Development and Validation of Stability Indicating RP-HPLC and UV Method for Simultaneous Quantitation of Repaglinide and Sitagliptin Phosphate in Combination

Nusrat K. Shaikh¹*, Rakeshkumar Jat², Jitendra O. Bhangale²
1. Shri JJT University, Churu-Jhunjhunu road, Jhunjhunu, Rajasthan 333001
2. Smt. N. M. Padalia Pharmacy College, Navapura, Ahmedabad, 382 210 Gujarat, India

ABSTRACT

The present work describes Stability indicating RP-HPLC and Simultaneous equation UV spectrophotometric method for the quantitative determination of Repaglinide and Sitagliptin phosphate. The parameters Specificity, linearity, accuracy, precision, detection limit, quantitation limit, Robustness and system suitability tests were studied and their results were complied to ICH guideline Q2 (R1). Chromatography was carried out by reverse phase technique on an RP-18 with mobile phase composed of Acetonitrile: Phosphate buffer (65:35; % v/v) adjusted to pH 3.5 with 10% orthophosphoric acid) with flow rate 1 ml/min. Both drugs were eluted, isocratically using detection wavelength 228 nm. Simultaneous equation UV spectrophotometric method was performed and two wavelengths 240 nm (λmax of Repaglinide) and 267 nm (λmax of Sitagliptin phosphate) were selected for the formation of simultaneous equation. The A (1%, 1cm) was determined at both the wavelengths selected for each drug. A set of two simultaneous equations were formed as Cx and Cy. Methanol used as Solvent (diluent) for UV method. For proposed methods, the linearity for both methods were obtained in the concentration range of 0.5-2.5 μg/ml for Repaglinide and 50-250 μg/ml for Sitagliptin phosphate. Statistical analysis by student’s t-test showed no significance difference between the results obtained by these two methods. The suitability of method for the quantitative determination of Repaglinide and Sitagliptin phosphate was proved by validation. The proposed methods and its results had been successfully applied and validated statistically to the simultaneous estimation of Repaglinide and Sitagliptin phosphate in their combination for quality analysis.

Keywords: Repaglinide, Sitagliptin phosphate, RP-HPLC method, UV method

*Corresponding Author Email: nusratshaikh.pharmacist@gmail.com
Received 28 October 2020, Accepted 07 November 2020
INTRODUCTION

Repaglinide (Figure 1), 2-ethoxy-4-[2-[(1R)-3-methyl-1-(2-piperidin-1-yl phenyl) butyl amino]-2-oxoethyl] benzoic acid\textsuperscript{1}, is an oral anti-hyperglycaemic agent which stimulate insulin release by binding to β cells of the pancreas\textsuperscript{2}. Sitagliptin phosphate (Figure 2), (3 R) - 3-amino -1- [3-(trifluoromethyl)-6,8-dihydro-5 H- [1,2,4] triazolo [4,3-a] pyrazin-7-yl]-4-(2,4,5-trifluorophenyl) butan-1-one; phosphoric acid\textsuperscript{3}, is dipeptidyl peptidase 4 (DPP-4) enzyme inhibitor. DPP-4 enzyme breaks the incretins Glyco Lipoprotein -1 (GLP-1), which is gastrointestinal hormones released in response to a meal. To prevent GLP-1 inactivation, Sitagliptin phosphate increase insulin secretion by suppressing glucagon release from the alpha cells in pancreas\textsuperscript{4}. Individually, Repaglinide and Sitagliptin phosphate are available in different dosage forms in market. Number of clinical trials on Repaglinide and Sitagliptin phosphate in combination has been performed using by Researchers. In view of Clinical Trials, Sitagliptin phosphate produces synergistic effect with Repaglinide in type 2 diabetes mellitus by stimulating decrease in glycated haemoglobin. Resulting none hypoglycemia (side effect of Repaglinide) observed in Type -2 diabetes mellitus patients\textsuperscript{5}. From the Exhaustive literature survey, Analysis of Repaglinide and Sitagliptin phosphate by various methods like Spectroscopic methods viz. UV and Mass Spectroscopy; and Chromatographic methods viz. High Performance Liquid Chromatography (HPLC); High Performance Thin Layer Chromatography (HPTLC) has been reported individually\textsuperscript{6-7} and also in different class of combination like Repaglinide alone\textsuperscript{8-12}, Sitagliptin alone\textsuperscript{13-14}, Repaglinide and Metformin\textsuperscript{15-18}, Sitagliptin and Metformin\textsuperscript{19-24} and many more. Since no method has been develop and validated for simultaneous estimation of Repaglinide and Sitagliptin phosphate in combination. Hence, the objectives of the present work was to develop and validate Stability indicating RP-HPLC and Simultaneous equation UV Spectrophotometric method for simultaneous estimation of Repaglinide and Sitagliptin phosphate in combination.

