Development and Validation of a Novel RP-HPLC Analytical Method for Sitagliptin Determination in Human Plasma

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ABSTRACT

Background: Different bio-analytic methods have been developed for determining drug concentration in plasma, but methods for sitagliptin determination are still very rare. In this study, RP-HPLC based method has been developed for assessing sitagliptin concentration in plasma.

Aim: To develop and validate RP-HPLC based analytical method for estimating sitagliptin in human plasma for pharmacokinetic applications.

Methods: In the present study, the mobile phase composed of acetonitrile: 0.5% triethanolamine (20:80) with pH 6.5 has been utilized. Samples of plasma containing sitagliptin and internal standard (IS)-rosiglitazone were extracted with dichloromethane:diethyl ether (4:6; v/v) at pH 7.4. The rate of flow was 1 ml/min. The retention time was about 5,232 and 6,903 minutes respectively for sitagliptin and rosiglitazone.

Results: At concentrations of 100-3200 ng/ml in plasma, calibration curves of sitagliptin were linear. The inter- and intra-day precision and accuracy ranged in between 93.56-98.56% and 1.09-

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1. INTRODUCTION

Sitagliptin, an oral antidiabetic agent, is chemically known as (2R)-4-Oxo-4-[(3-(trifluoromethyl))-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine [1]. Sitagliptin may be sometimes combined with other antihyperglycemic medications to lower blood glucose levels in type-2 diabetes patients [2]. Sitagliptin competitively represses Dipeptidyl Peptidase-4 (DPP-4) which leads to elevated levels of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), diminished levels of glucagon, and stronger insulin response to glucose [3,4,5]. Sitagliptin intake with/without food doesn't influence its pharmacokinetics, and its oral bioavailability is 87%.

Different bio-analytic methods have been developed for determining drug concentration in plasma, but methods for sitagliptin determination are still very rare. There are few studies which have developed analytical methods based on RP-HPLC [6,7] and LC-MS/MS principles [8-12] for the determination of sitagliptin in biological fluids. But LC-MS/MS methodological approaches are very expensive and require the availability of expensive equipments. So through this study, RP-HPLC based approach was explored as it is cost effective. As per literature review, two earlier studies have developed an analytical method for sitagliptin determination in human plasma. One study was conducted as per United States-Food and Drug Administration [7] guidelines and the other one has not specified any guidelines. The present study was performed as per International Conference on Harmonisation (ICH) guidelines with newly developed mobile phase by using an advanced RP-HPLC equipment and detector. Earlier studies were validated only with the parameters of selectivity, specificity, recovery, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) and stability (freeze thaw, short/long term) but the present study in addition to these conducted extensive validation parameters like calibration curves, ruggedness, dilution integrity etc. Thus the current work developed and validated a simple, sensitive, accurate, reproducible and cost effective RP-HPLC technique for estimating sitagliptin in human plasma even within a very low detection range [13].

2. MATERIALS

2.1 Chemicals and Reagents

Sitagliptin (Fig. 1a) standard and rosiglitazone (Fig. 1b) IS were received as gift samples from Dr. Reddy's Laboratories, Hyderabad, Telangana and SMS Pharmaceuticals Ltd, Hyderabad, Telangana respectively. Human Blood Unit were procured from Vuppala Venkaiyah Memorial Blood Bank. Acetonitrile and triethanolamine were purchased from Standard Reagents Pvt Ltd, Hyderabad, India and Vestro Solvents Pvt Ltd, Hyderabad, India respectively to prepare mobile phase solution. Acetonitrile was also used as buffer solution. Diethyl ether was purchased from Standard Reagents Pvt Ltd, Hyderabad, India and dichloromethane from Balai Formulations Pvt Ltd. Deionized distilled water used in the mobile phase was purchased from UCC fine Chem, Hyderabad, India.
2.2 Apparatus and Chromatographic Conditions

