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Quantitative determination of metformin, saxagliptin and 5-hydroxy saxagliptin simultaneously by hydrophilic interaction liquid chromatography - electrospray ionization mass spectrometry and its application to a bioequivalence study with a single-pill combination in human

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Abstract:

A simple, sensitive and specific hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometric (HILIC–MS) method was developed and validated to determine the plasma concentrations of metformin, saxagliptin and 5-hydroxy saxagliptin simultaneously in clinical studies. Plasma samples were first acidified and then protein precipitated with acetonitrile. Chromatographic separation was achieved on a HILIC Chrom Matrix HP amide column (5μm, 3.0 × 100 mm I.D.). The mobile phase consisted of acetonitrile and 5 mM ammonium formate buffer containing 0.1% formic acid. Multiple reaction monitoring transitions were performed on triple quadrupole mass spectrometric detection in positive-ion mode with an electrospray ionization source. The calibration curves showed good linearity ($r \geq 0.999$) over the established concentration range of 1.0 - 1000 ng/mL for metformin and 0.1 - 100 ng/mL for saxagliptin and its active metabolite 5-hydroxy saxagliptin. The extraction recovery for all of the analytes was more than 92 % and the matrix effect ranged from 91.0 to 110.0 %. After validation, the method was successfully applied to a bioequivalence study with a single-pill combination (SPC) consisting of 5mg saxagliptin and 500 mg metformin in 10 healthy Chinese subjects.

Keywords: HILIC-MS/MS; Metformin; Saxagliptin; 5-hydroxy Saxagliptin; Human plasma;

1 Introduction

Diabetes is one of the major chronic non-communicable diseases. The International Diabetes Federation (IDF) estimates the global prevalence to about 700 million by 2040 [1]. In the past decades of fighting diabetes, the development of the disease has been actively alleviating with the growing understanding of its pathophysiology and the development of a series of antihyperglycemic drugs [2]. Yet, many treatments commonly fail to reach the criteria required to control diabetes [3]. For each therapeutic agent, as monotherapy, the progressive deterioration of diabetes control resulted in approximately 50% of the patients achieving recommended glycemic treatment goals after 3 years while 9 years down to about 25% [4]. Diabetes is still a pervasive disease and has enormous public health consequences [5]. Most patients require multiple therapies (*eg*. add-on therapy or combined therapy) to achieve target levels over a long period of time, especially for type 2 diabetes mellitus (T2DM) in adults [6-9]. Now, a single-pill combination (SPC) with its features of enhanced, simplified and optimized has gained significant attention in clinical. SPC formulation commonly contains two or more therapeutic agent having complementary mode. The management of T2DM may be facilitated by the use of SPC, which can improve patient compliance by allowing patients to take fewer pills per day [10-13].

Metformin (MFM) is considered a first-line hypoglycemic agent in T2DM management [2, 14], which inhibits hepatic gluconeogenesis by activating AMPK through mitochondrial inhibition [15, 16]. However, for many patients, the blood glucose after the application of metformin is still unable to achieve effective control or maintain the target level over a long time [17]. Dipeptidyl peptidase-4 (DPP-4) inhibitor has attracted considerable interests as the most rational combination to be adding on top of metformin [18-22], because of their complementary or synergistic action on efficacy and durability when used in combination [6, 22, 23]. Among them, Saxagliptin (SXG) is an orally

effective DDP-4 inhibitor, which can selectively, reversibly and competitively act on DDP-4 [24]. Moreover, studies have shown that there is no significant interaction between SXG and MFM in pharmacokinetics in healthy subjects [25]. Therefore, combination of MFM and SXG can be considered as one of the preferred SPC therapies and a novel armament for the treatment of T2DM. Kombiglyze ™ XR (saxagliptin and metformin HCl extended-release) Tablet has been approved by FDA in the USA for the treatment of T2DM in adults [26].

After oral administration, SXG is rapidly absorbed and metabolized by CYP3A4/5 to a pharmacologically active metabolite 5-hydroxy saxagliptin (OH-SXG). In humans, the plasma systemic exposure of OH-SXG is about 3 times greater than that of SXG [27]. Therefore, the establishment of a simultaneous quantitative methods for MFM and SXG with its metabolite OH-SXG in biological matrices is quite essential and important for in vivo studies of saxagliptin/metformin SPC formulations. However, most analytical methods have focused on single detection of MFM [28-32], single detection of SXG [33-36], or detection of SXG and its metabolite OH-SXG [37-39]. The reported methods for simultaneous determination of MFM and SXG have been mostly applied to the chemical purity identification of SPC pharmaceutical preparations, which can not be used for in vivo studies due to the lower limits of quantitation up to a few hundred nanograms per milliliter [40, 41]. In bioequivalence studies, simultaneous detection of MFM, SXG and OH-SXG in biological matrices has been mentioned as well, but there is no detailed information on method establishment and method validation [42, 43]. Only one study reported detailed information on method development and validation for simultaneous quantification of MFM, SXG and OH-SXG in biological matrix [25]. However, the sample preparation in this published method is complicated by solid-phase extraction using sodium dodecyl sulfate as ion-pair reagent. Here, we present a simple, specific and sensitive HILIC-MS/MS

method for simultaneous determination of MFM, SXG and OH-SXG in human plasma by one-step protein precipitation. Moreover, this novel method was successfully applied to a bioequivalence study with saxagliptin/metformin (5mg/500mg) SPC tablets in healthy adult Chinese subjects.

2 Experimental

2.1 Chemicals and materials

Metformin (MFM) and 2-Chloroadenosine (IS, internal standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Saxagliptin (SXG) was purchased from Selleckchem (Houston, TX, USA). 5-hydroxy saxagliptin (OH-SXG) was purchased from Quality Control Chemical Inc. (Washington, DC, USA). Figure 1 shows the chemical structures. The acetonitrile and methanol of HPLC-grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid and ammonium formate were purchased from Acros Organics (Geel, Belgium). Ultrapure water was prepared by Milli-Q Ultrapure water purification system (Millipore, Bedford, USA).

