Development and Validation of Analytical Methods for Simultaneous Estimation of Sitagliptin Phosphate and Pioglitazone Hydrochloride

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ABSTRACT

Simple, rapid, accurate and precise UV spectrophotometric and RP-HPLC methods had been developed for simultaneous estimation of Sitagliptin Phosphate (STG) and Pioglitazone Hydrochloride (PIO). The method applied was Simultaneous Equation Method (Vierodt’s Method), based on measurement of absorbance of Sitagliptin Phosphate and Pioglitazone Hydrochloride at \( \lambda_{\text{max}} \) 267nm and 270nm respectively. Linearity was found in the concentration range of 50-250µg/ml for Sitagliptin and 5-25µg/ml for Pioglitazone with regression coefficient \( r^2 \)=0.9997 and \( r^2 \)=0.9995 respectively. RP-HPLC method was carried on Phenomenex C-18 column (150 mm × 4.6 mm, 5 µ) by using a mobile phase acetonitrile: methanol: water (30:30:40) as a mobile phase at 1.0 ml/min flow rate at 270 nm. The linearity was found to be in the range of 10-50 µg/ml and 3-15µg/ml with regression coefficient of \( r^2 \)= 0.9998, and \( r^2 \)=0.9996 for Sitagliptin Phosphate and Pioglitazone HCl respectively. The peak obtained were sharp having clear baseline separation with a retention time 5.6 and 2.8 min for Sitagliptin Phosphate and Pioglitazone HCl. This method is accurate and precise and can be employed for routine analysis of Sitagliptin Phosphate and Pioglitazone hydrochloride in different pharmaceutical dosage forms.

KEYWORDS

Sitagliptin Phosphate, Pioglitazone HCl, UV - spectrophotometry, RP-HPLC

INTRODUCTION

Sitagliptin Phosphate and Pioglitazone Hydrochloride are antidiabetic drugs. Sitagliptin belongs to the class of Dipeptidyl peptidase-4[DPP-4] inhibitors, a new class of anti-diabetic drugs which act by increasing glucose dependent insulin release. Therapeutically DPP-4 inhibitors are used to treat type 2 diabetes alone or combination with other drugs which increases the sensitivity of
insulin at target site DPP-4 inhibitors act by inhibiting the inactivation of enteroendocrine incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino-tropic (GIP) polypeptide. Pioglitazone is a drug belongs to the class of thiazolidinedione, which is used to decreases insulin resistance. It is an Antidiabetic agent to manage NIDDM [non-antihyperglycemic insulin-dependent diabetes mellitus, sugar diabetes] called type-2 diabetes.

There are various methods for analysis of the drugs like UV, HPLC or HPTLC. But there is no method reported for simultaneous estimation of STG and PIO in bulk and dosage form.

So, objective of the present study was to develop and validate simple, precise and accurate UV spectrophotometric and RP-HPLC method for simultaneous estimation of STG and PIO.

**MATERIAL & METHODS**

**Instruments**

UV- VIS double beam spectrophotometer of Systronic 2201, Mumbai, with spectral bandwidth of 2nm and a pair of matched quartz cells of length 1cm were used for analytical work. Younglin Acme 9000 HPLC was used for identification and separation. All the weighing was carried out on the Electronic Balance AY220, Shimadzu, Japan. Sonication was carried out by Microclean-103.

**Materials and Reagents**

Pioglitazone Hydrochloride was purchased from Swapnoop Drugs and Pharmaceuticals, Aurangabad. Sitagliptin Phosphate was supplied as a Gift sample by Torrent Pharmaceutical Ltd, Ahmadabad, Gujarat. The reagents used were Hydrochloric acid and Distilled water for UV-spectrophotometric method and for RP-HPLC method, Acetonitrile LiChrosolv®, Methanol LiChrosolv® and water LiChrosolv® which were procured from Merck specialities Pvt. Ltd., Mumbai.

**Methods**

**UV- Spectrophotometric Method**

**Preparation of 0.1N HCl**

0.85ml of concentrated Hydrochloric acid was diluted with Distilled water to make the volume of 100ml to obtain 0.1N HCl.

**Preparation of Standard Stock Solution**

10 mg each of STG and PIO was weighed separately and transferred to two different 10 ml volumetric flasks. Both the drugs were dissolved in 5 ml of 0.1N HCl separately in volumetric flasks and volume made up to mark with 0.1N HCl to make the concentration of 1000µg/ml. From these solutions 1ml was pipetted out separately from both the volumetric flasks and transferred to two another 10ml volumetric flasks. Made up the volume up to the mark with the same solvent to obtain the final concentration of 100µg/ml for both the drugs.