The symmetry shield RP18 HPLC system (Milford, MA, USA), a separation module of waters 2695 comprised of a quaternary pump, an auto-sampler, a column heater, a degasser and a 2998 PDA (Photo Diode Areditector) detector. Empower 3 software was used for data acquisition and computer processing. Chromatographic separations were performed on HILIC C18 HS column (250mm x 4.6mm x 5μm) (Kromatek, Great Dunmow, England) attached to a guard column Hypersil BDS C-18 (20mm x 4 mm) (Thermo Fisher Scientific India Pvt Ltd, Hyderabad, India). The mobile phase composed of acetonitrile and 0.05% TEA at pH 6.5 (20:80, v/v). Freshly prepared buffer (acetonitrile) solution was utilized for chromatographic purpose. Whatman membrane filters of size 0.45 μm Wipro GE Healthcare, Bangalore, India) were used to filter all solutions. The volume of injection was 8.0 μl maintained with the flow rate 1ml/min at an ambient temperature of the column. For detection purpose, diode array detector at 281 nm was utilized. Before injecting drug standards, equilibration of the column was made for at least 20 minutes with the mobile phase flowing through the system.

3. METHOD VALIDATION

3.1 Preparation of Stock and Working Solutions

Stock solutions of sitagliptin and rosiglitazone were prepared by dissolving 100mg of each drug in 10 mL of acetonitrile and volume was made up to 100mL by adding mobile phase to produce a concentration of 1000 μg/mL for each drug. 10 mL of the prepared solution of each drug was withdrawn and volumes made up to 100 mL to achieve 100 μg/mL of sitagliptin and rosiglitazone. This was considered as a stock solution. From this stock solution, working solutions were prepared with a concentration of 2, 4, 8, 16, 32 and 64 µg/mL. The working solutions were refrigerated at 4°C in a clear glass volumetric flask light protected with aluminium foil.

3.2 Preparation of Sample Solutions for Calibration Curve (CC) and Quality Control (QC)

Blank human plasma (0.95 mL) aliquots were spiked with the 50μL (0.05 mL) aliquots of prepared working solutions (2, 4, 8, 16, 32 and 64 μg/mL) to prepare different calibration standard concentrations like 100, 200, 400, 800,
1600 and 3200 ng/mL (Table 1). Similarly, different quality control samples were also prepared from the working solutions (Table 2). By further diluting stock solution in acetonitrile, fresh working solutions were prepared. Working solutions of rosiglitazone were prepared by diluting the stock solution with acetonitrile at a concentration of 500 ng/ml. Fifty microliters of rosiglitazone were used for every analysis.

3.2 Extraction Procedure

The liquid-liquid extraction method was used to prepare the sample. An aliquot of spiked human plasma (1 mL) was placed in stoppered test tube, then 50 mL of IS working solution (10 mg/mL) was added and vortex-mixed for 1 minute. 5 mL dichloromethane:diethyl ether (4:6, v/v) was added into this solution, and the contents of the tubes were mixed in a reciprocating shaker at 100 strokes per minute for 30 mins and centrifuged at 3000 rpm for 10 minutes to separate the phases. The supernatant organic layer (4 mL) was transferred to a new tube and allowed to evaporate under nitrogen stream. The residue was then reconstituted in 250 mL of mobile phase and analyzed chromatographically.

3.3 Method Validation

Plasma calibration assays were done in 6 runs to test linearity, accuracy, precision, selectivity, dilution integrity, ruggedness, stability studies, recovery study, the limit of detection LOD and LOQ.

4. RESULTS AND DISCUSSION

4.1 Optimization of Chromatographic Conditions

Several combinations of buffer and organic phase were tested for mobile phase. It was found that the combination of acetonitrile–0.05% and TEA (pH 6.5) (ratio 20:80, v/v) provided good peaks for sitagliptin. Fig. 2 shows the representative chromatograms of blank human plasma and plasma samples spiked with sitagliptin or rosiglitazone.

