Development and Validation of New Analytical LC-MS/MS Method for the Estimation of Antidiabetic Drugs Ertugliflozin and Sitagliptin in Combined Pharmaceutical Dosage form

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Authors’ contributions
This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Ertugliflozin and Sitagliptin is combination of Antidiabetic drug in tablet Steglujan 15 mg/100 mg film-coated tablets®, a member Antidiabetic drug, is a recent drug developed by Merck Sharp and Dohme Company for the treatment of Type 2 diabetes. Ertugliflozin and Sitagliptin can be used alone or in combination therapy. A highly sensitive, precise and accurate Liquid Chromatography with mass spectrometry (LC-MS/MS) method is developed and validated for the determination of Ertugliflozin and Sitagliptin in combined formulation. Chromatographic separation was carried out on Phenomenex Gemini, C18, (150 × 4.6 mm,5 μm) column. Isocratic method was based on 0.1% formic acid: acetonitrile (10:90, v/v)as mobile phase, column temperature at 40°C and flow rate at 0.6 mL/minuteswere utilized. The mass spectrometer was operated under multiple reactions monitoring (MRM) mode using electrospray ionization by monitoring the transition pair (precursor to product ion) of m/z 437.10-328.95in the positive mode for Ertugliflozin and transition pair (precursor to product ion) of m/z 408.10-234.95 in the positive mode for Sitagliptin. The method was found...
linear in the concentration range of 15 to 450 ng/mL and 100–3000 ng/mL for Ertugliflozin and Sitagliptin respectively. The optimized method was validated according to the International Conference on Harmonization (ICH) and FDA guidelines. The developed method was found suitable for the quantitation of Ertugliflozin and Sitagliptin in Pharmaceutical dosage form.

Keywords: Ertugliflozin; sitagliptin; LC-MS/MS; validation; assay; mass spectrometry.

1. INTRODUCTION

1.1 Drug Profile

Sitagliptin is an inhibitor of DPP-4, an enzyme that degrades the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Concentrations of the active intact hormones are increased by sitagliptin, thereby increasing and prolonging the action of these hormones. By increasing and prolonging active incretin levels, sitagliptin increases insulin release and decreases glucagon levels in the circulation in a glucose-dependent manner. Sitagliptin phosphate monohydrate is 7-[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8-tetrahydro-[3-(trifluoromethyl)-1,2,4-triazolo[4,3-a]pyrazine phosphate (1:1) monohydrate (Fig. 1) Molecular formula: C_{16}H_{15}F_{6}N_{5}O · H_{3}PO_{4} · H_{2}O molecular mass: 523.32 g/mol, Sitagliptin molecular mass: 407 g/mol.

Ertugliflozin: Ertugliflozin is an oral, selective inhibitor of sodium glucose co-transporter-2 (SGLT2) which inhibits renal glucose reabsorption and results in urinary glucose excretion (UGE) and reductions in plasma glucose and haemoglobin A1c (A1C) in patients with type 2 diabetes mellitus (T2DM).

Ertugliflozin is a new chemical entity with a chemical name of (1S,2S,3S,4R,5S)-5-[4-Chloro-3,4-ethoxybenzyl]phenyl]-1-hydroxymethyl-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol (Fig. 2). Ertugliflozin is included in the drug product as a co crystal with L-pyroglutamic acid (L-PGA), known as Ertugliflozin L-PGA. Molecular formula: C_{27}H_{32}C_{11}N_{10}. Ertugliflozin L-PGA Relative molecular mass: 566.00 g/mol. Ertugliflozin molecular mass: 436.9 g/mol.

Analytical method validation ensures that LC-MS/MS analytical techniques shall give reliable and reproducible results; it is a crucial step in developing new dosage forms as it provides information about accuracy, precision, linearity, detection, and quantitation limits. Robustness. According to the ICH guideline, “the objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose.” It is now mandatory in the process of drug development to supply the validation data to authorities. Guidelines for analysis method validation include ICH and USP guidelines.

Literature survey revealed a few methods reported for determination for Ertugliflozin and Sitagliptin by HPLC and other analytical method but no LC-MS/MS method developed till data in Pharmaceutical preparation [1-4,5-7].
2. MATERIALS AND METHODS

2.1 Instrumentation

Chromatographic Shimadzu LC/MS/MS -8030 system equipped with a Phenomenex Gemini, C18, 150 × 4.6 mm id, 5µm column.

