# LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY ASSAY METHOD FOR ESTIMATION OF SAXAGLIPTINE AND DAPAGLIFLOZIN IN HUMAN PLASMA

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## Abstract

There has been much research on both saxagliptin and dapagliflozin. However, only a few HPLC and LC-MS methods that use stable labeled isotopes to measure the amount of a drug alone or in combination with other drugs and have a longer chromatographic run time have been published. A quick and sensitive LC-MS/MS mass spectrometric test method has been made and fully validated to find dapagliflozin and saxagliptin in human plasma simultaneously. Solid-phase extraction on the Cleanert PEP-H extraction cartridge was used to separate the analyte and I.S. from the human plasma. The extracted samples were separated on an Ace Phenyl (150 X 4.6 mm, 5 m) column at a set flow rate of 0.8 mL/min using an isocratic mobile phase of acetonitrile and 5 mM ammonium acetate buffer (70:30 v/v). For dapagliflozin, the calibration curve was linear throughout a range of 0.502-227 ng/mL, while for saxagliptin, the range was 0.103-76.402 ng/mL. The procedure was validated in accordance with standards set by the U.S. Food and Drug Administration, and the outcomes are acceptable.

**Keywords:** saxagliptin and dapagliflozin, Solid-phase extraction, mobile phase.

## 1. INTRODUCTION

Reduced insulin synthesis and secretion due to pancreatic beta-cell malfunction characterize the chronic metabolic disorder known as diabetes mellitus type II. Because most people aren't moving about as much and getting heavier, this sickness is spreading quickly. [1] A novel reversible SGLT2 inhibitor called dapagliflozin helps maintain blood glucose levels by lowering glucose reabsorption and increasing glucose excretion through the kidneys. Forxiga, a new SGLT2 inhibitor, has won approval all around the world. [2] A novel class of DPP-4 inhibitor, saxagliptin, enhances glycemic management by preventing the inactivation of the incretin hormone GLP-1. This decreases postprandial glucagon and glucose levels and increases glucagon-like peptide-1, according to the third factor [3].

Because of the high risk of hypoglycemia and its associated cardiovascular complications, many medicines are often required to establish glycemic control. [4] It makes more sense to combine SGLT2 and DPP-4 inhibitors to treat hyperglycemia rather than using either alone. Intern, which consists of saxagliptine and dapagliflozin, is a combination medication for treating type 2 diabetes that has been approved by regulatory organizations. [5] Many HPLC [9-13] and a handful of LC-MS [6-8] techniques have been revealed for determining both medications separately and in combination. Phanindra [6] et al. and Shruti [7] et al. did not use deuterated substances as an internal standard in their published procedures. Swapna et al. reported a lengthy and laborious process for their liquid-liquid extraction approach. This paper reports the determination of Saxagliptine and dapagliflozin in human plasma that has been artificially spiked as part of our continuing research. To overcome the challenges encountered during this study, we developed a very sensitive LC-MS/MS capable of measuring both analytes at LLOQs of 0.50

and 0.10 ng/ml for Saxagliptine and dapagliflozin, respectively. The developed method was fast, taking only 2 minutes to complete a chromatographic run. Utilizing deuterated chemicals as I.S., the given method is superior to the approaches reported by Phanindra and Shruti. This paper details the use of SPE to isolate analyte and I.S. from human plasma that has been artificially spiked.

## 2. Experimental details:

### 2.1. Reagents and Chemicals

Reference samples of dapagliflozin (98.47%), dapagliflozin-d5 HCl (99.04%), saxagliptine HCl (97.85%), and saxagliptine-d2 HCl (99.14%) were supplied by Seldom Pharma Pvt Ltd (India, Yanam). Milli Q, a water purification system manufactured by Millipore, disinfected the water for the LC-MS/MS analysis (Bangalore, India). J.T. Baker supplied HPLC-quality acetonitrile, dimethyl sulfoxide, and methanol (Phillipsburg, USA). Merck provided analytical quality ammonium acetate and acetic acid (Mumbai, India). The human plasma control K2-EDTA sample was supplied by Deccan Pathological Lab (Hyderabad, India).

