small variations in the mobile phase composition, mobile phase pH and wavelength of detector did not influence the separation and detection of DGFZ and SGPT. The percentage of change in each condition was calculated for both DGFZ and SGPT and confirms that very small amount of changes were observed in all the changed conditions. Hence the method was found to be robust. Results were given in table 2.7

<table>
<thead>
<tr>
<th>Chromatographic condition</th>
<th>DGFZ (10.0 µg/ml)</th>
<th>SGPT (5.0 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Area</td>
<td>Peak Area</td>
<td>percentage of change</td>
</tr>
<tr>
<td>At normal conditions</td>
<td>1008292</td>
<td>0.0</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>991325</td>
<td>1.68</td>
</tr>
<tr>
<td>Mobile phase pH: 6.82</td>
<td>1020672</td>
<td>1.22</td>
</tr>
<tr>
<td>Mobile phase pH: 6.78</td>
<td>996804</td>
<td>1.13</td>
</tr>
<tr>
<td>P: Detector wavelength: 238nm</td>
<td>992875</td>
<td>1.52</td>
</tr>
<tr>
<td>Detector wavelength: 232nm</td>
<td>1004969</td>
<td>0.32</td>
</tr>
<tr>
<td>% of RSD</td>
<td>1019076</td>
<td>1.06</td>
</tr>
</tbody>
</table>

| % of RSD                  | 1.29              | % of RSD         | 0.91              |

\textbf{Table 2.7: Robustness results}

2.4.2.\textit{vii Sensitivity:}

Very sensitive detection limit of 0.15625µg/ml and 0.078125µg/ml, quantification limit of 0.3125µg/ml and 0.15625µg/ml was observed for DGFZ and SGPT in the developed method. Results confirmed that the method can be applicable for the quantification of DGFZ and SGPT up to a very low concentration of 0.15µg/ml. Hence the method was found to be sensitive. LOD and LOQ chromatograms were given in figure 2.6 and 2.7 respectively.
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HPLC Report

Figure 2.6: LOD chromatogram

HPLC Report

Figure 2.7: LOQ chromatogram
2.4.2.8 Forced degradation studies:

In all the stress degradation studies results shows clear separation of both the drugs DGFZ and SGPT along with degradation products formed. There is interference of degradation products and both standard drugs DGFZ and SGPT. In base degradation study (figure 2.8), the percentage of degradation was found to be 6.12% and 9.09% for DGFZ and SGPT respectively. Three degradation products were clearly separated at retention times of 2.16 min, 3.62min and 7.70min. In acidic degradation study (figure 2.9), three degradation products were formed and were detected at a retention time of 3.79 min, 6.04 min and 9.12min. The degradation was found to be 2.15% and 8.0% for DGFZ and SGPT respectively. In thermal degradation study (figure 2.10), both the drugs were found to be very stable and only one degradation product was formed. The percentage of degradation was found to be 8.88% and 3.88% for DGFZ and SGPT respectively. In oxidative degradation study (figure 2.11) the percentage of degradation was found to be 9.56%, 6.59% for DGFZ and SGPT respectively with two degradation products detected at 2.55 min and 5.95min. Very less percentage of degradation of 4.93% and 2.69% was observed for DGFZ and SGPT respectively in Photo Stability Studies (figure 2.12). Hence results confirmed that the method was found to be suitable enough for the separation and detection of degradation products and quantification of DGFZ and SGPT. Forced degradation study results were given in table 2.8.

<table>
<thead>
<tr>
<th>Chromatographic condition</th>
<th>DGFZ</th>
<th></th>
<th>SGPT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak Area</td>
<td>Percentage of Degradation</td>
<td>Peak Area</td>
<td>Percentage of Degradation</td>
</tr>
<tr>
<td>No degradation</td>
<td>1008292</td>
<td>0.0</td>
<td>553427</td>
<td>0.0</td>
</tr>
<tr>
<td>Oxidative degradation</td>
<td>911893</td>
<td>9.56</td>
<td>516932</td>
<td>6.59</td>
</tr>
<tr>
<td>Acid Degradation</td>
<td>986525</td>
<td>2.15</td>
<td>509110</td>
<td>8.0</td>
</tr>
<tr>
<td>Alkali Degradation</td>
<td>946549</td>
<td>6.12</td>
<td>503120</td>
<td>9.09</td>
</tr>
<tr>
<td>Thermal Degradation</td>
<td>918717</td>
<td>8.88</td>
<td>531924</td>
<td>3.88</td>
</tr>
<tr>
<td>Photo Stability Studies</td>
<td>958504</td>
<td>4.93</td>
<td>538534</td>
<td>2.69</td>
</tr>
</tbody>
</table>