2.2 Liquid chromatographic and mass spectrometric conditions

Chromatography was performed with a Shimadzu LC-20A system consisting of a Shimadzu LC-20AD XR binary pump, a Shimadzu SIL-20AC XR autosampler, a Shimadzu CTO-20A column oven and a Shimadzu DGU-20A5 online degasser (Shimadzu, Kyoto, Japan). A HILIC Chrom Matrix HP amide column (5μm, 10cm×3.0mm I.D.) was selected for chromatographic separation with gradient elution at a flow rate of 0.4 ml/min. The aqueous phase (A) was a buffer containing 0.1% formic acid and 5mM ammonium formate. The organic phase (B) selected acetonitrile. The gradient elution was applied: 0-6.5 min, 80% B; 6.5-7.0 mins, reduce B to 10%; 7.0-8.5 min, 10% B; 8.5-9.0 min, increase B to 80% B. The total runtime was 13.0 min. Retention times of MFM, SXG, OH-SXG and IS were 3.5,

3.0, 5.2 and 1.9 min, respectively. The column temperature was set at 40℃. The injection volume was set to 5μL.

Mass spectrometry was performed using a quadrupole tandem mass spectrometer (API 4000, Applied Biosystems, Foster City, CA) in a multi-reaction monitoring mode under positive electrospray ionization. The resolution of Q1 and Q3 was both set to a half-width of 0.7Da.

The MS/MS parameters were optimized, ion spray voltage was set to 5500V, ion spray temperature was set to 550℃, ion source gas 1 was set to 50 Arb, ion source gas 2 was set to 55 Arb, curtain gas (Nitrogen) was set to 30 Arb, collision gas (Nitrogen) was set to 10 Arb, entrance potential was set to 10 V, collision cell exit potential was set to 12 V. The optimized m/z transitions were as follows: 130.1 → 85.1 for MFM, 316.4 → 180.2 for SXG, 332.3 → 196.3 for OH-SXG, 302.1 → 170.1 for 2-chloroadenosine (IS). The product ion spectrums of MFM, SXG and OH-SXG are presented in Fig.1. The declustering potential (DP) was set to 60 V for MFM and SXG, 70 V for OH-SXG and 50V for IS. The collision energy (CE) was set at 22 eV, 33 eV, 30 eV and 23 eV for MFM, SXG , OH-SXG and IS respectively. Data acquisition and data analysis were performed using Analyst software version 1.5.1. (Applied Biosystems, Foster City, CA, USA).

2.3 Preparation of stock solutions, calibration standards and control samples

The stock solutions of MFM, SXG, OH-SXG and IS were prepared in acetonitrile at a final concentration of 1.0 mg/mL. A series of combined working solutions of MFM, SXG and OH-SXG were prepared by appropriately diluting the standard stock solutions to the desired concentrations with acetonitrile. All solutions were stored at 4℃ before use.

The calibration standard (CS) and quality control (QC) samples were prepared by spiking blank plasma with respective combined working solutions. The concentration of mixed CS samples in plasma were 1, 3, 10, 30, 100, 300, 1000 ng/mL for MFM, 0.1, 0.3, 1,

3, 10, 30, 100 ng/mL for SXG and OH-SXG. According to the pre-test results of some bioequivalence samples, the concentration range of MFM from 1 ng/ml to 1000 ng/ml and the concentration ranges of SXG and OH-SXG from 0.1 ng/ml to 100 ng/ml could cover over 98% of the sample concentration. QC samples were prepared at three levels of low, medium and high. For MFM, SXG and OH-SXG, the concentrations of low QCs were corresponding to 3.0, 0.3 and 0.3 ng/ml respectively, the concentrations of mid-QCs were corresponding to 30, 3 and 3 ng/ml respectively, and the high QCs were corresponding to 800, 80 and 80 ng/ml respectively. Plasma samples were stored at -80 ºC before analysis.

2.4 Sample preparation

The analytes were extracted from the plasma sample using one-step protein precipitation. 50 μL plasma sample was firstly acidified by 5μL HCl (5mM). The acidified sample was then vortexed briefly and 150 μL acetonitrile containing IS (50 ng/mL) was added for protein precipitation. Vortex for 5 min to make the plasma protein precipitation completely. The mixture was then centrifuged at 30000×g for 10 min (Sorvall Biofuge stratos, Germany), and 5 μL supernatant was injected for analysis.

2.5 Method validation

The method was validated in terms of selectivity, linearity, accuracy, precision, extraction recovery, matrix effect and stability according to US Food and Drug Administration guidelines for bioanalytical method validation (US Food and Drug Administration, 2001) [44].

2.5.1 Selectivity and specificity

This step was carried out to identify potential chromatographic interference from endogenous entities at the peak regions of the analyte and IS. The specificity was assessed by analyzing blank human plasma, blank plasma spiked with authentic standards of MFM,

SXG and OH-SXG, and plasma sample after oral administration of saxagliptin/metformin SPC tablet. Chromatographic peaks from plasma samples were compared with the authentic standards by the retention times and MRM responses.

2.5.2 Linearity

A seven-point linear calibration curve was constructed using a weighted (1/x) least squares linear regression by plotting the peak area ratios of analyte/IS versus nominated plasma concentrations over the range of 1.0 - 1000 ng/mL for metformin and 0.1 - 100 ng/mL for saxagliptin and 5-hydroxy saxagliptin. The lower limit of quantitation (LLOQ), which was defined as the lowest concentration on the calibration curve, should fulfill the analytical requirement that S/N > 10 and the accuracy and precision were up to ± 20% relative error (RE, %) and relative standard deviation (RSD, %), respectively. The calibration curve was considered to be adequate when at least 75% of the analyzed points were within RE ≤15%.

2.5.3 Accuracy and Precision

Accuracy and precision were assessed by determining QC samples at three concentration levels (low, medium and high) on three different validation days with six replicates of each QC level. The accuracy was expressed as RE (%), and the precision as RSD (%). Both RE and RSD were expected to be within ± 15% to be acceptable. During routine analysis, each analytical run included a set of calibration samples, a set of QC samples in triplicate and unknowns.