### 2.2. Instrumentation and conditions for chromatography

A Nexera XR UHPLC system was controlled by Analyst 1.6 and interfaced with a Shimadzu Corporation LCMS-8060 system in Kyoto, Japan. Workstations equipped with an Ace Phenyl (150 X 4.6mm, 5m) column were used for the analysis. The processed samples, totaling just 2 L, were put into a 40°C column. At a flow rate of 0.8 mL/min, the isocratic mobile phase was introduced into the electrospray ionization chamber of the mass spectrometer. This mobile phase is composed of acetonitrile and 5mM ammonium acetate in a (70:30, v/v) ratio. MS/MS detection in positive ion mode was used to determine the concentrations of the analytes and the internal standard using a Shimadzu LCMS fitted with a Turboionspray TM interface, with the temperature set to 550oC and the ion spray voltage set to 5500V. The flow rates for the nebulizing gas, auxiliary gas, curtain gas, and Collison gas were 45 liters per minute, 40 liters per minute, 40 liters per minute, and 7 litres per minute, respectively. Declustering potential, entry potential, and collision energy (C.E.) for dapagliflozin were 30, 25, 10 V; for dapagliflozin-d5, they were 24, 20, 14 V; for saxagliptine, they were 40, 25, 10 V; and for saxagliptine-d2, they were 35, 21, 14 V. By keeping an eye on the m/z 409 transition pairs, the ions were discovered in multiple-reaction monitoring mode (MRM). 14 precursor ion to m/z 135 for dapagliflozin, m/z 412.43 precursor ion to m/z 135 for dapagliflozin-d5, m/z 316. 20 precursor ion to m/z 180.13 product ion for saxagliptine and m/z 318.10 for saxagliptine- d2, Q1, and Q3 quadra. The analytical information was analyzed using the software Analyst 1.6.

### 2.3. Standard solution preparation:

The different weighing methods created primary stock solutions of dapagliflozin and saxagliptine, and then standard calibration curves and Q.C. samples were generated from these solutions. Dapagliflozin and saxagliptine 1000 g/mL stock solutions were prepared in methanol and shown to be stable for ten days when kept at 2-8 degrees Celsius. Standard dapagliflozin and saxagliptine were prepared by properly diluting their respective stock solutions with a diluent solution of methanol and water (60:40, v/v). Both dapagliflozin-d5 and saxagliptine-d2 were prepared as a 1000 g/mL primary stock solution in methanol. Using the diluent (methanol and water, 60:40, v/v), a solution of 0.55 g/mL of mixed internal standards was prepared.

### 2.4. Preparation of CC standards and Q.C. samples:

Calibration samples were mixed with 25 ul of dapagliflozin and 25 ul of saxagliptine with 950 ul of human control plasma. Standards for calibration curves were established, with dapagliflozin values spanning 0.502-227 ng/mL and saxagliptine values spanning 0.103-76.402 ng/mL.

Accuracy and precision were evaluated by spiking control K2-EDTA human plasma with known doses of dapagliflozin and saxagliptine and then dividing 300 L aliquots of the plasma samples among multiple test tubes. Dapagliflozin has an LLOQ of 0.501, an LQC of 1.51, an MQC1 of 37.750, an MQC2 of 151.00, an HQC of 961.354, and a DIQC of 227.07; saxagliptine has an LLOQ of 0.103, an LQC of 0.612, an MQC1 of 15.288, an MQC2 of 50.960, and (DIQC). All samples were stored at a temperature of -705 °C until further usage.