![Chemical structure of Repaglinide](image)

**Figure 1: Chemical structure of Repaglinide**
Figure 2: Chemical structure of Sitagliptin phosphate

MATERIALS AND METHOD

Chemicals and Reagents
The bulk drug, Repaglinide and Sitagliptin phosphate were procured as gift sample from West Coast Pharmaceuticals Ltd., Ahmedabad and Torrent Pharmaceuticals Ltd., Ahmedabad, respectively. Methanol, Acetone and Water used of HPLC grade were procured from Finar chemicals, Ahmedabad. Potassium dihydrogen phosphate and ortho phosphoric acid, 75 % (AR Grade) were purchased from Astron Chemicals Ltd., India. All solutions were prepared fresh on daily basis.

Instrumentation and Analytical condition
The HPLC method was performed on Systronic RP-HPLC (LC-138), UV Detector SPD-20 A, Rheodyne injector fitted with a 20 µl loop and used Clarify® software for determination. The method was conducted using Reverse phase techniques. Both drugs were eluted isocratically using Acetonitrile: Phosphate buffer (pH 3.5 adjusted with 10 % Ortho phosphoric Acid) (65:35; v/v) with flow rate 1 ml/min. The detection wavelength of UV-vis Detector was set to 228 nm. All solutions with mobile phase were prepared daily, which were filtered by 0.45 µm membrane filter (Millipore) and sonicated with Sonicator (Equitron, India) before use. A Kromstar® C18 (250 × 4.6 mm, 5 µm) Column and Systronics® pH meter were used. The HPLC system was operated at room temperature (25 ± 1°C).

UV Spectrophotometric method was performed on Shimadzu UV Visible double beam spectrophotometer (Model-1900) and using 1.0 cm quartz cells. UV Probe software was used for all absorbance measurements. All weighing were done on Digital Analytical balance (Wensar Dab 13-220).

Preparation of Standard Solution
Accurately weighed 10 mg of Repaglinide and 100 mg of Sitagliptin phosphate standard were transferred to 100 ml volumetric flask and dissolved in 100 ml methanol. The flasks were shaken and volume was made up to the mark with Methanol to give solution containing 100 µg/ml of Repaglinide and 1000 µg/ml of Sitagliptin phosphate. From this solution, Repaglinide was pipetted
out as aliquots 0.05, 0.1, 0.15, 0.2 and 0.25 ml and Sitagliptin phosphate was pipetted out as aliquots 0.5, 1.0, 1.5, 2.0, 2.5 ml of the stock solution were further diluted to 10 ml volumetric flasks individually with methanol to get concentrations 0.5, 1.0, 1.5, 2.0, 2.5 μg/ml for Repaglinide and 50, 100, 150, 200 and 250 μg/ml for Sitagliptin phosphate.

**Preparation of Sample solution**

Accurately weighed equivalently weight of Repaglinide (1 mg) and Sitagliptin Phosphate (100 mg) which transferred in 100 ml volumetric flask and make up half mark with Methanol. This solution was sonicated till the drug dissolves and was made upto mark with methanol. This solution was filtered through Whatmann filter paper. The concentration of Repaglinide was 10 μg/ml and Sitagliptin Phosphate was 1000 μg/ml. From above mixture solutions, take 1 ml and transferred in to a 10 ml volumetric flask and the volume was adjusted up to the mark with mobile phase to make final concentration of Repaglinide 1 μg/ml and Sitagliptin Phosphate 100 μg/ml.

**Selection of wavelength detection**

Repaglinide (1 μg/ml) and Sitagliptin phosphate (100 μg/ml) were used for the detection of wavelength.

**RP-HPLC Method**

The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. Repaglinide and Sitagliptin phosphate were observed good peak height resolution and shape at 228 nm. Hence, wavelength of 228 nm was selected for further study (Figure 3).

![Figure 3: Overlay UV Spectrum of Repaglinide (1 μg/ml) and Sitagliptin phosphate (100 μg/ml) in Methanol showing selected wavelength for HPLC and Maximum absorbance of Repaglinide and Sitagliptin phosphate at 240 nm and 267 nm for UV method](image_url)
The solutions were scanned and their spectra were recorded in the range of 200-400 nm against Methanol as a reagent blank. The overlain spectrums of Repaglinide and Sitagliptin phosphate at different concentration were recorded. From the figure 3, two wavelengths 240 nm (λmax of Repaglinide) and 267 nm (λmax of Sitagliptin phosphate) were selected for the determination of simultaneous equation. The A (1 %, 1 cm) was determined at both the wavelengths selected for each drug. A set of two simultaneous equations were formed as:

For Repaglinide,

\[ C_x = \frac{A_2 a y_1 - A_1 a y_2}{a x_2 a y_1 - a x_1 a y_2} \]

For Sitagliptin phosphate,

\[ C_y = \frac{A_1 a x_2 - A_2 a x_1}{a x_2 a y_1 - a x_1 a y_2} \]

Where, Cx and Cy are concentrations (µg/ml) of Repaglinide and Sitagliptin phosphate in sample solution, respectively. A₁ and A₂ are the absorbance of sample solutions at 240 nm (λ₁) and 267 nm (λ₂), respectively. ax₁ and ax₂ are the absorptivity of Repaglinide at 240 nm (λ₁) and Sitagliptin phosphate at 267 nm (λ₂), respectively. ay₁ and ay₂ are the absorptivity of Sitagliptin phosphate at 267 nm (λ₁) and Repaglinide at 240 nm (λ₂), respectively. The values of Cx and Cy were calculated by putting the values in these simultaneous equations.