2.2 Chemicals and Reagents

Acetonitrile, Formic acid, Methanol, Water were of HPLC Grade.

2.3 Chromatographic Conditions

Mobile phase comprising of mixture of two solutions i.e. A: 0.1% Formic acid in water & B: Acetonitrile (10:90 v/v) as described above. Mobile phase was delivered at flow rate of 0.6 mL/minutes. The chromatographic injection volume was 5.0 µL. Under these conditions, the retention time of Ertugliflozin and Sitagliptin was about 1.8 and 3.2. Run time 6.0 min. Column maintained at 40°C and Auto sampler temperature at 15°C using the Phenomenex Gemini, C18, 150 × 4.6 mm id, 5µm.

Tuning Parameter of MS/MS for drug

Model: - Shimadzu LC/MS/MS -8030
Operating Software: Lab Solution 5.53 SP3C
System Controller: Shimadzu CBM-20A, SPD-M20A

2.4 Preparations

2.4.1 Formic acid in water, 0.1% v/v

Pipette out 0.100 mL of formic acid in to a measuring cylinder containing 100 mL of Methanol. Mixed the contents thoroughly and transferred into a reagent bottle. Stored at ambient temperature. This solution was used within 3 days from the date of Preparation.

2.4.2 Mobile Phase (Formic acid in water, 0.1% v/v: Acetonitrile 10: 90% v/v)

In measuring cylinder 900 mL of Acetonitrile and 100 mL of formic acid in water, 0.1% v/v was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

2.4.3 Auto sampler rinsing solution

In measuring cylinder 500 mL of Methanol and 500 mL of water was taken, then transferred into a reagent bottle and mixed the contents thoroughly. Stored at ambient temperature. This solution was used within 3 days from the date of preparation.

2.4.4 Drug 1 (Ertugliflozin) stock solution, 1ng/mL

Ertugliflozin standard was weighed accurately equivalent to 10 mg of Ertugliflozin and appropriate volume of Methanol was added to make final concentration of Ertugliflozin equivalent to 1 ng/mL accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5±3°C. Use the solution within 7 days from date of preparation.

2.4.5 Drug 2 (Sitagliptin) stock solutions, 1 ng/mL

Sitagliptin was weighed accurately equivalent to 10 mg of Sitagliptin and appropriate volume of Methanol was added to make final concentration of Sitagliptin equivalent to 1 ng/mL accounting for its potency and the actual amount weighed. Solution was stored in refrigerator at 5±3°C. Use the solution within 7 days from date of preparation.

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization

Several physical and chemical properties of Ertugliflozin and Sitagliptin were available from the literature. The analytical method was developed to select preliminary reversed phase LC-MS/MS method chromatographic conditions, including MS Spectra, mobile phase, stationary phase, and sample preparation procedure. For this purpose, a series of trials were performed by varying the ratio of include trials.

Optimizing the chromatographic conditions on the Phenomenex Gemini, C18, 150 × 4.6 mm, 5µ column, the results of method optimization are summarized in Table 1 Mobile phase comprising of mixture of two solutions i.e. A: 0.1% Formic acid in water & B: Acetonitrile (10:90 v/v) as described above. Mobile
phase was delivered at flow rate of 0.6 mL/minutes. The chromatographic injection volumewas 5.0 μL. Under these conditions, the retention time of Ertugliflozin and Sitagliptin was about 1.8 and 3.2. Run time 6.0 min. Column maintained at 40°C and Auto sampler temperature at 15° Cusing the Phenomenex Gemini, C18, 150 × 4.6 mm id, 5μm retention time about 3.2 min for Ertugliflozin (ERTU) and 1.8 min for Sitagliptin (SITA) (Figs. 3-6). The chromatograms were acquired by using Lab Solution Software 5.60 SP2D Supplied by Shimadzu.

### 3.2 Method Validation

The method was validated as per ICH and FDA guidelines, and the validation parameters included specificity, linearity, range, accuracy and precision, sensitivity (LOQ and LOD) and robustness [8-10].

#### 3.2.1 Specificity

Specificity of the method was evaluated by injecting 5.0 μL solutions of standard, sample, blank, and placebo separately.