2.5.4 Extraction recovery and Matrix effect

The recovery and matrix effect from human plasma precipitation were determined at 3, 30, 1000 ng/mL (n = 6) for metformin and at 0.3, 3, 100 ng/mL (n = 6) for saxagliptin and

5-hydroxy saxagliptin by comparing three sets of samples: (A) analytes spiked into plasma before precipitation, (B) analytes spiked into the precipitate after precipitation of blank plasma, and (C) analytes spiked into the precipitate after precipitation of equivalent saline instead of blank plasma. Recovery was calculated as percentage of the peak area of set A compared to that of set B. Similarly, matrix effect was calculated as percentage of the peak area of set B compares to that of set C. According to the US-FDA bioanalytical guidelines, recovery of the analyte need not be 100%, but the extent of recovery should be consistent, precise, and reproducible.

2.5.5 Stability

Six replicates at low, medium and high concentrations were used for stability validation under a variety of storage and handling conditions for all of the analytes. Samples were subjected to three freeze–thaw cycles, and stored at room temperature for 8 h and -80℃ for nine days. Post-preparative stability was evaluated by reanalyzing post-extraction samples kept in the autosampler at 4℃ for 24 h.

The stability of stock solutions was evaluated at room temperature for 48 h and under 4℃ for three months. In order to adapt to the detection intensity of LC-MS/MS, the test stock solution (1 mg / ml) was first diluted to 1μg/ml working solution with acetonitrile and then injected in six consecutive injections to obtain the average peak area. The stability of the stock solution was evaluated by comparing the peak area obtained form the stock solution under different storage conditions with that obtained from the freshly prepared stock solution.

2.6 Bioequivalence Study

The developed method was applied to a bioequivalence study with a test saxagliptin/metformin (5mg/500mg) extended-release (XR) SPC tablet and a reference

Kombiglyze™ XR tablet (5mg saxagliptin + 500 mg metformin hydrochloride, AstraZeneca Pharmaceuticals LP Wilmington, DE, USA) in 10 healthy Chinese subjects. The study was conducted in accordance with the quality control standards of drug clinical trials (China Food and Drug Administration, 2003). This was a single-site, randomized, single-dose, two-period, cross-over and self-controlled pharmacokinetic study. Subjects underwent screening evaluations to determine their eligibility within three weeks prior to the first dose. On day 1 of period 1, the 10 subjects were randomized to receive a single oral dose either the test or the reference saxagliptin/metformin XR SPC tablet. After a washout period of two weeks, subjects were crossed over in period to receive the alternative treatment. Blood samples for determination of plasma concentrations of MFM, SXG and OH-SXG were collected into 5mL tubes containing dipotassium ethylenediaminetetraacetic acid (EDTA) before administration and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, 24, 36 and 48h after administration. Plasma was separated by centrifuging whole blood at 8000 rpm for 10 min. Plasma samples were stored at -80℃ until analysis.

The pharmacokinetic parameters of MFM, SXG and OH-SXG, such as C_{max}, t_{max}, AUC_{last}, $AUC_{0\sim\infty}$ and $t_{1/2}$, were calculated from plasma concentration-time data using standard non-compartmental methods with Phoenix WinNonlin v6.4 (Pharsight Corp., Mountain View, CA, USA). C_{max} represented the maximum plasma concentration, t_{max} represented the time to reach C_{max}, AUC_{last} represented the area under the plasma concentration-time curve from time zero to the time of the last measurable concentration, $AUC_{0\sim\infty}$ represented the AUC from time zero extrapolated to infinity, and $t_{1/2}$ represented the elimination half-life. These pharmacokinetic parameters were summarized by analyte and treatment using descriptive statistics (number of subjects, mean, SD, CV) with Phoenix WinNonlin v6.4. Among them, C_{max}, AUC_{last} and $AUC_{0\sim\infty}$ were used to establish bioequivalence between the test and reference formulation using cross-over bioequivalence

assessment with Phoenix WinNonlin v6.4. Bioequivalence was concluded if the 90% confidence intervals for the test to reference ratios of geometric means were entirely contained within 0.8-1.25 for both C_{max} and AUC.

3 Results and Discussion

3.1 Method development

In order to set the ESI-MS conditions, full scan ion spectra of precursor and product ions of the three analytes and IS were investigated in both positive and negative ion mode. The signal intensities found in the positive ionization mode were much more higher compared with the negative mode for all of the analytes and IS, because of alkyl amine groups in their structures. In the electrospray ionization (ESI) source, all analytes and IS predominantly form protonated molecules of $[M+H]^+$ in the full scan spectrum. No sodium or other solvent adducts or dimmers were observed. Figure 1 shows the product ion spectra of MFM, SXG, OH-SXG and IS. The major fragment ions were obtained at m/z 180.2, 196.3 and 170.1 for SXG, OH-SXG and IS, respectively. The mass spectral signals were optimized to achieve higher sensitivity. For MFM, a maximum intensity was obtained by the m/z 130.1 → 60.2 transition. However, due to a relatively high plasma concentration after oral administration of 500 mg MFM in human, a relatively weaker fragment ion at m/z 85.1 was selected for MFM quantification to avoid signal saturation at high concentration and reduce sample amounts for dilution in subsequent studies. 2-chloroadenosine was selected as internal standard (IS) because its m/z values of protonated ion and ion fragment were similar to those of SXG and OH-SXG. In addition, under the established chromatographic conditions, 2-chloroadenosine was completely separated from the compounds of interest.