Table 2.8: Forced degradation study results
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Simultaneous Analysis of Dapagliflozin and Saxagliptin in pharmaceutical dosage form

Figure 2.8: Chromatogram of DGFZ and SGPT in base degradation study

Figure 2.9: Chromatogram of DGFZ and SGPT in acid degradation study
Figure 2.10: Chromatogram of DGFZ and SGPT in Thermal degradation study

Figure 2.11: Chromatogram of DGFZ and SGPT in Oxidative degradation study
Figure 2.12: Chromatogram of DGFZ and SGPT in Photo Stability Studies

2.4.2.9 Sample analysis:
The formulation sample solution prepared from marketed formulation sample (Qtern) at 10.0 µg/ml and 5.0 µg/ml concentration of DGFZ and SGPT was analyzed in the developed method. Similar concentration of the bulk drug sample was also analyzed. The percentage of assay was found to be more than 98% was observed for both the drugs. There is no detection of interfering compounds or formulation excipients were detected in formulation chromatogram. Hence the developed method can successfully applicable for the estimation of DGFZ and SGPT in both bulk drug and pharmaceutical formulations. Formulation chromatogram was given in figure 2.13 and formulation results were shown in Table 2.9.

<table>
<thead>
<tr>
<th>Brand</th>
<th>Sample Concentration</th>
<th>Standard area</th>
<th>Formulation area</th>
<th>Amount of drug found</th>
<th>Percentage of accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qtern</td>
<td>10.0 µg/ml</td>
<td>1008292</td>
<td>1006488</td>
<td>9.982 µg/ml</td>
<td>99.82%</td>
</tr>
<tr>
<td>DGFZ - 10 mg</td>
<td>5.0 µg/ml</td>
<td>553427</td>
<td>551436</td>
<td>4.982 µg/ml</td>
<td>99.64%</td>
</tr>
<tr>
<td>SGPT - 5 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.9: Formulation analysis results for DGFZ and SGPT
Chapter 2  
Simultaneous Analysis of Dapagliflozin and Saxagliptin 
in pharmaceutical dosage form

Figure 2.13: Formulation chromatograms of DGFZ and SGPT

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Retain T</th>
<th>Height</th>
<th>Area</th>
<th>Conc</th>
<th>Tail Factor</th>
<th>Theo Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>4.672</td>
<td>74517</td>
<td>1006488.3</td>
<td>64.504</td>
<td>0.06</td>
<td>2385</td>
</tr>
<tr>
<td>2</td>
<td>Saxagliptin</td>
<td>6.749</td>
<td>45506</td>
<td>561436.4</td>
<td>35.396</td>
<td>1.10</td>
<td>6181</td>
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<tr>
<td>Sum</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

120023  1557924.7  100.0000
2.5 Discussion

The development of the method is aimed at the separation and simultaneous determination of DGFZ and SGPT using HPLC. Both drugs were eluted with different solvents with different ratio to evaluate and system suitability. Mobile phase pH is also optimized with various buffers and finally both drugs were found to be better separated in ammonium dihydrogen phosphate buffer (pH 6.8) and methanol in the ratio of 65:35 v/v as mobile phase. This separation was achieved on Intersil ODS C18 column (250 mm × 4.6 mm × 5 μ) at a flow rate of 1.5ml/min and UV detection were at a wavelength of 280nm. At proposed conditions, the retention time of DGFZ and SGPT were found to be 4.6 min and 6.7 min respectively. (Number of theoretical plates in the order >2500, <2 tailing factor and >2 resolution factor was considered as acceptable system suitability). The number of theoretical plates was found to be 2374, 6123 and tailing factor was found to be 0.02, 1.16 for DGFZ and SGPT respectively with resolution factor of 8.31 was observed. These results confirm the system suitability acceptance limit for the proposed method (table 2.2).