Method Validation

The Methods were validated as per ICH guideline Q2(R1)²⁵. The proposed method has been extensively validated in terms of Specificity, Linearity and range, Accuracy, Precision, Detection limit, Quantification limit, Robustness and System suitability tests.

Specificity

Sample solutions (Repaglinide 1 µg/ml and Sitagliptin Phosphate 100 µg/ml) were performed to verify degradation and interferences (Figure 4). None interference was found with the Chromatogram of Repaglinide, Sitagliptin Phosphate and blank resulted in method was Specific.
Figure 4: RP-HPLC Chromatogram for (a) Blank, (b) Sitagliptin phosphate (100 μg/ml) and (c) Repaglinide (1 μg/ml) in Acetonitrile: Phosphate buffer (pH 3.5): (65:35 % v/v) at 228 nm {Run time: 10 min, Flow rate: 1ml/min}

Linearity and Range

The Calibration curve was constructed with concentrations 0.5-2.5 μg/ml of Repaglinide and 50-250 μg/ml of Sitagliptin phosphate for RP-HPLC (Figure 5) and UV methods (Figure 6 and 7). Linearity was computed in term of slope, intercept and correlation coefficient.

Accuracy

Recovery study of RP-HPLC and UV method were conducted as per ICH guideline to determine accuracy at three different concentration levels i.e. 50 %, 100 % and 150 %. Accuracy was calculated in percentage of recovery.
Figure 5: Overlain RP-HPLC Chromatogram of Sitagliptin phosphate (50-250 µg/ml) and Repaglinide (0.5-2.5 µg/ml) in Acetonitrile: Phosphate Buffer (pH=3.5) (65: 35 % v/v) at 228 nm {Run time: 10 min, Flow rate: 1 ml/min}

Figure 6: Overlain UV Spectra of Repaglinide (Linearity) (0.5 – 2.5 µg/ml) at 240 nm

Figure 7: Overlain UV Spectra of Sitagliptin phosphate (Linearity) (50 - 250 µg/ml) at 267 nm
Figure 8: Optimized RP-HPLC Chromatogram of Sitagliptin phosphate (100 μg/ml) and Repaglinide (1 μg/ml) in ACN: Buffer (KH₂PO₄) (pH 3.5) (65:35 % v/v); Flow rate: 1 ml/min at 228 nm

**Precision**

The precision studies of RP-HPLC and UV method were conducted at three levels like Intraday precision, Interday precision, and Repeatability. In Intraday precision, solutions containing 0.5, 1, 1.5 μg/ml of Repaglinide and 50, 100, 150 μg/ml of Sitagliptin phosphate were analyzed three times on the same day. In Interday precision, solutions containing 0.5, 1, 1.5 μg/ml of Repaglinide and 50, 100, 150 μg/ml of Sitagliptin phosphate were analyzed on three different successive days and in Repeatability, solutions containing 1 μg/ml of Repaglinide and 100 μg/ml of Sitagliptin phosphate were analyzed for six times. All the results were expressed in % R.S.D.

**Detection Limit (DL) and Quantification Limit (QL)**

Detection limit and Quantification limit of RP-HPLC and UV method were calculated using following equation as per ICH guidelines.

\[
\text{Detection limit} = 3.3 \times \left(\frac{\sigma}{S}\right)
\]

\[
\text{Quantification limit} = 10 \times \left(\frac{\sigma}{S}\right)
\]

Where,

\(\sigma\) = standard deviation of the Y intercept of calibration curve

\(S\) = Mean slope of the corresponding calibration curve.

**Robustness**
The Robustness of the RP-HPLC method was determined by analysis of samples under a variety of conditions as flow rate (± 0.2 ml/min), wavelength (± 2 nm), and mobile phase ratio (± 2 % v/v).

**System suitability tests**
A system suitability test (Resolution, Column efficiency, tailing factor and Theoretical plates) were performed to verify resolution and reproducibility of chromatography system.

**Forced degradation studies**
Selectivity was assessed by performing Forced degradation studies. Combination of Repaglinide (1 μg/ml) and Sitagliptin Phosphate (100 μg/ml) used as sample was stressed under various conditions like acid, alkaline, oxidative, photo and thermal to conduct forced degradation studies. Although, Repaglinide and Sitagliptin Phosphate are practically soluble in Acetonitrile: Phosphate Buffer (pH 3.5) (65:35 % v/v) was used as a solvent throughout studies.

**Acid degradation**
Accurately 1 ml of sample solution pipetted out from mixture solution in to 10 ml volumetric flask. 1 ml of 0.1 N Hydrochloric acid added to each flask and kept for 2 h at 40 °C. To neutralize, 1 ml of 0.1 N Sodium hydroxide was added in each flask and dilute upto volume with methanol. Filter the solution through 0.45 micron membrane filter and injected into chromatography and chromatogram has been recorded.