#### Table 1. LC parameter

| Parameters          | Ertugliflozin | Sitagliptin |
|---------------------|--------------|-------------|
| Ion Source          | Electro Spray ionization |              |
| Polarity            | Positive     |             |
| Parent Ion          | 437.10       | 408.10      |
| Daughter Ion        | 328.95       | 234.95      |
| Dwell Time(msec)    | 50           | 50          |
| Collision Energy (CE) | -10         | -20         |

Source Dependent Parameters

| Parameters          | Used |
|---------------------|------|
| DL Temperature      | 300 °C |
| Nebulizing Gas Flow | 3.0 L/min |
| Heat Block Temperature | 500 °C |
| Drying Gas Flow     | 15.0 L/min |

#### MS Spectra

![MS Spectra Ertugliflozin - Parent Ion](Ertugliflozin MS Spectra.png)

![Ertugliflozin chromatogram](Ertugliflozin Chromatogram.png)

![MS Spectra Sitagliptin- Parent Ion](Sitagliptin MS Spectra.png)

![Sitagliptin chromatogram](Sitagliptin Chromatogram.png)
3.2.2 Linearity

To evaluate the linearity and range of the method, direct standard solutions were prepared by diluting the standard stock solution with the diluent in different concentrations of Ertugliflozin: 15-450 ng/ml, and Sitagliptin 100-3000 ng/ml. From the regression analysis, a linear equation was obtained and the goodness-of-fit (r²) was found to be 0.99 which cover the target concentration, respectively. Three replicate injections from each concentration were analysed under the same conditions. Linear regression analysis was used to evaluate the linearity of the calibration curve by using the least square linear regression method.

3.2.3 Sensitivity

Limit of detection (LOD)/limit of quantitation (LOQ) of Ertugliflozin and Sitagliptin were determined by measuring the signal-to-noise ratio. limit of detection (LOD) is the concentration that gives a signal-to-noise ratio of approximately 3:1, while the limit of quantification (LOQ) is the concentration that gives a signal-to-noise ratio of approximately 10:1 with %RSD (n = 3) of less than 10%. The LOD’s were 568.50 ng/mL and 3796.35 ng/mL for Ertugliflozin and Sitagliptin respectively. The LOQ’s were 5685.24 ng/mL and 37963.54 ng/mL for Ertugliflozin and Sitagliptin respectively.

3.2.4 Accuracy

The accuracy of the assay method was determined by recovery studies at three concentration levels (80%, 100%, and 120%) each concentration were injected. percentage recovery of Ertugliflozin and Sitagliptin added and RSD were calculated for each of the three replicate samples.

3.2.5 Precision

System precision was established by six measurements of the standard solution at the 100% concentration levels on the same day. Method precision was established by six assay determinations of the sample solution at the 100% concentration levels on the same day [11-14]. The RSD of obtained results was calculated to evaluate repeatability results.

3.2.6 Robustness

Robustness of the method was verified by applying minor and deliberate changes in the experimental parameters. Obtained data for each case was evaluated by calculating % RSD and percent of recovery.

3.3 Method Validation

3.3.1 Specificity

Specificity was evaluated by comparing the chromatograms of Diluent, standard solution, and sample solution (Ertugliflozin and Sitagliptin). the chromatogram results are shown in Figures 7–11. It can be observed that there no coeluting peaks at the retention time of Ertugliflozin and Sitagliptin interference. This result indicates that the peak of the analyte was pure and this confirmed the Specificity of the method.

3.3.2 Linearity and range

Analytical method linearity is defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a specific range. The mean peak area obtained from the HPLC was plotted against corresponding concentrations to obtain the calibration graph. The results of linearity study (Figs. 12 and 13) gave linear relationship over the concentration range of Ertugliflozin: 15-450 ng/ml, and Sitagliptin 100-3000 ng/ml. From the regression analysis, a linear equation was obtained and the goodness-of-fit (r²) was found to be 0.99, indicating a linear relationship between the concentration of analyte and area under the peak.
Table 2. Linearity

| Level | Ertugliflozin | | | |  | Sitagliptin | | | |
|-------|---------------|---|---|---|---|---|---|---|---|
|       | Conc (ng/ml)  | Area | Conc (ng/ml) | Area | | Correlation | | Slope | Intercept |
| 1     | 15            | 76  | 100          | 22653 | | 0.999823113 | | 13.38894506 | 148.9539939 |
| 2     | 30            | 234 | 200          | 44612 | | 0.998193 | | 250.5908 | -14242.7 |
| 3     | 75            | 796 | 500          | 112072 | | | | | |
| 4     | 150           | 1929 | 1000 | 216777 | | | | | |
| 5     | 300           | 3859 | 2000 | 464744 | | | | | |
| 6     | 450           | 5869 | 3000 | 757703 | | | | | |

Fig. 9. Standard SITA

Fig. 10. Mixture Solution ERTU Peak

Fig. 11. Mixture solution SITA peak

Fig. 12. Linearity of Ertugliflozin

Fig. 13. Linearity of Sitagliptin
3.3.3 Limit of detection and limit of quantification (LOD and LOQ)

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, while the limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision.