MFM is a highly polar biguanide compound (octanol : water partition coefficient 0.01 [45]). When the reverse phase chromatographic analysis was applied, the retention time of

MFM was very short [28, 31, 46] and may be consistent with the rapid elution of endogenous substances in the biological matrix. However, SXG and OH-SXG can be adequately retained on conventional C18 reversed-phase columns within acceptable run times [34, 37, 38]. Adequate retention for all of the analytes was a tough challenge in the simultaneous analysis of MFM, SXG and OH-SXG, because of their varying polarities. Due to high polarity of MFM, a polar column namely Synergi 4u Polar-RP 80A filled with an ether - phenyl bonded phase was firstly tested. It was indeed satisfactory for the retention of MFM, but the chromatographic behavior of SXG and OH-SXG was unsatisfactory, including the peak shape, the peak intensity and the resolution of the analyte. HILIC-based chromatography is another logical choice because it provides the ideal selectivity for the retention and separation of MFM in biological matrix which containing other various polar compounds. In this manuscript, we selected a polyacrylamide immobilized silica column (namely Chrom-Matrix HP amide column) to developed a hydrophilic interaction liquid chromatogram method. In order to avoid deformation of the chromatogram, acetonitrile was used instead of methanol as the mobile phase to ensure that the organic content of the mobile phase was approximately equal to that of the sample. However, acetonitrile is a weak solvent in the hydrophilic interaction chromatography mode, while water is a strong solvent. Increasing the proportion of acetonitrile in the mobile phase can prolong the retention time. However, a high proportion of acetonitrile could result in a broadened peak shape [47]. This phenomenon occurred on the OH-SXG, which has a peak width of nearly 1 min, but its peak shape was symmetrical. The modifier of formic acid and ammonium formate in the mobile phase can improve the peak shape and increase the sensitivity of detection. In addition, a gradient elution mode was optimized to avoid some interference from endogenous matrix components and to ensure excellent injection-to-injection

reproducibility. Under the established chromatographic conditions, the retention factors (k') of MFM, SXG and OH-SXG were all greater than one.

Because of its high polarity, MFM is extremely difficult to extract from biological matrices [48]. In order to overcome this difficulty, protein precipitation is the most commonly used method for sample preparation. Acetonitrile was selected as a protein precipitant because of its high efficiency of precipitating [49, 50]. During the development of the method, it was found that even if the plasma concentration of the analyte was equivalent, the extraction recovery of the analyte in plasma from different individuals was different. After alkalization with 5μL of 0.1 mol/L sodium hydroxide solution to a 50μL of plasma sample, the performance of SXG and OH-SXG could ameliorate, but no effect on MFM. This might be related to their varying basic nature (pKa 12.3, 7.9 and 7.6 for MFM, SXG and OH-SXG, respectively [25]). However, after acidified with 5μL of 0.005 mol/L hydrochloric acid to a 50μL of plasma sample, individual variation was significantly reduced for all of the analytes. Acidification might make the molecular / ionic state of the analyte in human plasma matrix to be consistent between individuals. The treatment of acidified plasma was also reported in published literatures [51].

3.2 Method validation

3.2.1 Specificity

Figure 2 shows typical chromatograms of a blank matrix, a blank matrix spiked with IS, a blank matrix spiked with analytes and IS, and a sample after oral administration of a SPC tablet (saxagliptin/metformin, 5mg/500mg). The retention time for MFM, SXG, OH-SXG and IS were 3.5, 3.0, 5.2 and 2.0 min, respectively. No interference from endogenous components or other impurities was observed during the retention time of the analytes and IS, indicating that the method had good specificity.

3.2.2 Linearity and sensitivity

The calibration curves exhibited a good linearity over a concentration range of 1.0 ~ 1000 ng/ml for MFM and 0.1 ~ 100 ng/mL for SXG and OH-SXG. The regression equation with weighting factor of 1/x was y=0.0141x+0.009 for MFM, y=0.0238x-0.0001 for SXG, and y=0.0139x-0.0002 for OH-SXG, respectively. The correlation coefficient (*r*) was 0.9996 for MFM, 0.9994 for SXG and 0.9998 for OH-SXG, respectively. Detailed data was listed in Table 1. The REs of all the analysis points of the calibration curve were in the range of -8.33% ~ 6.10%, indicating that the established calibration cuvre was adequate.

The LLOQ in human plasma was 1ng/ml for MFM, 0.1 ng/ml for SXG and OH-SXG, respectively. At this LLOQ, signal noise ratio was greater than 10, and the accuracy and precision were 6.95% and 1.78% for MFM, 6.04% and -5.06% for SXG, 4.61% and -1.33% for OH-SXG, respectively. The LLOQ of 1.0 ng/ml established for MFM was lower than the LLOQs established previously for the single determination of MFM in the plasma matrix (10-50 ng/ml) [28, 46, 51]. The LLOQ of 0.1 ng/ml established for both SXG and OH-SXG were equivalent or lower than the LLOQs (0.1 ng/ml for SXG and 0.2 ng/ml for OH-SXG) established by Xu *et al* for clinical studies [37]. Even with the latest co-detection method of MFM (LLOQ, 1.5 ng/ml), SXG (LLOQ, 0.1 ng/ml) and OH-SXG (LLOQ, 0.2ng/ml) in human plasma [25], the LLOQs we established also had a significant advantage.

3.2.3 Precision and accuracy

The accuracy was calculated by comparing the average measured value with the nominal value. The precision was evaluated by calculating the relative standard deviation. Accuracy and precision data for MFM, SXG and OH-SXG at three QC levels are summarized in Table 2. The intra-day precision was ranged from 3.3 % to 8.2 %, accuracy ranged from -6.3 % to 2.2 %. The inter-day precision was ranged from 3.5 % to 5.9 %,

accuracy ranged from -5.6 % to 4.6 %. All intra- and inter-day variations, as well as the accuracy, were within the acceptable range according to the FDA Guidance for Industry on Bioanalytical Method Validation, indicating that the method was reliable and reproducible for the determinination of MFM, SXG and OH-SXG in human plasma.

3.2.4 Recovery and Matrix effect

Table 3 summarizes the extraction recovery and matrix effect of MFM, SXG and OH-SXG in human plasma. The mean recovery of MFM is ranged from 101.0% to 104.8% and the matrix effect ranged from 93.3% to 98.8%. The mean recovery of SXG is ranged from 92.8% to 95.0% and the matrix effect ranged from 91.0% to 98.8%. The mean recovery of OH-SXG is ranged from 98.3% to 110.0% and the matrix effect ranged from 101.7% to 106.7%. Obviously, no significant matrix interference was found. The recovery was good and was consistent at low, medium and high concentrations.