**Base degradation**
Accurately 1 ml of sample solution pipetted out from mixture solution in to 10 ml volumetric flask. 1 ml of 0.1 N Sodium hydroxide added to each flask and kept for 2 h at 40 °C. To neutralize, 1 ml of 0.1 N Hydrochloric acid was added in each flask and dilute upto volume with methanol. Filter the solution through 0.45 micron membrane filter and injected into chromatography and chromatogram has been recorded.

**Oxidative degradation**
Accurately 1 ml of sample solution pipetted out from mixture solution in to 10 ml volumetric flask. 1 ml of 3 % Hydrogen peroxide added to each flask and kept for 2 h at 40 °C. Filter the solution through 0.45 micron membrane filters and injected into chromatography and chromatogram has been recorded.

**Photolytic degradation**
Drugs were placed in a photo stability chamber and exposed to direct UV light for 2 h. At different time intervals the drugs were taken out, dilute appropriately and injected into chromatography to determine the amount of degradation of the drugs.

**Thermal degradation**
Accurately 1 ml of sample solution pipetted out from mixture solution in to 10 ml volumetric flask and exposed under heat at 80 °C for 2 h. At different time intervals, make volume up to the mark with methanol and injected into chromatography to determine the amount of degradation of the drugs.

**Statistical comparison of RP-HPLC and UV Method**

The Student’s t-test calculated using following formula:

\[ t = \frac{\bar{x}_1 - \bar{x}_2}{s \sqrt{\left( \frac{1}{n_1} + \frac{1}{n_2} \right)}} \]

where, \( t \) is the t-value, \( \bar{x}_1 \) and \( \bar{x}_2 \) are the means of HPLC and UV respectively, \( s^2 \) is the pooled standard error of the two groups, and \( n_1 \) and \( n_2 \) are the number of observations in each of the groups.

**RESULTS AND DISCUSSION**

**RP-HPLC method**

In order to select mobile phase, various solvents with different proportions as Acetonitrile: Water, Methanol: Water, Acetonitrile: Phosphate buffer were used. Resulting, Acetonitrile: Potassium dihydrogen phosphate Buffer (pH 3.5) (65:35 %v/v) has been selected as optimized mobile phase based on peak parameters which obeyed ideal system suitability parameters like proper migration, separation and resolution at flow rate (1 ml/min) at 228 nm of Repaglinide and Sitagliptin phosphate (Figure 8). Figure 8 showed, Sitagliptin phosphate and Repaglinide were eluted and forming symmetrical peaks, also well separated from solvent front. The Retention time of Sitagliptin phosphate and Repaglinide were observed at 2.5 and 4.6 min, allows a rapid determination of the drugs, which was important for routine analysis. The results of system suitability parameters were tabulated in table 1.

![Figure 9: RP-HPLC Chromatogram of Acid Degradation for Sitagliptin phosphate (100 µg/ml) and Repaglinide (1 µg/ml) Sample at 2 h at 228 nm (Run time: 10 min, Flow rate:](image-url)
Figure 10: RP-HPLC Chromatogram of Base Degradation for Sitagliptin phosphate (100 µg/ml) and Repaglinide (1 µg/ml) Sample at 2 h at 228 nm {Run time: 10 min, Flow rate: 1ml/min}

Figure 11: RP-HPLC Chromatogram of Oxidative Degradation for Sitagliptin phosphate (100 µg/ml) and Repaglinide (1 µg/ml) Sample at 2 h at 228 nm {Run time: 10 min, Flow rate: 1ml/min}

Figure 12: RP-HPLC Chromatogram of Photolytic Degradation for Sitagliptin phosphate
(100 µg/ml) and Repaglinide (1 µg/ml) Sample at 2 h at 228 nm (Run time: 10 min, Flow rate: 1 ml/min)

Figure 13: RP-HPLC Chromatogram of Thermal Degradation for Sitagliptin phosphate (100 µg/ml) and Repaglinide (1 µg/ml) Sample at 2 h at 228 nm (Run time: 10 min, Flow rate: 1 ml/min)

Calibration curve were constructed by plotting average Peak area versus Concentration. Straight line equations were obtained from calibration curve. The linear regression equation for Repaglinide was $y = 29.971x + 48.316$, with correlation coefficient ($r = 0.9972$), and $y = 26.321x + 2.304$, with correlation coefficient ($r = 0.998$) for Sitagliptin phosphate which showed highly significant for the method (Table 2). The % recovery of Repaglinide and Sitagliptin phosphate was found to be 99.66 - 100.50 and 99.94 - 100.16, respectively (Table 3). From the results, good sensitivity has been achieved which reflects the high efficiency of the separation methods. The intraday, interday and repeatability precision of Repaglinide and Sitagliptin Phosphate were expressed in % RSD and indicated in acceptable limits. This result indicates that the method is precise and accurate. The precision data of Repaglinide and Sitagliptin phosphate showed in table 4 and table 5, respectively.