**Assay of Tablet**

The fixed dose combination of these drugs is 100mg of STG with 30mg of PIO. Due to unavailability of the dosage form of this combination, standard STG drug 100mg is added to the tablets of PIO of 15mg (PIOZ 15) to simulate the condition of actual product. The required number of tablets were taken, weighed
and their average weight was determined. The tablets were crushed to fine powder and from the triturate, tablet powder equivalent to 30mg of PIO was weighed and to this weighed powder, 100mg of standard STG drug was added to get 100mg:30mg combination. Now all this weighed powder was transferred to 100ml volumetric flask containing 50ml of 0.1N HCl, dissolved, made the volume up to 100ml to get the concentration of 300µg/ml and 1000µg/ml of PIO and STG respectively filtered through whatman filter paper. Now from this solution 1ml was diluted to 10 ml in other volumetric flask with same solvent to get the concentration of 30µg/ml and 100µg/ml. From above solution 5ml was diluted to 10 ml with the same solvent to get final concentration of 15µg/ml of PIO and 50µg/ml of STG.

**Simultaneous Equation Method (Vierodt’s Method)**

If sample contains two absorbing drugs (X and Y) each of which absorbs at the $\lambda_{max}$ of other. It may be possible to determine both the drugs by the technique of simultaneous equations if criteria below are met;

i. The $\lambda_{max}$ of two components is reasonably dissimilar.

ii. The two components do not interact chemically.

iii. The two absorbing drugs (X and Y), each of which should absorb at the $\lambda_{max}$ of other.

Two equations are constructed based upon the fact that at $\lambda_1$ and $\lambda_2$ the absorbance of the mixture is the sum of the individual absorbance of X and Y.

$$C_x = \frac{A_2ay_1 - A_1ay_2}{ax_2ay_1 - ax_1ay_2}$$

$$C_y = \frac{A_1ax_2 - A_2ax_1}{ax_2ay_1 - ax_1ay_2}$$

Where, $A_1$ and $A_2$ are absorbances of diluted mixture at $\lambda_1$ and $\lambda_2$ respectively.

$C_x$ and $C_y$ are the concentrations of x and y respectively.

$ax_1$ and $ax_2$ are absorptivities of x at $\lambda_1$ and $\lambda_2$ respectively.

ay_1 and ay_2 are absorptivities of y at $\lambda_1$ and $\lambda_2$ respectively.
Combined Standard Stock Solution of STG and PIO

1ml of ‘Std Stock STG’ (1000µg/ml) and 0.3ml of ‘Std Stock PIO’ (300µg/ml) transferred to 10 ml volumetric flask and diluted to 10 ml with methanol to get ‘Std Stock MIX AP’ (100µg/ml STG and 30µg/ml PIO).

Selection of Analytical Wavelength

To investigate the appropriate wavelength for simultaneous determination of STG (10µg/ml) and PIO (10µg/ml) individual solutions in the mobile phase were scanned in the range of 200-400nm.

Selection of Mobile Phase and its Strength

The solutions of STG (10µg/ml) and PIO (10µg/ml) were prepared in water and methanol filtered through syringe filter, then injected into HPLC system. The chromatograms were analysed using different combination of Acetonitrile: Methanol: Water (30:30:40) at a flow rate of 1ml/min for 10-30min at 270nm. Mixed solution of STG (10µg/ml) and PIO (10µg/ml) was prepared in methanol and filtered through syringe filter, then injected into the HPLC system, after the column saturated with mobile phase and constant back pressure.

Selection of Column (Stationary Phase)

To get well resolved, symmetric peak with highest no. of theoretical plates the solution of the STG and PIO were analysed using C18 column as a stationary phase.

Chromatographic Conditions

- **Analytical Column:** Phenomenex C18 column (150 mm × 4.6 mm, 5 µm)
- **Mobile Phase:** Acetonitrile: Methanol: Water (30:30:40)
- **Flow Rate:** 1ml/min
- **Injection Volume:** 20 µl
- **Detection Wavelength:** 270nm

Identification of Separated Peak of the Drugs

For identification of peak of the drugs; the standard solutions of STG (10µg/ml) and PIO (10µg/ml) were injected separately into HPLC system and retention time were matched with retention time of mixture.