2.5. Sample preparation:

A 20 L aliquot of a 0.50 g/mL dapagliflozin-d5 and Saxagliptine-d2 working solution was added to a 200 L aliquot of human control plasma. To this, 200 l of formic acid at 0.2% concentration was added to each sample, and the vials were vortexed to ensure the complete mixing of the contents. We used a Cleanert PEP-H extraction cartridge that had been pre-washed in 1.0 mL methanol and 1.0 mL water, and then we placed the material onto it. The extraction cartridge was given a 1.0 mL water rinse before being subjected to a 1.0 mL treatment with a 5% methanol in water solution. Under positive pressure, let the area dry for about a minute. Autosampler vials were used to store the eluted medicines after they had been washed with 1 ml of mobile phase solution and given a vortex. The LC-MS/MS machine was fed a 2 L injection of the solution.

2.6. Validation of developed method:

Extensive validation was conducted under the criteria of the US FDA [28]. In addition, six human plasma matrix samples were evaluated to check for selectivity.

Retention time interferences for dapagliflozin and saxagliptine are permitted if they contribute less than 20% of the response at the LLOQ. In general, as long as it accounts for less than twenty percent of the response at the LLOQ or lowest standard curve point, background noise at the retention time of dapagliflozin and Saxagliptine is acceptable. Examining the matrix effect necessitates tests at two concentrations (LQC and HQC) for each analyte and internal standard. Six normal plasmas and one each of lipemic, low, and high hemolytic plasmas were treated as blank matrices in two sets of nine. One set of blank matrices with a spiked LQC and internal standard, and another set of blank matrices with a spiked HQC and internal standard, were examined with a reconstitution solution. The IS-normalized MF for each injection for two levels was determined using the following formula; samples were prepared by mixing analyte and internal standard with the diluting solution and injecting in 6 duplicates each to match LQC and HQC concentrations.

$$\text{I.S. normalized M.F.} = \frac{\text{M.F. of Analyte}}{\text{M.F. of ISTD}}$$

The I.S. normalized matrix factor was determined using the %CV and found to be less than 15%, which is within the allowance for both the LQC and HQC levels. The linearity of dapagliflozin and saxagliptine was evaluated at doses ranging from 0.502-227 and 0.103-76.402 ng/mL, respectively. Standard calibration curves were plotted with a minimum of eight points and then confirmed to establish linearity. However, while the evaluation of blank plasma samples confirmed the absence of direct interferences, these data could not be used to generate calibration curves. The precision limit for LLOQ was set at 20%, whereas the precision limit for each back-calculated concentration was 15%. At least 67% of the time, the calibration curve will be accepted if the LLOQ and ULOQ are within the acceptance range. Five independent investigations were performed on each calibration standard. All of the samples were processed from least concentrated to most concentrated. To evaluate the reliability and accuracy of intra-day assays, six duplicates were analyzed at five Q.C. levels throughout two runs on the same day. The precision within 15% RSD and accuracy within 15% S.D. from nominal values were also required as acceptance requirements, except for LLOQ QC, which required a 20% departure from nominal values. However, batch acceptance criteria need 67% of all quality control samples and 50% of each stage to be acceptable before moving on to the next.

Spiked plasma samples (six each of low, moderate, and high Q.C.s) and samples produced by spiking the extracted drug-free plasma samples with the same concentrations of the analytes just before chromatography were compared for their peak areas of the analytes. Similar to the determination of extraction efficiency, internal standard recovery was calculated by comparing the mean peak areas of the six extracted Q.C. samples to those of the internal standards in samples prepared by spiked the six extracted drug-free plasma samples with the exact amounts of internal standards just before chromatography.

The dilution integrity test is performed during real-time analysis of samples to verify the results of the dilution test performed on higher analyte concentrations than the ULOQ. Three times the ULOQ concentration of each analyte was used in a dilution integrity experiment. Three different CC8 concentrations were used to assess the dilution integrity of both dapagliflozin (961.35 ng/mL) and saxagliptine (346.402 ng/mL). There was a 1:4 dilution of the materials before analysis, and the dilution factor was 5.