The Detection and Quantitation limit of Repaglinide were found to be 0.144 µg/ml and 0.478 µg/ml, respectively and for Sitagliptin phosphate, Detection and Quantitation limit were found to be 0.254 µg/ml and 0.840 µg/ml, respectively at 228 nm which were within the acceptable limits. The % assay of Repaglinide and Sitagliptin phosphate were found to be 99.75 and 99.84, respectively. The Robustness was determined under a variety of conditions as flow rate (± 0.2 ml/min), wavelength (± 2 nm), and mobile phase ratio (± 2 % v/v) and results were expressed in % RSD. The Robustness data showed in table 6.
Table 1: System suitability parameter

| Parameters                  | Retention Time | Tailing Factor | Number of Theoretical plates | Resolution |
|-----------------------------|----------------|----------------|------------------------------|------------|
| Sitagliptin phosphate       | 2.5            | 0.743          | 6854                         | 2.5        |
| Repaglinide                 | 4.6            | 1.496          | 7708                         |            |

Table 2: Regression analysis of data for the quantitation of Repaglinide and Sitagliptin phosphate by the proposed methods

| Statistical parameters | HPLC Method | UV Method |
|-----------------------|-------------|-----------|
|                       | Repaglinide | Sitagliptin phosphate | Repaglinide | Sitagliptin phosphate |
| Concentration range(µg/ml) | 0.5-2.5 | 50-250 | 0.5-2.5 | 50-250 |
| Wavelength (nm)        | 228 nm | 240 nm | 267 nm | 267 nm |
| Regression equation    | y = 29.971x + 48.316 | y = 26.321x + 2.304 | y = 0.06x + 0.0056 | y = 0.0168x - y = 0.0046x - y = 0.006x - y = 0.0004 |
| Correlation coefficient (r) | 0.9972 | 0.998 | 0.9988 | 0.997 | 0.998 |

Table 3: Recovery test for Repaglinide and Sitagliptin phosphate

| Name of Drug | % Level Of Recovery | Test Amount (µg/ml) | Amount of drug taken (µg/ml) | Spiked Std Amount (µg/ml) | Total amount Recovered (µg/ml) | % Recovery ±S.D. (n=3) | Total amount Recovered (µg/ml) | % Recovery ±S.D. (n=3) |
|--------------|---------------------|---------------------|----------------------------|--------------------------|-------------------------------|------------------------|-------------------------------|------------------------|
| Repaglinide  | 50                  | 1                   | 0.5                        | 1.5                      | 1.495                         | 99.66±0.0057           | 1.49                         | 99.33±0.01               |
|              | 100                 | 1                   | 1                          | 2                        | 2.01                          | 100.50±0.0115          | 1.98                         | 99.00±0.02               |
|              | 150                 | 1                   | 1.5                        | 2.5                      | 2.51                          | 100.40±0.0152          | 2.48                         | 99.20±0.01               |
|              | 50                  | 100                 | 50                         | 150                      | 149.90                        | 99.94±0.0115           | 147.83                       | 98.55±0.1322             |
| Sitagliptin phosphate | 100     | 100                 | 100                        | 200                      | 200.20                        | 100.10±0.0208          | 197.66                       | 98.83±0.2328             |
|              | 150                 | 100                 | 150                        | 250                      | 250.40                        | 100.16±0.0230          | 246.83                       | 98.73±0.2421             |

Table 4: Precision for Repaglinide

| Conc. (µg/ml) | HPLC Method | UV Method |
|---------------|-------------|-----------|
|               | Mean Peak area ± SD (n=3) | % R.S.D. | Mean Absorbance ± SD (n=3) | % R.S.D. |
| 228 nm        | 0.5         | 99.07 ± 1.140 | 1.15 | 0.037±0.0006 | 0.013 ± 0.0002 | 1.62 | 1.53 |
| 240 nm        | 1           | 171.26 ± 1.651 | 0.96 | 0.063±0.0009 | 0.022 ± 0.0003 | 1.42 | 1.36 |
| 267 nm        | 1.5         | 234.42 ± 1.926 | 0.82 | 0.097±0.0010 | 0.031 ± 0.0001 | 1.03 | 0.96 |
### Interday Precision of Repaglinide

| Conc. (µg/ml) | Mean Peak area ± SD (n=3) | % R.S.D. | Mean Absorbance ± SD (n=3) | % R.S.D. |
|--------------|---------------------------|----------|-----------------------------|----------|
|              | 228 nm                    | 240 nm   | 267 nm                      | 240 nm   | 267 nm |
| 0.5          | 98.85 ± 1.351             | 1.36     | 0.038 ± 0.0006              | 1.57     | 1.42   |
| 1            | 171.03 ± 1.930            | 1.12     | 0.063 ± 0.0008              | 1.26     | 1.25   |
| 1.5          | 235.25 ± 2.199            | 0.93     | 0.098 ± 0.0009              | 0.91     | 0.93   |

### Repeatability of Repaglinide

| Conc. (µg/ml) | Mean Peak area ± SD (n=6) | % R.S.D. | Mean Absorbance ± SD (n=6) | % R.S.D. |
|--------------|---------------------------|----------|-----------------------------|----------|
|              | 228 nm                    | 240 nm   | 267 nm                      | 240 nm   | 267 nm |
| 1            | 173.84 ± 1.483            | 0.86     | 0.063±0.0004                | 0.63     | 0.45   |