Method Validation

Method A

The UV spectrophotometric method was validated as per ICH guidelines Q2 R1 for method validation. The parameters evaluated were linearity, precision, accuracy, LOD and LOQ.

Linearity

This was studied by diluting standard stock solution (1000µg/ml) of STG to 50-250µg/ml and PIO (100µg/ml) to 5-25 µg/ml concentrations. Concentration curves of concentration against absorbance were plotted at their respective wavelengths for both the drugs and the obtained data was subjected to regression analysis. The standard curves for STG and PIO are shown in figure 5 and 6.

Accuracy

Recovery studies were carried out by standard addition method by adding known amount of SIT and PIO (working standard) to pre-analysed sample at three different concentration levels that is 80%, 100% and 120% of assay concentration and percent recoveries were calculated.

![Calibration Curve of STG](image)

**Figure 5:** Calibration curve of STG of UV-Spectrophotometric method at $\lambda_1$ (267nm) and $\lambda_2$ (270nm)
Development and Validation of Analytical Methods for Simultaneous Estimation of Sitagliptin Phosphate and Pioglitazone Hydrochloride

Figure 6: Calibration curve of PIO of UV-Spectrophotometric method at $\lambda_1$ (267nm) and $\lambda_2$ (270nm)

Table 1: Standard absorptivity for STG

| Concentration ((µg/ml)) | Absorbance $\lambda_1$ (267 nm) | Absorbance $\lambda_2$ (270 nm) | Specific Absorptivity Values $\lambda_1$ (267 nm) | Specific Absorptivity Values $\lambda_2$ (270 nm) |
|------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| 50                     | 0.181                            | 0.162                            | 36.2                             | 32.4                             |
| 100                    | 0.352                            | 0.314                            | 35.2                             | 31.4                             |
| 150                    | 0.517                            | 0.462                            | 34.47                            | 30.8                             |
| 200                    | 0.697                            | 0.622                            | 34.85                            | 31.1                             |
| 250                    | 0.860                            | 0.771                            | 34.4                             | 30.84                            |
| Mean                   | 0.35024                          | 0.3131                           | 35.024                           | 31.31                            |

Table 2: Standard absorptivity for PIO

| Concentration ((µg/ml)) | Absorbance $\lambda_1$ (267 nm) | Absorbance $\lambda_2$ (270 nm) | Specific Absorptivity Values $\lambda_1$ (267 nm) | Specific Absorptivity Values $\lambda_2$ (270 nm) |
|------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| 5                      | 0.100                            | 0.103                            | 200                              | 206                              |
| 10                     | 0.180                            | 0.186                            | 180                              | 186                              |
| 15                     | 0.273                            | 0.284                            | 182                              | 189.33                           |
| 20                     | 0.363                            | 0.376                            | 181.5                            | 188                              |
| 25                     | 0.450                            | 0.466                            | 180                              | 186.4                            |
| Mean                   | 0.35024                          | 0.3131                           | 35.024                           | 31.31                            |

Precision

Precision was ascertained by determination of six replicates of same concentrations of sample and standard for method precision and system precision. Both intraday and inter-day precisions were carried out.

Table 4: Repeatability study data for STG and Pio (n=6)

| Sr N o. | Concentration of STG (µg/ml) | Absorbance of STG | Concentration of PIO (µg/ml) | Absorbance of PIO |
|---------|-------------------------------|-------------------|-----------------------------|-------------------|
| 1       | 100                           | 0.352             | 15                          | 0.273             |
| 2       |                               | 0.345             |                             | 0.271             |
| 3       | 15                            | 0.355             | 0.267                       |
| 4       |                               | 0.349             |                             | 0.270             |
| 5       |                               | 0.353             | 0.269                       |
| 6       |                               | 0.349             |                             | 0.267             |
| SD      |                               | 0.0036            |                             | 0.0023            |
| RSD     |                               | 1.03              |                             | 0.852             |

LOD

Detection limit was determined based on the standard deviation of peak areas of same concentrations that is standard solutions of STG (150µg/ml) and PIO (15µg/ml) prepared six times and LOD calculated by

$$\text{LOD} = 3.3 \times (\text{SD}/\text{S})$$

Where, SD= Standard Deviation; S= Slope of Curve

LOQ

Detection limit was determined based on the standard deviation of peak areas of same concentrations that is standard solutions of STG
(150µg/ml) and PIO (15µg/ml) prepared six times and LOQ calculated by

\[ \text{LOQ} = 10 \times \left( \frac{\text{SD}}{\text{S}} \right) \]

where, SD= Standard Deviation; S= Slope of Curve

Method B:

Specificity
The chromatogram of standard solution of mixture of STG and PIO was compared with formulation to observe the interference of excipient.