Analyte stability in stock solutions and plasma samples was evaluated by conducting stability tests under various conditions. The area response of the analytes (stability samples) was compared to the response of a fresh stock solution sample to establish the stability of the stock solution at room temperature after 12 hours. Six replicates were examined at the LQC and HQC levels for benchtop stability (16 h), processed sample stability (autosampler stability for 51 h, wet extract stability for 45 h, and reinjection stability for 24 h), freeze-thaw stability (five cycles), and long-term stability (30 days). The sample was considered stable if the assay values fell within the allowable ranges for accuracy (15 percent S.D.) and precision (15 percent RSD).

## 3. Results

### 3.1. Mass spectrometry

Analyte mass parameters were established for both positive and negative ionization modalities. A satisfactory response was achieved in the positive ionization mode. In addition, evidence from the MRM mode was considered to enhance selectivity. The protonated forms of the analyte and internal standard ions were used as the parent ions in the Q1 spectra, which were then used to generate the Q3 product ion spectra. From m/z 409.14 to 135, m/z 412.43 to 135, m/z 316.2 to 180.13, and m/z 318.10 to 180.13 were the most sensitive mass transitions recorded for dapagliflozin, dapagliflozin-d5, saxagliptine, and saxagliptine-d2, respectively.

### 3.2. Method development

Several iterations were performed to optimize the chromatographic parameters, particularly the mobile phase composition, to attain high resolution, symmetric peak morphologies for the analytes, and rapid run times. Columns from Chromolith, Hypersil, Ace Phenyl, Zorbax, Kromasil, and Intersil were used in conjunction with acetonitrile and buffer solutions of varying concentrations to try separation. As a result, the mobile phase consisted of acetonitrile and 0.1% ammonia in 5mM ammonium acetate buffer (70:30, v/v). Peak shape and response for both analytes and internal standards were satisfactory at LLOQ using ace phenyl(150 x 4.6mm, 5um). Furthermore, it only took 3.0 minutes to complete the run since the retention durations of dapagliflozin and saxagliptine were so low.

In this investigation, excellent drug recoveries were achieved using a straightforward solid-phase extraction (SPE) method for sample preparation. It is advised that stable labeled isotopes of the analyte be used as internal standards in bioanalytical research to increase assay precision and decrease variable recovery between the analyte and the internal standard. Stable isotope-labeled medicines are used as an internal standard in LC-MS/MS analyses where a significant matrix effect is possible. Dapagliflozin-d5 and saxagliptine-d2 stable labeled isotopes were determined to be perfect for the current purpose after several compounds were investigated in the early phases of this work to locate an excellent internal standard.

### 3.3. Selectivity and Specificity chromatography

The interference of endogenous plasma constituents with the analytes and I.S. was evaluated by visual inspection of chromatograms generated from processed blank plasma samples. As shown in Fig. 2, there was no apparent direct interference from endogenous chemicals in drug-free plasma in the blank plasma traces during the retention time of the analytes and I.S.

No interference was noticed at the retention time of dapagliflozin, saxagliptin, saxagliptin-d2, or any other drug when a working concentration of dapagliflozin-d5 was provided. For example, when injecting the active concentration of saxagliptin-d2, no interference was seen at the retention time of dapagliflozin, dapagliflozin-d5, and saxagliptin (see fig. 3).

### 3.4. Sensitivity

For the analytes, the concentration of the LLOQ was selected as the lowest limit of quantifiable interest. At the LLOQ concentration, the accuracy and precision for dapagliflozin and saxagliptine, respectively, were 4.51% and 93.38%.

### 3.5. Matrix effect

No significant matrix effect was seen for dapagliflozin or saxagliptin in eight batches, including lipemic or Haemolysed plasma, at low or high concentrations (LQC or HQC). I.S. normalized factor was 0.99 for LQC and 1.00 for HQC, while the precision for I.S. normalized matrix factor was 3.21% and 1.88%, respectively, for dapagliflozin.