**Table 5: Precision for Sitagliptin phosphate**

### Intraday Precision of Sitagliptin phosphate

| Conc. (µg/ml) | Mean Peak area ± SD (n=3) | % R.S.D. | Mean Absorbance ± SD (n=3) | % R.S.D. |
|--------------|---------------------------|----------|-----------------------------|----------|
|              | 228 nm                    | 267 nm   | 240 nm                      | 267 nm   |
| 50           | 78.20 ± 1.069             | 0.192    | 0.032 ± 0.0005              | 1.51     |
| 100          | 155.47 ± 2.016            | 0.385    | 0.059 ± 0.0007              | 0.98     |
| 150          | 250.86 ± 3.018            | 0.598    | 0.087 ± 0.0008              | 0.78     |

### Interday Precision of Sitagliptin phosphate

| Conc. (µg/ml) | Mean Peak area ± SD (n=3) | % R.S.D. | Mean Absorbance ± SD (n=3) | % R.S.D. |
|--------------|---------------------------|----------|-----------------------------|----------|
|              | 228 nm                    | 267 nm   | 240 nm                      | 267 nm   |
| 50           | 77.63 ± 1.147             | 0.193    | 0.033 ± 0.0005              | 1.55     |
| 100          | 155.60 ± 2.167            | 0.387    | 0.060 ± 0.0008              | 1.03     |
| 150          | 249.67 ± 3.233            | 0.599    | 0.088 ± 0.0007              | 0.66     |

### Repeatability of Sitagliptin phosphate

| Conc. (µg/ml) | Mean Peak area ± SD (n=6) | % R.S.D. | Mean Absorbance ± SD (n=6) | % R.S.D. |
|--------------|---------------------------|----------|-----------------------------|----------|
|              | 228 nm                    | 267 nm   | 240 nm                      | 267 nm   |
| 100          | 156.94 ± 1.311            | 0.386±0.003| 0.059±0.0005                | 0.77     |
Table 6: Robustness Study for Repaglinide and Sitagliptin phosphate

| Sr. No. | Parameter                      | Variation | Area ± S.D. | % R.S.D. |            |            |
|---------|--------------------------------|-----------|-------------|-----------|------------|------------|
|         |                                |           | Repaglinide | Sitagliptin phosphate | Repaglinide | Sitagliptin phosphate |
| 1       | Flow rate                      |           |             |           |            |            |
|         | (1 ml/min)                     | 0.8 ml/min| 171.72 ± 1.061| 154.87 ± 0.912| 0.61       | 0.58       |
|         | (± 0.2 ml/min)                 | 1.0 ml/min| 172.01 ± 1.717| 155.78 ± 1.158| 0.99       | 0.74       |
|         |                                | 1.2 ml/min| 171.49 ± 0.940| 155.51 ± 0.808| 0.54       | 0.51       |
| 2       | Detection wavelength           |           |             |           |            |            |
|         | (228 nm) (± 2 nm)              | 226 nm    | 172.06 ± 1.069| 155.80 ± 1.150| 0.62       | 0.73       |
|         | (228 nm) (± 2 nm)              | 228 nm    | 172.76 ± 1.494| 156.50 ± 1.279| 0.86       | 0.81       |
|         |                                | 230 nm    | 171.66 ± 0.946| 155.87 ± 1.095| 0.55       | 0.70       |
| 3       | Mobile phase (65:35 %v/v)      |           |             |           |            |            |
|         | (± 2 %v/v)                     | 63:37 %v/v| 172.32 ± 1.126| 155.67 ± 1.150| 0.65       | 0.73       |
|         |                                | 65:35% v/v| 172.96 ± 1.654| 156.74 ± 1.365| 0.95       | 0.87       |
|         |                                | 67:33 % v/v| 171.72 ± 1.061| 155.14 ± 1.095| 0.61       | 0.70       |
UV Method

A reliable, precise and accurate UV spectrophotometric method was developed and validated for simultaneous estimation of Repaglinide and Sitagliptin phosphate in combination. Repaglinide (1 µg/ml) and Sitagliptin phosphate (100 µg/ml) solutions were scanned between 200-400 nm. The λmax for Repaglinide and Sitagliptin phosphate were found to be 240 nm and 267 nm respectively. These wavelengths were used for all measurements. The spectra of Repaglinide (1 µg/ml) and Sitagliptin phosphate (100 µg/ml) were constructed and the linearity range were observed (Figure 6 and 7). Calibration curves were constructed and Beer’s law was obeyed over the concentration range of 0.5-2.5 µg/ml for Repaglinide and 50-250 µg/ml for Sitagliptin phosphate. The linear regression equation (correlation coefficient) for Repaglinide were $y = 0.06x + 0.0056$ at 240 nm ($r = 0.9988$) and $y = 0.0168x + 0.0048$ at 267 nm ($r = 0.9994$); and for Sitagliptin phosphate $y=0.0046x - 0.0584$ at 267 nm ($r = 0.997$) and $y=0.006x - 0.0004$ at 240 nm ($r = 0.998$). The results of linearity were tabulated in table 2. The % recovery of Repaglinide and Sitagliptin phosphate was found to be 99.20 - 99.33% and 99.55 - 99.83%, respectively (Table 3). Results were obtained lie in acceptable limits. The intraday, interday and repeatability precision of Repaglinide and Sitagliptin phosphate were expressed in % RSD and indicated in acceptable limits. This result indicates that the method is precise and accurate. The precision data of Repaglinide and Sitagliptin phosphate showed in table 4 and table 5, respectively.