Figure 7. Overlain Chromatograms of sample and standard solution of drugs

Table 3. Accuracy for UV-spectrophotometric Method

| Sr. No. | Level of % Recovery | Amount of ‘Sample Stock-A’ (ml) | Amount of Standard Drug Added (µg/ml) | Total Amount Found (µg/ml) | Amount Recovered (µg/ml) | % Recovery |
|---------|---------------------|-------------------------------|-------------------------------------|---------------------------|--------------------------|------------|
|         |                     | STG  | PIO  | STG  | PIO  | STG  | PIO  | STG  | PIO  | STG  | PIO  |       |
| 1       | 0                   | 0    | 0    | 0    | 0    | 4.88 | 1.52 | 0    | 0    | 0    | 0    |       |
| 2       | 80                  | 0.5  | 4    | 1.2  | 8.8  | 2.7  | 3.92 | 1.18 | 98   | 98.33|
| 3       | 100                 | 0.5  | 5    | 1.5  | 10   | 3.1  | 5.12 | 1.58 | 102.4| 105.33|
| 4       | 120                 | 0.5  | 6    | 1.8  | 10.92| 3.34 | 6.04 | 1.82 | 100.66| 101.11|

Linearity
1, 2, 3, 4 and 5ml of ‘Std Stock MIX AP’ were transferred in a series of 10ml volumetric flasks. The volume was made up to the mark with methanol to obtain the conc. of 10, 20, 30, 40 and 50µg/ml of STG and 3, 6, 9, 12 and 15µg/ml of PIO. The solutions were filtered through 0.45µ syringe filter and 20µl injected into the HPLC system and their chromatogram were recorded for 10mins under the chromatographic conditions as described above after getting a stable baseline. Peak areas were recorded for all the peaks. Calibration curves of STG and PIO were constructed by plotting the peak area of STG v/s conc. of PIO and peak area of STG v/s conc. of PIO, respectively. The correlation coefficient \( (r^2) \) of least square linear regression for STG and PIO was calculated.

Table 5. Response of STG at various linearity levels

| Conc. of STG (µg/ml) | Peak Area (mV) |
|----------------------|---------------|
| 0                    | 0             |
| 10                   | 48            |
| 20                   | 92            |
Development and Validation of Analytical Methods for Simultaneous Estimation of Sitagliptin Phosphate and Pioglitazone Hydrochloride

### Table 6: Response of PIO at various linearity levels

| Sr. No. | Conc. of PIO (µg/ml) | Peak Area (mV) |
|---------|----------------------|----------------|
| 1.      | 3                    | 54             |
| 2.      | 6                    | 109            |
| 3.      | 9                    | 160            |
| 4.      | 12                   | 217            |
| 5.      | 15                   | 266            |

**Accuracy**

20 Tablets (PIOZ 30) were weighed and finely powdered, an accurately weighed tablet powder (183.1mg) equivalent to 15 mg of PIO was dissolved and diluted to 100ml with methanol. 0.5 ml of above solution was transferred in four different 10ml volumetric flask labeled as 0%, 80%, 100% and 120%. Then 0, 0.8, 1, 1.2ml of ‘Std Stock MIX AP’ (100µg/ml STG and 30µg/ml PIO) were added and volume was made up to the mark with mobile phase. All the solutions were filtered through syringe filter and injected into the HPLC system and their chromatograms were recorded under the same chromatographic conditions after getting a stable baseline. Peak areas were recorded and percent recoveries were calculated.

Figure 8: Calibration curve of STG of RP-HPLC method

Figure 9: Calibration curve of PIO of RP-HPLC method

Figure 10: Overlaid Chromatograms of serial dilutions of STG and PIO in optimized chromatographic conditions
Development and Validation of Analytical Methods for Simultaneous Estimation of Sitagliptin Phosphate and Pioglitazone Hydrochloride

Figure 11: Chromatogram of STG (10 µg/ml) in optimized chromatographic conditions

Figure 12: Chromatogram of PIO (10 µg/ml) in optimized chromatographic conditions

Figure 13: Chromatogram of combination of STG (10µg/ml) & PIO (10µg/ml) in optimized chromatographic conditions

**Precision**

The precision of an analytical method was studied by performing Repeatability and intermediate precision.