At both the LQC and HQC levels, the I.S. normalized matrix factor for saxagliptine was 1.00, with 2.95 and 0.57 percent precision, respectively.

### 3.6. Linearity

Dapagliflozin's eight-point calibration curve was linear over a concentration range of 0.502-227 ng/mL, and saxagliptin's was linear over a concentration range of 0.103-76.402 ng/mL. Comparisons of the two weighting models revealed that the concentration-detector response relationship in human plasma for both analytes was best fit by a regression equation with a weighting factor of 1/x2 of the drug to the I.S. concentration. Validation yields weighted calibration curves with an average correlation coefficient of 0.99.

### 3.7. Precision and accuracy

Accuracy and precision data for dapagliflozin and saxagliptin plasma samples taken at various times of the day are displayed in Table 1. Both assay results were within the allowable range of variation (intra- and inter-day).

### 3.8. Extraction efficiency

A simple solid-phase extraction using Cleanert PEP-H extraction cartridges showed the most resilient and gave the cleanest samples. The analyte and internal standard recoveries were good and reproducible. The mean overall recoveries for dapagliflozin, saxagliptin, dapagliflozin-d5, and saxagliptin-d2 were 99.46%, 89.65%, 91.23%, and 90.61%, respectively.

### 3.9. Dilution integrity

Three ULOQ concentrations were used to test the dilution integrity (606.582 ng/mL for dapagliflozin and 609.342 ng/mL for saxagliptin). Before analysis, the samples were diluted by a factor of 5, from 1:4 to 1:4.

The requirements for dapagliflozin were an accuracy of 87% and a CV of 2.04% for 1:4 dilutions.

For saxagliptin, the requirements for % accuracy and % CV for 1:4 dilutions were determined to be 103% and 3.25 %, respectively, which are acceptable.

### 3.10. Run Size Evaluation

There were 155 different samples of run sizes established. Using a calibration curve, we determined that our analysis of the 36 LQC, MQC1, MQC2, and HQC samples was within the allowable range.

### 3.11. Stability studies

Benchtop stability (16 h), autosampler stability (51 h), freeze-thaw stability (5 cycles h), reinjection stability (24 h), wet extract stability (45 h at room temperature), whole blood stability (30 min, 60 min), and long-term stability at -70 degrees Celsius all showed mean percent nominal values of the analytes within 15% of their predicted concentrations for the analytes at their LQC and HQC l. (Table 2). Thus, the validation procedure yielded results that were within the acceptable range.

## 4. Conclusions

This study describes a rapid, simple, specific, and sensitive LC-MS/MS assay that has been extensively validated according to FDA criteria for the simultaneous measurement of dapagliflozin and saxagliptin in human plasma. Due to its low cost, ease of use, and reliance on solid-phase extraction, this method is attractive for high-throughput bioanalysis of dapagliflozin and saxagliptin because of its rapid sample turnover (less than 3.0 minutes per sample). Based on the totality of the validation parameters' results, we can infer that the developed method can be applied to BA/BE studies and routine therapeutic medication monitoring with the necessary rigor and accuracy.

Table 1 Precision and accuracy of the method for determining dapagliflozin and saxagliptine in plasma samples.

| Analyte | Concentration added (ng/mL) | Intra-batch precision and accuracy range (4 from each batch) | | Inter-day precision and accuracy (*n*=24; 4 from each batch) | |
|---|---|---|---|---|---|
| | | Precision range (%) | Accuracy range (%) | Precision (%) | Accuracy (%) |
| Dapagliflozin | 0.502 | 5.08-17.59 | 93.38-105.23 | 11.57 | 100.71 |
| | 1.51 | 1.16-9.03 | 95.99-101.65 | 5.79 | 102.49 |
| | 37.75 | 0.47-1.68 | 105.47-106.64 | 2.67 | 106.69 |
| | 151 | 0.21-2.12 | 102.64-109.58 | 3.51 | 105.94 |
| | 227 | 0.50-1.54 | 91.48-92.29 | 5.09 | 98.24 |
| Saxagliptine | 0.103 | 4.64-8.01 | 85.33-109.11 | 7.75 | 97.04 |
| | 0.612 | 1.91-3.57 | 98.51-108.55 | 2.63 | 96.54 |
| | 15.288 | 0.77-2.58 | 101.58-106.56 | 2.13 | 104.58 |
| | 50.960 | 0.47-2.25 | 101.95-108 | 2.70 | 103.79 |
| | 76.402 | 0.63-5.18 | 102.53-107.83 | 9.45 | 110.37 |