The organic buffer, i.e., acetonitrile, was identified as the most suitable buffer that doesn’t absorb at low wavelength [14]. Acetonitrile was selected as it was adequate to avoid band tailing. At low pH (pH < 2) silica-based particles were unstable [15] while at pH 6.5, best area counts with the least band tailing were observed, and so pH was fixed as 6.5. The run time was short and required just 10 min (retention times for sitagliptin and rosiglitazone were approximately 5.2 min and 6.9 min, respectively). This short analytical time was considered good for plasma samples.

| Working solution utilised (µg/mL) | Volume of aliquot withdrawn (mL) | Volume of human plasma added (mL) | Obtained final concentration (ng/mL) |
|----------------------------------|----------------------------------|----------------------------------|-------------------------------------|
| 2                                | 0.05                             | 0.95                             | 100                                 |
| 4                                | 0.05                             | 0.95                             | 200                                 |
| 8                                | 0.05                             | 0.95                             | 400                                 |
| 16                               | 0.05                             | 0.95                             | 800                                 |
| 32                               | 0.05                             | 0.95                             | 1600                                |
| 64                               | 0.05                             | 0.95                             | 3200                                |

| Working solution utilised (µg/mL) | Volume of aliquot withdrawn (mL) | Volume of human plasma added (mL) | Obtained final concentration (ng/mL) | Level |
|----------------------------------|----------------------------------|----------------------------------|-------------------------------------|-------|
| 2                                | 0.05                             | 0.95                             | 100                                 | LLOQ  |
| 5                                | 0.05                             | 0.95                             | 250                                 | LQC   |
| 20                               | 0.05                             | 0.95                             | 1000                                | MQC   |
| 60                               | 0.05                             | 0.95                             | 3000                                | HQC   |
| 80                               | 0.05                             | 0.95                             | 4000                                | ULOQ  |
4.2 Liquid-Liquid Extraction

In this experiment, the liquid-liquid extraction method was chosen. To develop a single step liquid-liquid extraction procedure with good recovery, a large range of extraction solvents such as dichloromethane, diethyl ether, and a mobile phase (acetonitrile: 0.05% of TEA) were investigated. As per literature [16], extractions of medications from biological fluids are typically the most difficult step in any analysis because of the interference’s presence. The absolute recoveries of sitagliptin after single extraction from plasma using these organic solvents were less than 95% unless diethyl ether used where the absolute recovery was high (over 95% for both sitagliptin and rosiglitazone). Diethyl ether was considered better extracting solvent, most likely because of its moderate polarity, low cost, and volatility [17]. Diethyl ether was consequently selected to further explore the impact of pH on extraction efficiencies. It was found that the plasma pH of 7.4 gave the most noteworthy and best percentage recovery for sitagliptin (96%) and rosiglitazone (97%). This pH was chosen for liquid-liquid extraction method.

4.3 Recovery

Recovery was quantified by finding the ratio of the slopes of the calibration curves for extracted to non-extracted samples. Recovery for sitagliptin in plasma was found to be 96.42%.

4.4 Calibration Curves, Linearity, Accuracy and Precision

The calibration curve for sitagliptin was linear in 100–3200 ng/ml concentration range in human plasma, and linearity is shown in Fig. 3. The inter-and intra-day calibration curves showed consistent linearity, as seen in the consistency of intercept, slope and coefficient of correlation. A typical concentration curve for sitagliptin had a slope of 0.0013, an intercept of 0.0088, and r² = 0.9983. An accuracy and precision assay determined from the low (250 ng/ml), medium (1000 ng/ml) and high (3000 ng/ml) QC plasma samples. The inter-day assay estimated analysis of 6 QC samples and investigated on four different days. Intra-day estimated for each 6 QC sample on a single day. The study of inter- and intra-day precision was showed as percent of the
coefficient of variation (CV) and accuracy showed as the mean percentage of the analyte. According to the ICH [13], the estimated precision should not overtake 20% of the CV at each concentration. The accuracy and precision results of the intra- and inter-days are expressed in Table 3.

4.5 Specificity and Selectivity

In this HPLC study, selectivity was evaluated by analyzing the six different plasma lots and no co-eluting peaks occurred with IS and rosiglitazone. Fig. 4 shows the chromatogram of one of the tested drugs (rosiglitazone) with sitagliptin and the peaks of interests. The results showed no percentage of interferences at analyte retention in presence of rosiglitazone. Therefore, it was concluded that this method is selective and suitable for sitagliptin quantification in human plasma samples.