3.3.4 Accuracy

Accuracy describes the closeness of mean results obtained to the true value of the concentration while precision describes the closeness of individual measures when the procedure is applied repeatedly. The laboratory-prepared mixtures prepared under section 'Method validation and application to Steglujan® tablets were analysed. Accuracy was checked by percent recoveries of the samples. The mean of the percent recoveries was found to be 99 - 102 % and 99 - 102 % for ERTU and SITA, respectively. Table 3. The results of percentage recovery and %RSD were within the accepted limits from 98.0% to 102.0% and not more than 2.0%, respectively, which indicates the applicability of the method for routine drug analysis.

Table 3. Accuracy data

| Sample Name (Level) | ID#1 Compound Name: ERTU m/z: 437.10>328.95 | ID#2 Compound Name: SITA m/z: 408.10>234.95 |
|---------------------|---------------------------------------------|---------------------------------------------|
|                     | Area | Mean Area | % Recovery | Area | Mean Area | % Recovery |
| 80%                 | 3300 | 3264      | 102        | 323065 | 325439 | 100        |
|                     | 3258 | 101       |            | 324689 | 328562 | 102        |
|                     | 3235 | 100       |            | 328562 | 328562 | 100        |
| 100%                | 3999 | 4047      | 99         | 398962 | 402199 | 99         |
|                     | 4068 | 101       |            | 405500 | 405500 | 101        |
|                     | 4073 | 101       |            | 402136 | 402136 | 100        |
| 120%                | 4785 | 4853      | 99         | 485658 | 483993 | 101        |
|                     | 4856 | 100       |            | 479963 | 479963 | 101        |
|                     | 4918 | 102       |            | 486285 | 486358 | 101        |

Table 4. Precision data

| Sample Name | Drug Area | Mean Area | Drug Area | Mean Area |
|-------------|-----------|-----------|-----------|-----------|
| STD-1       | 4090      | 4016      | 405722    | 409307    |
| STD-2       | 4154      | 4051      | 402827    | 409307    |
| STD-3       | 4026      | 4001      | 405678    | 403109    |
| STD-4       | 4167      | 4048      | 400390    | 409507    |
| STD-5       | 4061      | 4167      | 401426    | 413553    |
| STD-6       | 4109      | 3983      | 407412    | 402202    |
| Acceptance Criteria – % | SD | 28.00271151 | SD | 3267.179719 |
| RSD NMT 2.0 | % RSD | 0.64 | % RSD | 0.81 |
| Table 5. Variation in column temperature 35°C |
|---------------------------------------------|
| **CT-35°C** | Ertugliflozin | Sitagliptin |
| Assay | Area | Area |
| Sample 1 | 4140 | 425255 |
| Sample 2 | 4168 | 425214 |
| Sample 3 | 4194 | 425210 |
| Mean | 4167.333 | 425226.3 |
| SD | 27.00617 | 24.90649 |
| % RSD | 0.65 | 0.01 |

| Table 6. Variation in column temperature 45°C |
|---------------------------------------------|
| **CT-45°C** | Ertugliflozin | Sitagliptin |
| Assay | Area | Area |
| Sample 1 | 4150 | 420054 |
| Sample 2 | 4204 | 414407 |
| Sample 3 | 4130 | 421647 |
| Mean | 4161.333 | 418702.7 |
| SD | 38.27967 | 3804.468 |
| % RSD | 0.92 | 0.91 |

| Table 7. Variation in flow rate 0.5 ml/Min |
|-------------------------------------------|
| **0.5ml/min** | Ertugliflozin | Sitagliptin |
| Assay | Area | Area |
| Sample 1 | 5084 | 503125 |
| Sample 2 | 4938 | 508619 |
| Sample 3 | 4982 | 509203 |
| Mean | 5001.333 | 506982.3 |
| SD | 74.89548 | 3353.286 |
| % RSD | 1.50 | 0.66 |