Table 2 Stability samples result for dapagliflozin and saxagliptine in human plasma (n=6).

| Stability test | Dapagliflozin | | | | Saxagliptine | | | |
|---|---|---|---|---|---|---|---|---|
| | Concentration added (ng/mL) | Mean±SD (ng/mL) | Accuracy / Stability (%) | Precision (%) | QC (spiked concentration, ng/mL) | Mean±SD (ng/mL) | Accuracy / Stability (%) | Precision (%) |
| Aautosampler stability (at 10°C for 51 h) | 1.51<br>227 | 1.615±0.37<br>236.70±12.68 | 99.5<br>100.17 | 2.33<br>0.54 | 0.612<br>76.402 | 0.600±0.01<br>79.541±0.560 | 98.18<br>104.42 | 2.68<br>0.70 |
| Wet extract stability ( at room temperature for 45 h) | 1.51<br>227 | 1.630±0.33<br>236.73±10.93 | 108.01<br>104.26 | 2.02<br>0.46 | 0.612<br>76.402 | 0.602±0.02<br>80.130±0.60 | 98.37<br>104.88 | 3.41<br>0.76 |
| Bench top stability (16 h) | 1.51<br>227 | 1.591±0.320<br>236.418±8.748 | 105.41<br>104.12 | 2.01<br>0.37 | 0.612<br>76.402 | 0.623±0.02<br>81.093±0.59 | 101.82<br>106.14 | 3.84<br>0.74 |
| Freeze-thaw stability | 1.51<br>227 | 1.6183±0.434<br>237.367±8.48 | 107.17<br>104.54 | 2.69<br>0.36 | 0.612<br>76.402 | 0.6150±0.022 | 100.49<br>104.78 | 3.68<br>1.23 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| (five cycles) | | | | | | 80.052±0.986 | | |
| Reinjection stability (24 h) | 1.51<br>227 | 1.627±0.408<br>236.90±13.98 | 107.52<br>104.06 | 2.51<br>0.59 | 0.612<br>76.402 | 0.6005±0.03<br>81.333±0.86 | 97.96<br>106.25 | 5.98<br>1.07 |
| Long-term Stability (at –70 °C for 30 days) | 1.51<br>227 | 1.572±0.416<br>236.181±8.37 | 96.85<br>99.95 | 2.65<br>0.35 | 0.612<br>76.402 | 0.616±0.03<br>80.371±0.69 | 100.71<br>105.91 | 102.80<br>99.01 |