3.2.4 Stability

The stability of MFM, SXG and OH-SXG under various storage conditions, including room temperature for 8 h, frozen at -80℃ for 9 days, three freeze-thaw cycles and autosampler stability at 4℃ for 24h was evaluated at low, medium and high concentrations (Table 4). All of the RE (%) were ranged from -10.7 % to 10.8 %, indicating that MFM, SXG and OH-SXG were stable under the conditions of sample storage and processing. In addition, the peak area obtained from the stock solution stored at room temperature for 48 h was about 98.9%, 102.6% and 99.5% of that obtained from the freshly prepared stock solution of MFM, SXG and OH-SXG, respectively. Similarly, the peak area obtained from the stock solution stored under 4℃ for three months was about 99.9%, 103.6% and 103.3% of that obtained from the newly prepared MFM, SXG and OH-SXG stock solutions, respectively. It could be seen that the stock solutions were stable under the above two

storage conditions. The stability results of the MFM, SXG and OH-SXG in stock solutions and in plasma samples were consistent with those previously published by Shah *et al* [39].

3.3 Bioequivalence Study

The validated HILIC-MS/MS method was successfully applied to determine MFM, SXG and OH-SXG simultaneously in human plasma of a bioequivalence study. The mean plasma concentration-time profiles for MFM, SXG and OH-SXG obtained following single administration of a test and a reference saxagliptin/metformin XR 5/500mg SPC tablets to ten healthy Chinese subjects are illustrated in Figure 3. These figures show that the mean plasma profiles were highly similar for all three analytes following administration of a test and a reference saxagliptin/metformin XR SPC tablets. Concentrations were quantifiable in most subjects through 48 h post-dose for all three analytes. Summary statistics for C_{max}, t_{max}, AUC_{last}, $AUC_{0\sim\infty}$ and $t_{1/2}$ for MFM, SXG and OH-SXG are presented in Table 5. Comparisons of a test and a reference SPC tablets expressed as geometric mean ratios and their associated 90% confidence intervals (CIs) for C_{max}, AUC_{last} and $AUC_{0\sim\infty}$ of all three analytes are also shown in Table 5. In our study, the results of C_{max} and $AUC_{0\sim\infty}$ for SXG after oral administration of the reference Kombiglyze™ saxagliptin/metformin XR 5/500mg SPC tablets were alomost identical to those obtained by Gummesson *et al* in healthy adult chinese subjects [42], while the results of C_{max} and $AUC_{0\sim\infty}$ for MFM and OH-SXG were about 0.3 and 1 times higher than the previous results, respectively. This may be related to the number of cases of two experimental protocols and the difference in the metabolic capacity of the subjects in two experiments.

In this manuscript, with respect to the C_{max}, AUC_{last} and $AUC_{0\sim\infty}$ values, the geometric mean ratios and 90% CIs were entirely contained within the bioequivalence limits of 80-125% (81.5-113.1% for all of the analytes) that were necessary to conclude bioequivalent between the test saxagliptin/metformin 5/500mg XR SPC tablet and the reference Kombiglyze™ XR

SPC tablet. Furthermore, t_{max} and $t_{1/2}$ values were also very similar for all three analytes after administration of the test and reference saxagliptin/metformin XR SPCs. These results demonstrated that the test and the reference SPC tablet were pharmacokintic equivalence both in the absorption extent and in the absorption rate as well as in the elimination rate.

4 Conclusion

A sensitive and specific HILIC-MS/MS method has been developed and validated to determine metformin, saxagliptin and 5-hydroxy saxagliptin simultaneously in human plasma. It was successfully applied to a bioequivalence assessment of a test and a reference saxagliptin/metformin extended-release single-pill combination (5mg/500mg) in 10 healthy Chinease subjects. Acidified samples of plasma were deproteinized with acetonitrile and this method guaranteed acceptable recovery and selectivity, as well as good sensitivity, precision, accuracy and stability. The LLOQs established for all of the analytes are lower compared with several published methods. This method does not have any difficulty in the application of the sample analysis process, making it very suitable for clinical pharmacokinetic studies of metformin and saxagliptin.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig.1 Product ion mass spectra of (a) Metformin (m/z 130.1→85.1), scan range 50-150amu; (b) Saxagliptin (m/z 316.4→180.2), scan range 50-350 amu, (c) 5-Hydroxy Saxagliptin (m/z 332.3→196.3), scan range 50-400 amu and (d) IS (m/z 302.1→170.1), scan range 50-350 amu in the positive ionization mode.

Fig.2 Multiple reaction monitoring ion-chromatograms of (A) blank plasma; (B) blank plasma precipitation with acetonitrile containing IS (50 ng/mL); (C) blank plasma spiked with combined working solution of metformin, saxagliptin and 5-hydroxy saxagliptin (30ng/mL, 3 ng/mL and 3 ng/mL in plasma, respectively) which followed by precipitation with acetonitrile containing 50 ng/mL of IS; (D) human plasma sample at 1.5 h after oral administration of a single-pill combination (5 mg saxagliptin + 500 mg metformin hydrochloride XR tablet).

Fig.3 Mean plasma concentration-time profiles of (A) Metformin, (B) Saxagliptin and (C) 5-Hydroxy Saxagliptin after oral administration of a test and a reference SPC formulation of saxagliptin/metformin XR tablet (5mg/500mg) to 10 healthy Chinese subjects. The inserted represented for the semi-log graph.

Tables

Table 1 Linearity and LLOQs of metformin, saxagliptin and 5-hydroxy saxagliptin in human plasma(n = 6).

Measured concentration (ng/ml)		MFM / SXG / OH-SXG Spiked Concentration (ng/ml)						
		1/0.1/0.1	3/0.3/0.3	10/1/1	30/3/3	100/10/10	300/30/30	1000/100/100
Metformin (MFM)	Mean	0.96	2.79	10.10	30.70	105.99	308.47	984.83
	SD	0.07	0.11	0.15	1.80	1.94	7.86	9.08
	RSD (%)	6.95	3.87	1.52	5.87	1.83	2.55	0.92
	RE (%)	1.78	1.16	-2.62	0.98	-0.70	-0.54	0.22
Saxagliptin (SXG)	Mean	0.11	0.30	1.02	2.73	9.50	29.40	101.34
	SD	0.01	0.03	0.08	0.13	0.67	0.85	1.25
	RSD (%)	6.04	9.95	7.83	4.71	7.04	2.91	1.23
	RE (%)	-5.06	-8.33	6.10	5.64	7.12	-1.59	-0.32
5-Hydroxy Saxagliptin (OH-SXG)	Mean	0.10	0.31	0.97	2.97	10.04	30.39	99.63
	SD	0.00	0.02	0.02	0.11	0.15	1.22	1.08
	RSD (%)	4.61	4.97	2.48	3.62	1.52	4.01	1.09
	RE (%)	-1.33	-0.67	0.72	-0.55	1.03	1.41	-0.51

Table 2 The intra-day and inter-day precision and accuracy of metformin, saxagliptin and 5-hydroxy saxagliptin (n = 6).