Proposed method was also subjected to either validation parameters like linearity, ruggedness, recovery, precision etc according to ICH guidelines. A good range for analysis of DGFZ and SGPT was achieved by linear calibration curve when constructed at a concentration range of 2.5μg/ml – 40μg/ml of DGFZ and 1.25 μg/ml – 20μg/ml of SGPT. Linear calibration curve was found to be $Y = 37678.69x + 609348.4$ ($r^2 = 0.9992$), $y = 26255.1x + 415025$ ($r^2 = 0.9990$) for DGFZ and SGPT respectively. Recovery test was conducted at the levels of 50%, 100%, 200% levels of calibration curve concentrations of DGFZ and SGPT. The %RSD of each recovery level was calculated (table 2.4) and it was found to be within the acceptable range of 98%-102% for DGFZ and SGPT. So, it is ratified that the developed method was found to be accurate and precise. The average percentage of recovery was found to be 99.66%, 100.87%, 100.21% for DGFZ and 99.79%, 100.07% and 100.44% for SGPT in 50%, 100% and 150% spiked levels respectively.
Good precision results were achieved by the repeatability and reproducibility of the developed method and the precision results are as follows: at 10μg/ml of DGFZ and 5μg/ml of SGPT. RSD % of six replicates was found 0.759 and 0.583 for DGFZ and SGPT for intraday precision and 1.29 and 0.91 were found for interday precision study (table 2.5). The % RSD was found to be less on change in the analyst for ruggedness study i.e 0.880 and 1.745 for DGFZ and SGPT respectively (table 2.6). Percentage change for robustness experiment was found within the limit of acceptance criteria, when small changes have been made for mobile phase ratio, pH of the mobile phase and detector wavelength. Percentage of change was observed as, 0.32%-1.68% for DGFZ and 0.004%-1.57% for SGPT respectively. Method sensitivity was observed at a detection limit of 0.15625μg/ml and 0.078125μg/ml, quantification limit of 0.3125μg/ml and 0.15625μg/ml was observed for DGFZ and SGPT in the developed method (figure 2.6 and 2.7). Solution stability studies reveals that the method was found to be stable upto 24 hours of the preparation based on the percentage assay change found within the limit.

The method successfully separated the degraded compounds along with standard drugs when subjected to various forced degradation studies like oxidation, acidic, alkali, thermal, heat photo stability studies. Hence the method can be applied for the separation and detection of degradation products and quantification of DGFZ and SGPT. Forced degradation study results were presented in figure 2.8-2.12 and table 2.8. In all the stress degradation studies, results show that drug DGFZ was found to be sensitive in the oxidative condition and stable at the acid condition. And drug SGPT found to be sensitive in alkali condition and stable in sunlight condition. The proposed method application was also studied in bulk drug and formulation analysis at 10μg/ml DGFZ and 5μg/ml of SGPT along with stress degradative studies. The formulation Qtern (consists of 5 mg of SGPT and 10 mg of DGFZ) was successfully analyzed by the proposed method. The percentage of assay was found to be more than 98% was for both the drugs. There is no detection of interfering compounds or formulation excipients in formulation chromatogram. Hence the developed method can be successfully applicable for the estimation of DGFZ and SGPT in both bulk drug and pharmaceutical formulations (figure 2.13) (Table 2.9).
The proposed method was a novel approach to develop a method for separation and
determination of DGFZ and SGPT in bulk and pharmaceutical dosage form along with
stability indicating application. Later on, various other methods have been reported with a
similar approach. Even though they are reported, the method developed with a similar
approach has been reported by Singh N et al [15], Advaita B. P et al [16], Gandla Kumara
Swamy [17] and Patel P.D et al [18]. The proposed method has wide range of analysis
where Singh N et al [15] proposed the linearity at 2 μg/ml -14μg/ml for both drugs. A
narrow linearity range was observed for the method proposed by Advaita B. P et al [16]
i.e from 56 μg/ml -84μg/ml of Saxagliptin hydrochloride and 112 μg/ml -168μg/ml of
Dapagliflozin where the proposed method have less concentration range. Another
disadvantage of the Advaita B. P et al [16] is separation required column oven temperature
at 40°C. This method failed to achieve good percentage assay when applied to formulation
analysis where the author found 97.92 ± 0.32 % assay for SGPT which is above the
acceptance limit (98%-102%).

The mobile phase condition of the method proposed by the Gandla Kumara Swamy
et al [17] is found quite similar to the mobile phase proposed by the Advaita B. P et al [16]
where both authors are reported the mobile phase of Potassium dihydrogren phosphate
Buffer (pH 6.0): acetonitrile (45:55 v/v). Another ratio of the mobile phase Acetonitrile:
Potassium dihydrogren phosphate (75:25 v/v) also described by Gandla Kumara Swamy et
al [17] in his research article. The proposed method was advantage over method proposed
by Patel P.D et al [18] where his method was applied to stress degradation studies and also
limit of detection of the method was also found better than his method. The present method
is a novel method and though the methods were proposed after its development, the method
has advantages over the results as well as applications compared to the reported methods.
2.6 Conclusion

Now a days, more innovative methods have been carried out in the field of pharmaceutical science to improve the efficacy of the drug analysis. The process worked out here for the simultaneous separation and determination of DGFZ and SGPT in bulk drug and pharmaceutical dosage form is one of its kind.

This is a reliable, an isocratic method where we can notice that validation study was found to be within the limits. As discussed earlier, it can be practically applied for stress degradation studies also. Due to its merits over the existing methods, the proposed method is apt for the estimation of DGFZ and SGPT in pharmaceutical formulations and individually in bulk drug.
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2.7 References

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