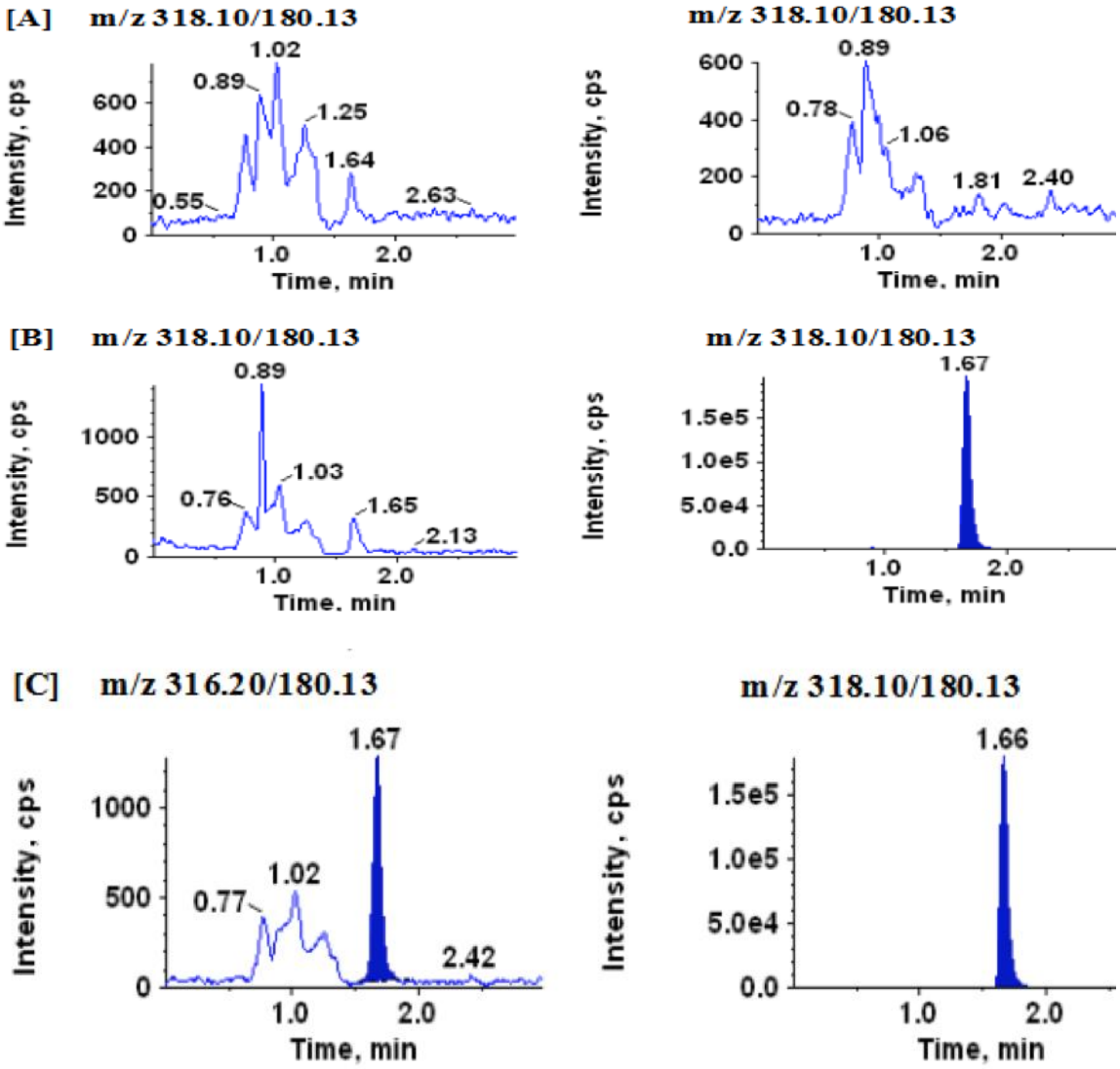


Figure 1: [A] Typical MRM chromatograms of saxagliptine (upper panel) and I.S. (lower panel) in human blank plasma. [B] Typical MRM chromatograms of saxagliptine (upper panel) and I.S. (lower panel) in human blank plasma with the I.S. [C] Typical MRM chromatograms of saxagliptine (upper panel) and I.S. (lower panel) in LLOQ sample.