4.6 Stability

As per ICH guidelines, stability studies were conducted [13]. Three concentrations of sitagliptin in plasma were prepared in six QC samples at low QC (250ng/ml), medium QC [1000ng/ml], and high QC (3000ng/ml). Three different stability studies were carried out, and results are shown in Table 4.

![Fig. 3. Linearity graph of sitagliptin](image)

**Table 3. Accuracy and precision of sitagliptin in human plasma**

| Concentration ng/ml | Mean ng/ml | S. D | Precision (%CV) | Accuracy % | Percentage difference |
|---------------------|------------|------|-----------------|------------|-----------------------|
| **Sitagliptin Intraday (within batch) (n=6)** | | | | | |
| Single day | | | | | |
| 250 | 244.83 | 3.56 | 1.09 | 97.52 | -2.48 |
| 1000 | 934.76 | 4.51 | 2.61 | 93.56 | -6.44 |
| 3000 | 2798.33 | 3.65 | 4.55 | 95.64 | -4.36 |
| **Sitagliptin Interday (between batch) (n=24)** | | | | | |
| Day 1 | | | | | |
| 250 | 245.72 | 3.158 | 1.29 | 98.56 | -1.44 |
| 1000 | 932.6 | 26.53 | 2.85 | 94.59 | -5.41 |
| 3000 | 2834 | 6688 | 2.36 | 95.94 | -4.06 |
| Day 2 | | | | | |
| 250 | 243.89 | 2.95 | 1.80 | 97.65 | -2.35 |
| 1000 | 938.82 | 12.58 | 3.54 | 93.65 | -6.35 |
| 3000 | 2796.56 | 4541 | 2.94 | 95.35 | -4.65 |
| Day 3 | | | | | |
| 250 | 241.80 | 2.65 | 1.76 | 97.75 | -2.25 |
| 1000 | 356.6 | 26.03 | 2.35 | 94.49 | -5.51 |
| 3000 | 2845 | 6645 | 2.36 | 96.94 | -3.06 |
| Day 4 | | | | | |
| 250 | 241.89 | 2.85 | 1.76 | 97.65 | -2.35 |
| 1000 | 978.82 | 12.79 | 3.66 | 93.65 | -6.35 |
| 3000 | 2745.56 | 4591 | 2.24 | 96.39 | -3.61 |
Room temperature stability studies for sitagliptin and IS were performed with medium QC samples for six hours at room temperature. The result indicated the room temperature stability for sitagliptin and IS as 98.06% and 100.79% respectively.

Refrigerator stability studies were performed with medium QC by storing the stock solutions at 2-8°C for four days in refrigerator. The study reported refrigerated stability of sitagliptin and IS as 98.19% and 96.59% respectively.

Table 4. Stability study of sitagliptin and IS

| Run | Room temperature stock solution stability of Sitagliptin and IS | Refrigerator stock solution stability of Sitagliptin and IS | Freeze-Thaw Stability Study of Sitagliptin |
|-----|---------------------------------------------------------------|----------------------------------------------------------|-------------------------------------------|
|     | 6 hours room temperature stock solution at MQC level (Peak area) | Comparison standard solution at MQC level (Peak area) | 250 ng/ml %Accuracy | 3000 ng/ml %Accuracy |
| 1   | 33123                                                  | 34183                                                     | Mean 251.56 101.56 | Mean 2856.22 90.56 |
| 2   | 32145                                                  | 33951                                                     | 248.16 98.65       | 2976.49 98.56       |
| 3   | 33085                                                  | 32224                                                     | 239.59 96.70       | 2902.06 97.34       |
| 4   | 33884                                                  | 33745                                                     | 241.96 89.51       | 2930.31 97.00       |
| 5   | 32873                                                  | 34154                                                     | 246.03 94.22       | 2894.77 96.38       |
| 6   | 33201                                                  | 33823                                                     | 245.26 96.54       | 2961.11 98.53       |
| Mean| 33457                                                  | 33650                                                     | Mean 243.88 2936.45| Mean 2936.45 90.56 |
| SD  | 612.07                                                 | 732.44                                                    | 0.0154 9.69        | 0.467 95.76         |
| %CV | 1.648                                                  | 2.187                                                     | % Nominal 97.44 | % Stability 98.26 97.45|
| % stability | 98.06 | 100.79 |
Freeze-thaw stability studies were performed with low & high QC by storing stock solutions at -70°C in a deep freezer for 24 hours. Low QC was thawed again at room temperature and refreezed. It was determined by four freeze-thaw cycles. The study reported freeze-thaw stability low QC & high QC samples as 98.26% and 97.45% respectively.