The Detection and Quantitation limit of Repaglinide were found to be 0.319 µg/ml and 0.967 µg/ml at 240 nm; 0.983 µg/ml and 2.977 µg/ml at 267 nm, respectively; and for Sitagliptin phosphate, Detection and Quantitation limit were found to be 0.431 µg/ml and 1.305 µg/ml at 267 nm; 0.275 µg/ml and 0.834 µg/ml at 240 nm which were within the acceptable limits. The % assay of Repaglinide and Sitagliptin phosphate were found to be 99.00% and 99.49%, respectively.

FORCED DEGRADATION STUDIES

Peak area of Sitagliptin phosphate and Repaglinide were found to be 153.26 and 170.02, respectively. % degradation of Sitagliptin phosphate and Repaglinide were calculated using this equation,

$$\text{% degradation} = 100 - \left( \frac{\text{Degradation area}}{\text{Standard area}} \right) \times 100$$

Acid degradation study

The combination showed sufficient degradation within 2 h with 0.1 N Hydrochloric acid at 40°C. Sitagliptin phosphate showed 9.08 and 18.19 % degradation at 1 and 2 h, respectively; whereas
Repaglinide showed 8.31 and 17.94 % degradation at 1 and 2 h, respectively (Figure 9).

**Base degradation study**
Similar to acid, sufficient degradation was observed within 2 h with 0.1 N Sodium Hydroxide at 40°C. Sitagliptin phosphate showed 6.03 and 13.67 % degradation at 1 and 2 h, respectively; whereas Repaglinide showed 1.57 % and 2.06 % degradation at 1 and 2 h, respectively (Figure 10).

**Oxidative degradation study**
Degradation was observed within 2 h after heating with 3 % Hydrogen peroxide at room temperature. Sitagliptin phosphate showed 3.09 and 7.18 % degradation at 1 and 2 h, respectively; whereas Repaglinide showed 2.33 and 6.14 % degradation at 1 and 2 h, respectively (Figure 11).

**Photolytic degradation study**
Drugs were exposed to direct UV light for 2 h. Sitagliptin phosphate showed 8.93 and 17.04 % degradation at 1 and 2 h, respectively; whereas Repaglinide showed 8.08 and 15.46 % degradation at 1 and 2 h, respectively (Figure 12).

**Thermal degradation study**
Drugs were exposed under heat at 80 °C for 2 h. Sitagliptin phosphate showed 5.63 and 10.06 % degradation at 1 and 2 h, respectively; whereas Repaglinide showed 2.87 and 5.95 % degradation at 1 and 2 h, respectively (Figure 13).

**Statistical comparison of RP-HPLC and UV Method**
The proposed analytical methods were compared using Statistical analysis. The student’s t-test was applied and did not showed significant difference between experimental values obtained in sample analysis by the two methods. The calculated t-value (t_calculated) was smaller than critical t-value (t_tabulated / t_critical), at 5 % significance level.

**CONCLUSION**
Simple, rapid, sensitive, accurate and precise RP-HPLC and UV spectroscopic methods has been developed and validated for routine analysis of Repaglinide and Sitagliptin phosphate. These proposed methods were suitable for simultaneous estimation of Repaglinide and Sitagliptin phosphate in bulk drug and synthetic mixture without any interference. The developed and validated methods were successfully applied in combination. Comprehensive stress testing to mixture of Repaglinide and Sitagliptin phosphate was carried out according to ICH guideline Q1A (R2) under various stress conditions in the presence of degradation products. During degradation study, the results obtained were found within the acceptance criteria. Validation of proposed methods was also carried out according to ICH guideline Q2 (R1). Hence, the proposed stability
indicating RP-HPLC assay method and UV method might be applied and utilized for the routine analysis for the estimation of Repaglinide and Sitagliptin phosphate in combination. Statistical analysis proved that the proposed methods were repeatable and selective for the analysis of Repaglinide and Sitagliptin phosphate in combination.