Analyte	Spiked concentration (ng/ml)	Intra-day			Inter-day		
		Measured concentration (ng/ml)	Precision (RSD%)	Accuracy (RE%)	Measured concentration (ng/ml)	Precision (RSD%)	Accuracy (RE%)
Metformin	3	2.8 ± 0.2	5.5	-6.2	2.7 ± 0.2	5.5	4.0
	30	28.1 ± 1.3	4.7	-6.2	29.0 ± 1.7	5.9	-5.6
	800	787.3 ± 37.0	4.7	-1.6	805.7 ± 37.7	4.7	-1.3
Saxagliptin	0.3	0.28 ± 0.02	8.1	-5.1	0.29 ± 0.02	5.5	-1.7
	3	2.8 ± 0.1	4.5	-6.3	2.8 ± 0.1	3.9	4.6
	80	81.1 ± 5.1	6.3	1.4	80.8 ± 3.7	4.6	0.04
5-Hydroxy Saxagliptin	0.3	0.31± 0.03	8.2	2.2	0.31 ± 0.02	5.5	1.3
	3	2.9 ± 0.1	3.3	-1.9	3.0 ± 0.1	4.9	-0.4
	80	81.7 ± 3.9	4.8	2.1	83.6 ± 3.0	3.5	1.3

Table 3 Extraction recovery and matrix effect data for metformin, saxagliptin and 5-hydroxy saxagliptin (n = 6).

Analyte	Spiked concentration (ng/mL)	Recovery (%)	Matrix effect (%)
Metformin	3	102.6 ± 1.0	98.8 ± 3.3
	30	101.0 ± 1.4	97.6 ± 0.5
	1000	104.8 ± 1.1	93.3 ± 0.7
Saxagliptin	0.3	94.8 ± 0.6	91.0 ± 1.0
	3	95.0 ± 0.8	98.8 ± 0.8
	100	92.8 ± 0.9	93.3 ± 0.1
5-Hydroxy Saxagliptin	0.3	110.0 ± 1.4	101.7 ± 0.5
	3	98.3 ± 1.0	106.7 ± 0.2
	100	99.2 ± 1.1	103.8 ± 0.3

Table 4 Stability of metformin, saxagliptin and 5-hydroxy saxagliptin in human plasma under different storage conditions (n = 6).

Storage conditions	Spiked concentration (ng/mL)	Metformin		Saxagliptin		5-Hydroxy Saxagliptin	
		Measured concentration (ng/ml)	Accuracy (RE%)	Measured concentration (ng/ml)	Accuracy (RE%)	Measured concentration (ng/ml)	Accuracy (RE%)
Room temperature for 8 h	3	3.3 ± 0.1	10.8	0.29 ± 0.01	-4.3	0.32 ± 0.01	6.3
	30	30.7 ± 0.9	2.4	2.7 ± 0.1	-9.8	2.7 ± 0.1	-10.2
	1000	1020.0 ± 28.9	2.0	99.8 ± 4.4	-0.2	106.8 ± 3.8	6.8
Frozen (-80℃) for 9 days	0.3	3.1 ± 0.1	4.6	0.29 ± 0.02	-1.8	0.29 ± 0.02	-4.6
	3	28.6 ± 1.1	-4.8	2.8 ± 0.1	-8.3	2.7 ± 0.1	-9.9
	100	910.8 ± 21.3	-8.9	102.4 ± 6.6	2.4	92.0±4.3	-8.0
Three freeze-thaw cycles	0.3	3.3 ± 0.1	10.2	0.28 ± 0.02	-7.3	0.32±0.01	5.0
	3	28.0 ± 0.6	-6.6	2.8 ± 0.1	-5.4	2.7±0.1	-10.7
	100	934.0 ± 21.5	-6.6	101.4 ± 6.2	1.4	93.0±2.2	-7.0
Autosampler stability (24 h at 4℃)	3	3.3 ± 0.1	9.9	0.31 ± 0.03	1.7	0.31±0.02	4.6
	30	29.9 ± 2.3	-0.2	2.8 ± 0.1	-7.7	2.8±0.1	-6.9
	1000	997.5 ± 26.2	-0.3	108.7 ± 3.3	8.7	105.4±4.1	5.8

Table 5 Summary statistics and bioequivalence for the plasma pharmacokinetic parameters of metformin and saxagliptin given as a test and a reference extented-release tablet of saxagliptin/metformin single-pill combination (5mg/500mg).