| Table 8. Variation in flow rate 0.7 ml/Min |
|-------------------------------------------|
| **0.7 ml/min** | Ertugliflozin | Sitagliptin |
| Assay | Area | Area |
| Sample 1 | 3196 | 347440 |
| Sample 2 | 3163 | 349970 |
| Sample 3 | 3164 | 347718 |
| Mean | 3174.333 | 348376 |
| SD | 18.77054 | 1387.425 |
| % RSD | 0.59 | 0.40 |

| Table 9. Variation in mobile phase ratio (88:12) |
|-----------------------------------------------|
| **Mobile Phase (88:12)** | Ertugliflozin | Sitagliptin |
| Assay | Area | Area |
| Sample 1 | 4226 | 333183 |
| Sample 2 | 4231 | 336716 |
| Sample 3 | 4216 | 337104 |
| Mean | 4224.333 | 335667.7 |
| SD | 7.637626 | 2160.512 |
| % RSD | 0.18 | 0.64 |
Table 10. Variation in mobile phase ratio (92:8)

| Mobile Phase (92:8) | Ertugliflozin | Sitagliptin |
|---------------------|---------------|-------------|
| ASSAY               | Area          | Area        |
| Sample 1            | 5094          | 594208      |
| Sample 2            | 5083          | 598190      |
| Sample 3            | 5024          | 596317      |
| Mean                | 5067          | 596238.3    |
| SD                  | 37.64306      | 1992.165    |
| % RSD               | 0.74          | 0.33        |

Table 11. Assay

| Sample name                  | Drug m/z                  | Ret. Time (Min) | Drug Area |
|------------------------------|---------------------------|-----------------|-----------|
| Steglujan® tablets 15 mg/100 mg | ERTU m/z: 437.10>328.95 | 3.198           | 4557      |
|                              | SITA m/z: 408.10>234.95  | 1.598           | 437345    |

Table 12. Summary parameter

| Sr.No | Parameter         | Result                                      |
|-------|-------------------|---------------------------------------------|
| 1     | Specificity       | No Inference observed                       |
| 2     | Linearity and Range | Linear Response                             |
| 3     | Limit of Detection (LOD) | Ertugliflozin: 51.10 ng/mL | Sitagliptin: –66.79 ng/mL |
| 4     | Limit of Quantification (LOQ) | Ertugliflozin: 154.86 ng/mL | Sitagliptin: 202.40 ng/mL |
| 5     | Accuracy          | Ertugliflozin: 99.0 % to 102.0 %           |
| 6     | Precision         | % RSD is within limit of NMT 2.0 %          |
| 7     | Robustness        | % RSD is within limit of NMT 2.0 %          |

3.3.5 Precision

The precision of the method is “the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions,” and it is normally expressed as the relative standard deviation [11-15]. The results of precision showed that the method is precise within the acceptable limits. The RSD were calculated for solutions; all the results are within limits. Acceptable precision was not more than 2.0% for the RSD as shown in Table 4.

3.3.6 Robustness

The analytical method robustness was tested by evaluating the influence of minor modifications in LC conditions on system suitability parameters of the proposed method, as mentioned in Section 2.6.6. The results of robustness testing showed that a minor change of method conditions, such as the variation of the temperature, flow rate and mobile phase ratio, is robust within the acceptable limits [16-23]. The results are summarized in Tables 5-10. In all modifications, good separation of Ertugliflozin and Sitagliptin was achieved, and it was observed that the percent of recovery was within acceptable limits and the %RSD is within limit of not more than 2.0%. Acceptable limits as well. The results are shown in Tables 5-10.

4. CONCLUSION

The proposed method proved to be sensitive, accurate and reproducible as the first LC–MS/MS method for determination of Ertugliflozin and Sitagliptin. The validated LC–MS/MS method was applied successfully on the pharmaceutical dosage form. The developed method should be of interest to the analysts in the area of drug control and can be conveniently used by quality control laboratories for the recently approved Steglujan® tablets 15 mg/100 mg. All the objectives of the study were met including fast, simple, accurate and precise determination of the drugs under investigation. Development and validation of a new LC–MS/MS method increases sensitivity with further possible applications and enhances the diversity of the available analytical
procedures for the drugs giving the analyst in quality control laboratories the chance to select the most appropriate method according to the underlying investigation. Furthermore, mass detection enhances the selectivity of detection especially when using MRM mode that may be further the method is suitable for further application of into pharmaceutical dosage form and can be applied on pharmacokinetic studies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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