REFERENCES

1. Maryadele NJ. The Merck Index, An encyclopedia of chemicals, drugs and biological. 14th ed., UK: Merck Research Laboratories: 2006, 567.
2. Tripathi KD. Essential of Medical pharmacology. 6th Ed. New Delhi: Jaypee Brother Medical publishers Limited; 2008. 254-270. 273.
3. Maryadele NJ. The Merck Index, An encyclopedia of chemicals, drugs and biological. 14th Ed. UK: Merck Research Laboratories; 2006. 798.
4. Rang H, Dale M. Rang and Dale’s pharmacology. 7th Ed. Toronto: Elsevier Publication; 2012. 377-378.
5. Kohei kaku S, Katsuhsia L, Shigeru K, Negishi H. Clinical study of Repaglinide efficacy and safety in type 2 diabetes mellitus patients with blood glucose levels inadequately controlled by Sitagliptin. J Diabetes Inv 2015; 2(5): 1-7.
6. Sethi PD. High performance liquid chromatography: Quantitative Analysis of Pharmaceutical Formulations. 1st Edn; CBS publishers: New Delhi, 2001. 182-184, 211-214.
7. Snyder LR, Kirkland JL, Glajch JL. Practical HPLC Method Development; 2nd Edn; Wiley Interscience, New York, 1997; 205-227, 234, 266.
8. Patel DR, Patel LJ, Patel MM. Development and Validation of stability indicating method for the determination of Repaglinide in Pharmaceutical dosage form using High Performance Liquid Chromatography. Int J Chemtech Res 2011; 5(3): 11-40.
9. Rajput SJ, Chaudhary BG. Validated Analytical Method of Repaglinide in Bulk and Tablet Formulation. Ind J Pharm Sci 2006; 2(1): 130-132.
10. Ahir KB, Patelia EM, Shah A. Simultaneous estimation of Metformin hydrochloride and Repaglinide in Pharmaceutical Formulation by HPTLC- Densitometry method. J Chromatogr Sep Tech 2014; 9(3): 225-296.
11. Fayyad MK, Ghanem EH. Liquid Chromatography Tandem Mass Spectrometry Method for Determination of Anti-Diabetic Drug Repaglinide in Human Plasma. American J Anal Chem 2014; 5(3): 281-290.
12. Akhtar J, Aqil M. Stability-indicating assay of Repaglinide in bulk and optimized nano emulsion by validated high performance thin layer chromatography technique. J Pharm Bioallied Sci 2013; 5(2): 10-36.

13. Kashid AM, Dhange AA, Gawande VT, Miniyar PB, Datar PA, Dhwale SC. RP-HPLC Method Development and Validation for Sitagliptin in Human Plasma. American J Pharmtech Res 2012; 2(5): 805-811.

14. Nirogi R, Kandikere V, Koteshwara M, Prashanth K, Raghupathi A, Boggavarapu R. Sensitive liquid chromatography tandem mass spectrometry method for the quantification of Sitagliptin, a DPP-4 inhibitor, in human plasma using liquid-liquid extraction. Biomed Chrom 2008; 22(1): 214-222.

15. Aslan SS, Yilmaz B. Derivative Spectrophotometric and Isocratic High-Performance Liquid Chromatographic Methods for Simultaneous Determination Repaglinide and Metformin Hydrochloride in Pharmaceutical Preparations. American J Anal Chem 2017; 8(1): 541-552.

16. Aslan SS, Yilmaz B. Derivative Spectrophotometric and Isocratic High-Performance Liquid Chromatographic Methods for Simultaneous Determination of Repaglinide and Metformin Hydrochloride in Pharmaceutical Preparations. American J Anal Chem 2017; 8(1): 541-552.

17. Tatiparthi R, Bannoth CK. Method Development and Validation of Metformin and Repaglinide in Rabbit Plasma by RP-HPLC. Int J Pharm Pharma Sci 2010; 35(1): 69-75.

18. Fouad MM, Rashed NS. Development and validation of Chromatographic and Spectroscopic methods for estimation of Repaglinide and Metformin HCl in Combined Dosage form. J Global Trends Pharm Sci 2012; 5(3): 1844-1848.

19. Karimulla SK, Vasanth PM, Ramesh T, Ramesh M. Method development and validation of Sitagliptin and Metformin using reverse phase HPLC method in bulk and tablet dosage form. Der Pharmacia Lett 2013; 5(5): 168-174.

20. Prasad PBN, Satyanarayana K, Krishnamohan G. Development and Validation of a Method for Simultaneous Determination of Metformin Hydrochloride and Sitagliptin phosphate in a Formulation by RP-HPLC. American J Anal Chem 2014; 5(11): 737-742.

21. Sachin LP, Jayant RB, Chetan MB. Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous determination of Sitagliptin and Metformin in tablet dosage form. Int J Res Pharm Bio Med Sci 2013; 4(2): 590-596.
22. Lathareddy Y, Rao NS. Stability-indicating RP-HPLC method and its validation for analysis of metformin & sitagliptin in bulk and pharmaceutical dosage form. World J Pharm Pharma Sci 2012; 2(5): 3691-3709.

23. Chellu SN, Malleswararao, Suryanarayan MV, Khagga M. Determination of Sitagliptin phosphate and Metformin hydrochloride in tablets by a validated UPLC method. Scientia Pharmaceutica 2012; 80(2): 139-152.

24. Moataz SH. Improved Chromatographic Separation of Sitagliptin phosphate and Metformin Hydrochloride. Int J Biomed Sci 2015; 11(4): 190-194.

25. ICH, Q2 (R1) Validation of Analytical Procedures: Text and Methodology International Conference on Harmonization, IFPMA, Geneva, Switzerland, November 2007: 6.