Parameter [a]	Test	Reference	BE [b]
Metformin			
C_{max}(ng/mL)	801.8 (15.0)	770.7 (17.6)	1.040 (0.979, 1.106)
t_{max}(h) [median (range)]	6.0 (4.0-8.0)	6.0 (3.0-6.0)	
AUC_{last} (ng*h/mL)	7547.8 (23.1)	7193.0 (17.7)	1.049 (0.975, 1.129)
$AUC_{0\sim\infty}$ (ng*h/mL)	7661.6 (22.9)	7253.4 (17.8)	1.056 (0.986, 1.131)
$t_{1/2}$(h) [median (range)]	8.5 (5.4-15.4)	6.2 (5.7-12.8)	
Saxagliptin			
C_{max}(ng/mL)	25.2 (44.0)	27.2 (41.1)	0.928 (0.858, 1.004)
t_{max}(h) [median (range)]	0.9 (0.5-3.0)	0.9 (0.5-2.0)	
AUC_{last} (ng*h/mL)	103.7 (30.8)	102.8 (20.3)	1.009 (0.934, 1.090)
$AUC_{0\sim\infty}$ (ng*h/mL)	105.4 (31.1)	104.4 (20.1)	1.010 (0.935, 1.090)
$t_{1/2}$(h) [median (range)]	8.3 (4.2-13.7)	6.8 (4.3-10.9)	
5-Hydroxy Saxagliptin			
C_{max}(ng/mL)	77.7 (19.3)	86.2 (20.4)	0.902 (0.815, 0.997)
t_{max}(h) [median (range)]	2.0 (1.0-3.0)	2.0 (1.0-3.0)	
AUC_{last} (ng*h/mL)	488.8 (19.5)	510.4 (17.0)	0.958 (0.902, 1.017)
$AUC_{0\sim\infty}$ (ng*h/mL)	495.7 (19.4)	516.5 (16.5)	0.960 (0.904, 1.019)
$t_{1/2}$(h) [median (range)]	8.0 (4.2-12.0)	8.1 (4.2-12.4)	

C_{max}, maximum plasma concentration; t_{max}, time to the maximum plasma concentration; AUC_{last}, area under the plasma concentration-time curve from time zero to the time of the last quantifiable concentration; $AUC_{0\sim\infty}$, area under the plasma concentration-time curve extrapolated to infinity; $t_{1/2}$, elimination half-life.

[a] Values are expressed as geometric mean (% coefficient of variation) except where stated otherwise.

[b] Geometric mean ratio (90 % confidence interval) of test vs reference formulation.

Highlights

- Simultaneous determination of metformin, saxagliptin and 5-hydroxy saxagliptin.
- A novel hydrophilic interaction liquid chromatography system using polyacrylamide.
- A specific and sensitive HILIC–MS method was well validated in human plasma.
- A bioequivalence study with saxagliptin/metformin single-pill combination in human.

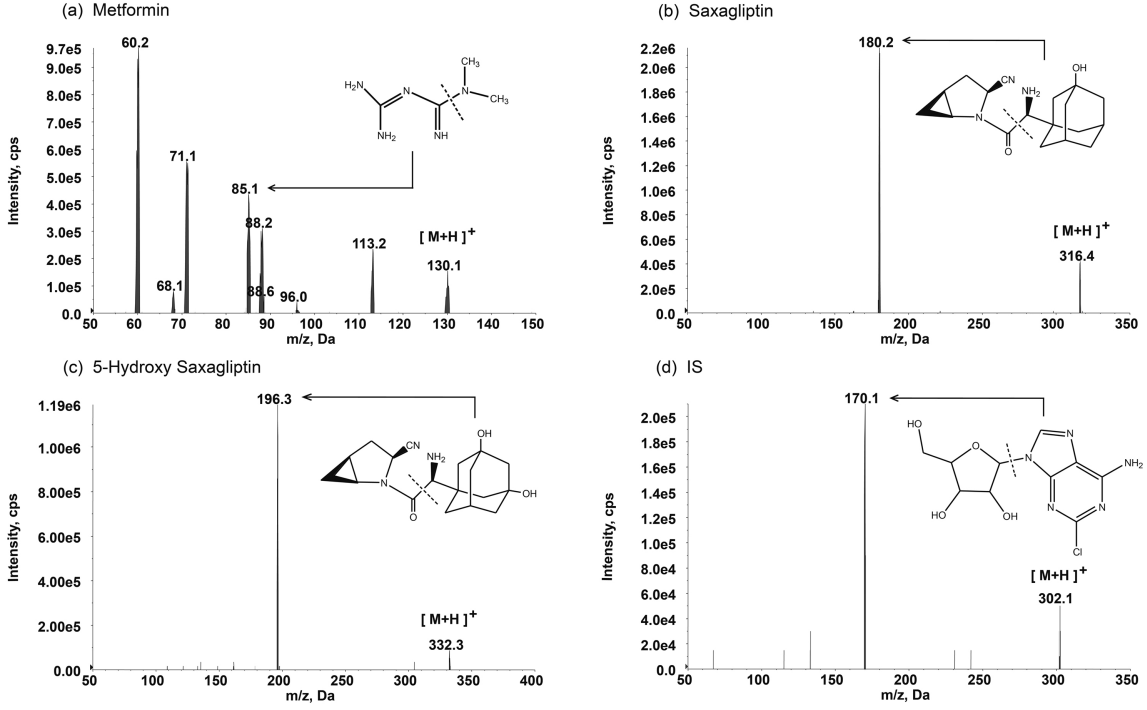
(a) Metformin
Intensity, cps
m/z, Da
60.2
71.1
85.1
88.2
68.1
88.6
96.0
113.2
130.1
[M+H]+
(b) Saxagliptin
180.2
316.4
[M+H]+
(c) 5-Hydroxy Saxagliptin
196.3
332.3
[M+H]+
(d) IS
170.1
302.1
[M+H]+