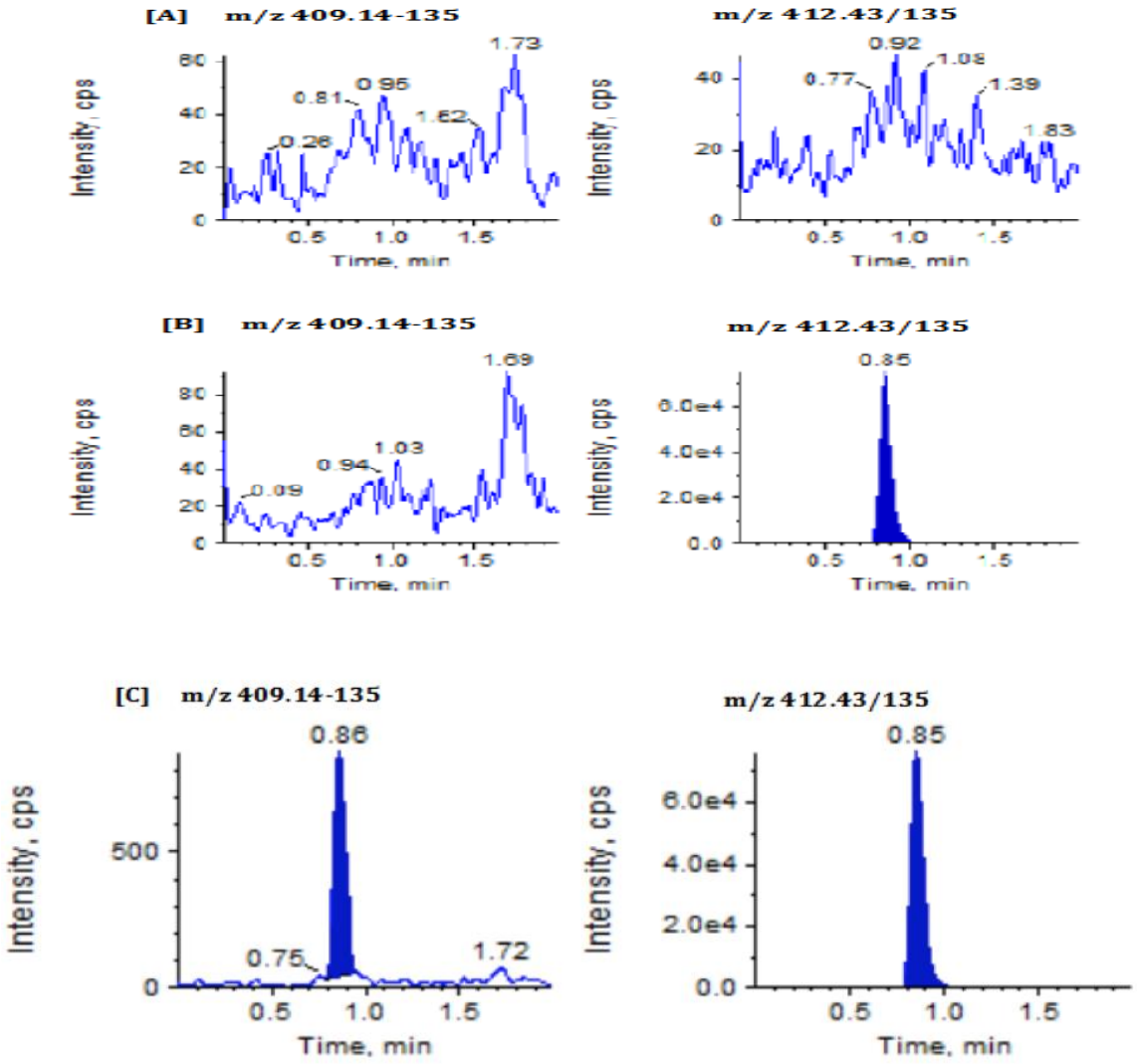


Figure 2: [A] Typical MRM chromatograms of dapagliflozin (upper panel) and I.S. (lower panel) in human blank plasma. [B] Typical MRM chromatograms of dapagliflozin (upper panel) and I.S. (lower panel) in human blank plasma with the I.S. [C] Typical MRM chromatograms of dapagliflozin (upper panel) and I.S. (lower panel) in LLOQ sample.

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