In these stability studies, sitagliptin and rosiglitazone did not show any significant degradation (Table 4). When plasma was stored at -70°C, sitagliptin and rosiglitazone peaks didn’t have any interference during storage with the matrix components. These results suggest that under conditions of storage and during injections, sitagliptin was stable in plasma.

4.7 Ruggedness

The ruggedness of the developed method, accuracy and precision were examined with the same make of the different column and with different reagent lots. The ruggedness of analyzed sitagliptin QC samples were accurate and precise as given in Table 5.

![Fig. 4. The chromatogram of rosiglitazone (10 mg/mL) [6.903 min] and sitagliptin (10 mg/mL) [5.232 min]](image)

**Table 5. Ruggedness study of sitagliptin**

| Concentration ng/ml | Mean ng/ml | S. D | Precision (%CV) | Accuracy % | Percentage Difference |
|---------------------|------------|------|-----------------|------------|-----------------------|
| 100                 | 93.08      | 0.0211 | 11.89           | 94.56      | -5.44                 |
| 250                 | 246.34     | 0.0556 | 11.27           | 102.22     | 2.22                  |
| 1000                | 938.82     | 0.1063 | 4.348           | 95.89      | -4.11                 |
| 3000                | 2756.00    | 0.3808 | 7.101           | 95.59      | -4.41                 |

**Chart 1. The developed method’s dilution integrity test**

| Run | Two times dilution | Four times dilution |
|-----|---------------------|---------------------|
|     | 100 ng/ml | % accuracy | 100 ng/ml | % accuracy |
| 1   | 101.23   | 101.23     | 96.12     | 96.12      |
| 2   | 98.56    | 98.56      | 95.68     | 95.68      |
| 3   | 89.56    | 89.56      | 96.44     | 96.44      |
| 4   | 89.44    | 89.44      | 96.92     | 96.92      |
| 5   | 87.65    | 87.65      | 94.88     | 94.88      |
| 6   | 101.24   | 101.24     | 96.20     | 96.20      |
| Mean| 94.22    | 95.95      |           |            |
| SD  | 0.7793   | 0.202      |           |            |
| % CV| 3.179    | 0.84       |           |            |
| % Nominal | 98.04 | 96.08      |           |            |
4.8 Dilution Integrity

The developed method's dilution integrity was tested by using six samples diluted 2 times and another six samples diluted 4 times. The precision and accuracy for sitagliptin at 2-fold dilution were found to be 3.179 and 98.04%, and at 4-fold dilution were 0.84 and 96.08%, respectively.

4.9 LOD and LOQ of Sitagliptin

LOD and LOQ are the lowest analyte concentration at which detection is feasible. LOD and LOQ of sitagliptin were found to be 8.592 ng/ml and 28.641 ng/ml by using signal-to-noise method.

5. CONCLUSION

The newly developed method for sitagliptin was validated using RP-HPLC. Sample preparation and quantification of sitagliptin is simple and convenient for human plasma samples. The validated method has good linearity, precision, accuracy, stability, selectivity, ruggedness, LOD and LOQ over the relevant therapeutic concentration range. Considering these points, the developed method is preferable for clinical monitoring of sitagliptin levels in human plasma and can be applied for pharmacokinetic studies.

DISCLAIMER

The products used for this research are commonly and predominantly used products in the area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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