**Robustness**

Combined standard solution of STG (20µg/ml), PIO (6µg/ml) was prepared and analysed at different flow rates (0.9, 1.0, 1.1 ml/min) and different wavelengths (269, 270, 271nm) separately.

**Table 7. Recovery Studies**

| Sr. No. | Level of % Recovery | Amount of ‘Sample Stock-A’ (ml) | Amount of Standard Drug Added (µg/ml) | Total Amount Found (µg/ml) | Amount Recovered (µg/ml) | % Recovery |
|---------|---------------------|---------------------------------|-------------------------------------|---------------------------|-------------------------|------------|
| 1       | 0                   | 0.5                             | 0 STG 0 PIO                          | 0 STG 7.63                | 0 STG 0 PIO             | 0 STG 0 PIO |
| 2       | 80                  | 0.5                             | 8 STG 2.4 PIO                        | 8.1 STG 9.93              | 8.1 STG 2.3             | 101.25 95.83 |
| 3       | 100                 | 0.5                             | 10 STG 3 PIO                         | 9.56 STG 10.75            | 9.56 STG 3.12           | 95.6 104    |
| 4       | 120                 | 0.5                             | 12 STG 3.6 PIO                       | 11.94 STG 11.05           | 11.94 STG 3.42          | 99.5 95     |
Table 8: Results of Repeatability Study for STG and PIO

| Inj. | Peak Area (mV) of STG | Peak Area (mV) of PIO |
|------|-----------------------|-----------------------|
| 1    | 95                    | 110                   |
| 2    | 93                    | 109                   |
| 3    | 93                    | 106                   |
| 4    | 96                    | 108                   |
| 5    | 95                    | 109                   |
| 6    | 92                    | 109                   |
| SD   | 1.54                  | 1.37                  |
| RSD  | 1.5                   | 1.2                   |

**System Suitability**

Chromatograms were studied for different parameters such as tailing factor, resolution and theoretical plates to see that whether they comply with the recommended limit or not.

**RESULTS AND DISCUSSION**

The method has been employed successfully for quantitative determination of STG and PIO by UV spectrophotometric method and Reverse Phase High Performance Liquid Chromatographic method for the simultaneous estimation of STG and PIO and to validate the developed methods according to ICH Q2 (R1) guidelines.

Table 9: Summary of UV- Spectrophotometric Method of STG and PIO

| Sr. No. | Parameters                          | STG              | PIO              |
|---------|-------------------------------------|------------------|-----------------|
| 1.      | Linearity Range (μg/ml)             | 50-250           | 5-25            |
| 2.      | Regression Equation (y = mx+c)      | y=0.0034x+0.0059 | y=0.0179x+0.004 |
| 3.      | Correlation Coefficient (r²)        | 0.9997           | 0.9995          |
| 4.      | LOD (μg/ml)                         | 3.6              | 0.413           |
| 5.      | LOQ (μg/ml)                         | 10.90            | 1.26            |
| 6.      | % Recovery                          | 98-101           | 98-102          |
| 7.      | Repeatability(%RSD)                 | 1.03             | 0.852           |

Table 10: Summary of RP-HPLC Method of STG and PIO

| Sr. No. | Parameters                          | STG              | PIO              |
|---------|-------------------------------------|------------------|-----------------|
| 1.      | Linearity Range (μg/ml)             | 10-50            | 3-15            |
| 2.      | Regression Equation (y = mx+c)      | y=4.61x+0.7      | y=17.73x+1.6    |
| 3.      | Correlation Coefficient (r²)        | 0.9998           | 0.9996          |
| 4.      | LOD (μg/ml)                         | 1.1              | 0.25            |
| 5.      | LOQ (μg/ml)                         | 3.34             | 0.77            |
| 6.      | % Recovery                          | 95-104           | 95-102          |
| 7.      | Repeatability(%RSD)                 | 1.5              | 1.2             |
CONCLUSION

The UV- spectrophotometric and HPLC methods are simple, accurate, precise for estimation of STG and PIO in bulk and pharmaceutical formulation. The short chromatographic time makes this method suitable for processing of multiple samples in short time. The method shows no interference by the excipients. The statistical parameters and recovery data reveals the good accuracy and precision. This method can be useful and suitable for the estimation of the STG & PIO in bulk and pharmaceutical formulations.

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