Figure 1

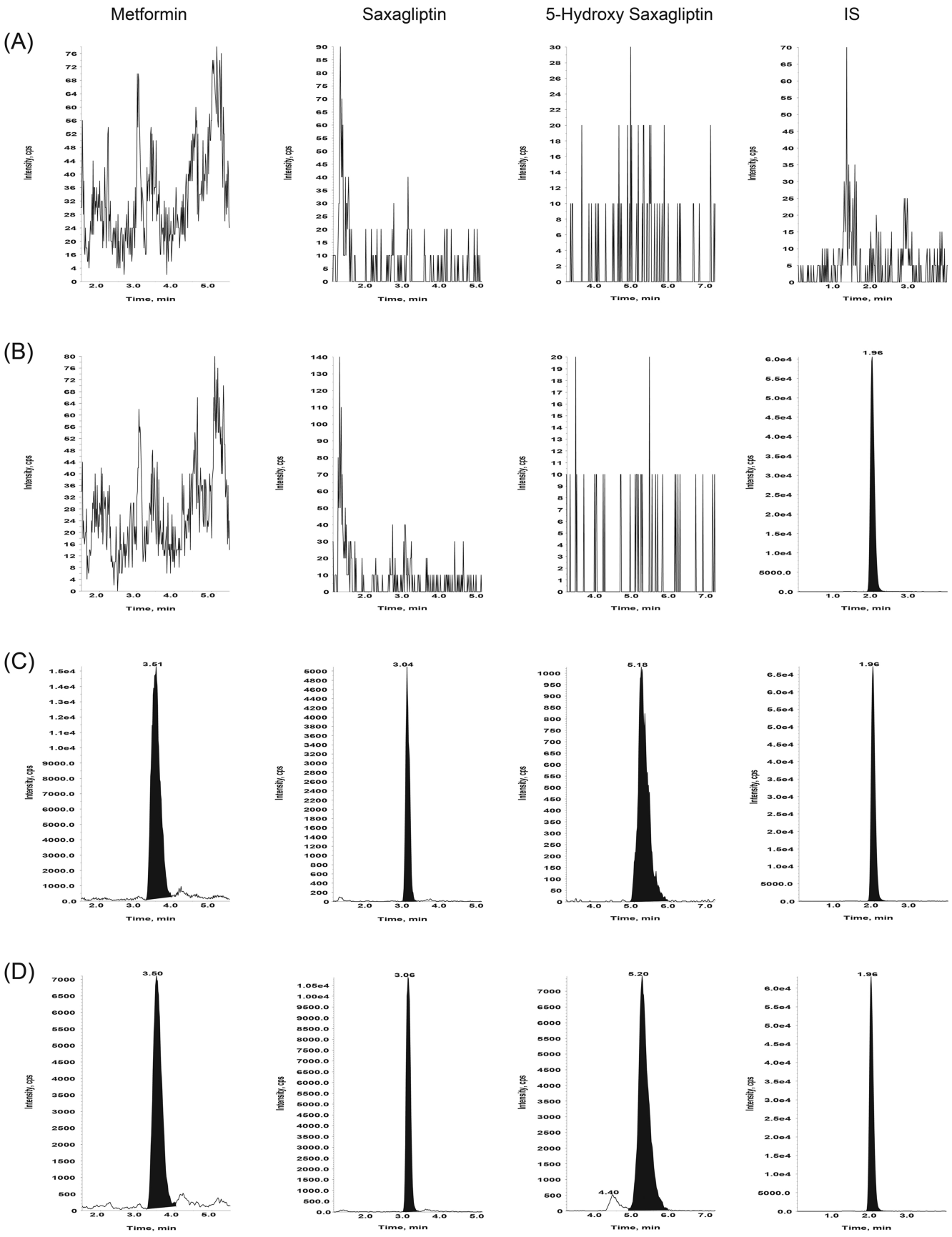
Metformin
Saxagliptin
5-Hydroxy Saxagliptin
IS
(A)
(B)
(C)
(D)
Intensity, cps
Time, min
3.51
3.04
5.18
1.96
3.50
3.06
5.20
4.40

Figure 2

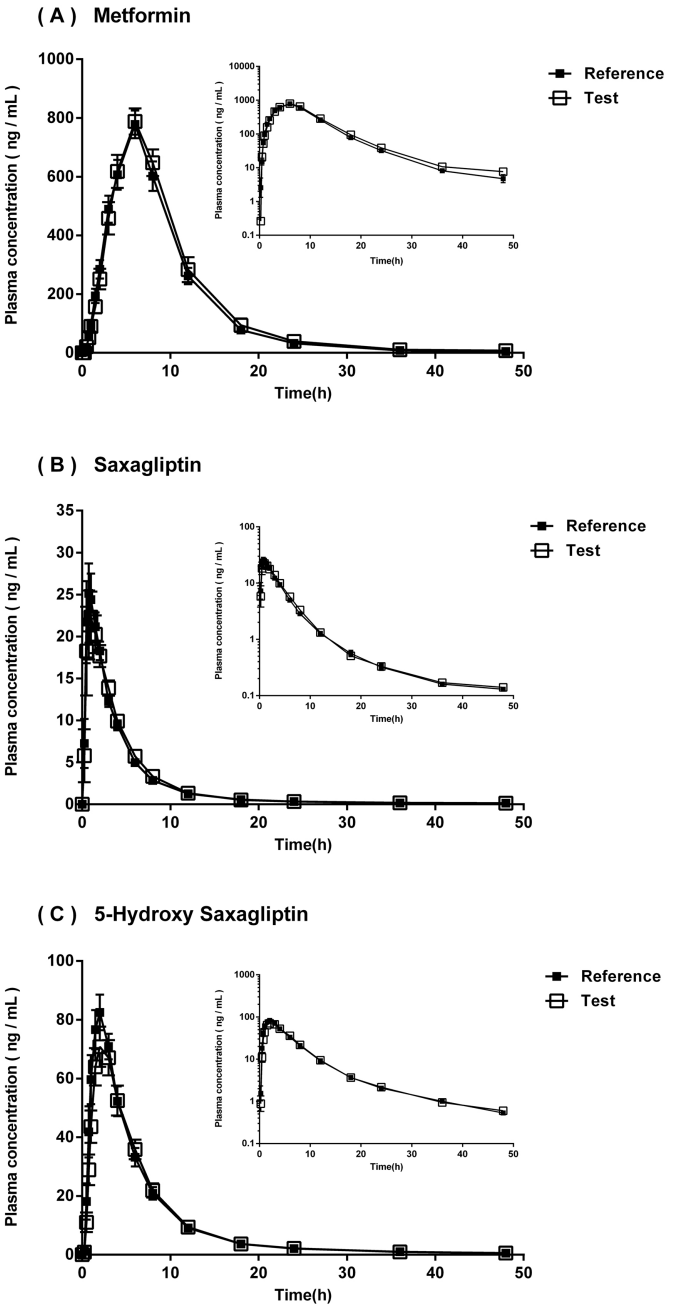
(A) Metformin
Plasma concentration (ng / mL)
Time(h)
Reference
Test
(B) Saxagliptin
Plasma concentration (ng / mL)
Time(h)
Reference
Test
(C) 5-Hydroxy Saxagliptin
Plasma concentration (ng / mL)
Time(h)
Reference
Test

Figure 3