An HPLC Method for the Determination of Saxagliptin in Human Plasma with Fluorescence Detection

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ABSTRACT

A sensitive and selective HPLC method with fluorometric detection was developed for the determination of saxagliptin (SGX) in human plasma and applied to a pharmacokinetic study. SGX was precolumn derivatized with fluorescamine, and the fluorescent derivative was separated on an RP C18 column using a mobile phase composed of acetonitrile-10 mM orthophosphoric acid by isocratic elution with flow rate of 1.3 ml/min. The method was based on the measurement of the derivative using fluorescence detection at 378 nm, with excitation at 463 nm. The calibration curve was linear over the range of 3.0–100.0 ng/ml. LOD and LOQ were found to be 0.15 and 0.5 ng/ml, respectively. Intraday and interday RSD values were less than 2.84%. The plasma concentration–time profile and pharmacokinetic parameters such as AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, $t_{1/2}$, were calculated according to the assays.

Indexing terms/Keywords

Fluorescamine; HPLC; pharmacokinetic; pre-column derivatization; saxagliptin; plasma

Academic Discipline And Sub-Disciplines

Analytical chemistry

SUBJECT CLASSIFICATION

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TYPE (METHOD/APPROACH)

Pharmaceutical analysis

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INTRODUCTION

Saxagliptin (SGX), (1S, 3S, 5S) - 2 - [(2S) - 2 - amino - 2 - (3 - hydroxy - 1 - adamantyl) acetyl] - 2 - azabicyclo [3.1.0] hexane - 3 - carbonitrile, is a widely used drug substance for treatment of type 2 diabetes mellitus [1]. It is a reversible dipeptidyl peptidase 4 (DPP4) inhibitor. DPP4 inhibitors facilitate glucose-dependent insulin secretion. Moreover, glucagon release decreases, gastric emptying slows and appetite reduces by increasing the levels of active glucagon-like peptide 1 (GLP-1) [2]. To date two liquid chromatography with tandem mass spectrometry (LC–MS/MS) methods were developed and validated for SGX in plasma, one of them describes the assay for human plasma [3] and the other one is for rat plasma [4]. However, they are fairly sensitive, accurate and precise methods, a simplier and more cost reduced method is required for the analysis of SGX in human plasma.

For this aim an HPLC method with fluorimetric detection has been developed and validated for human plasma assays. The plasma levels of the drug was monitorized in order to investigate the pharmacokinetic parameters. The fluorimetric detection is provided by a pre-column derivatization process with a fluorogenic reagent; fluorescamine, which is frequently preffered because of its basic derivatization procedure, fast reactivity and high sensitivity [5],[6],[7],[8]. The derivatization reaction is shown in Figure 1.
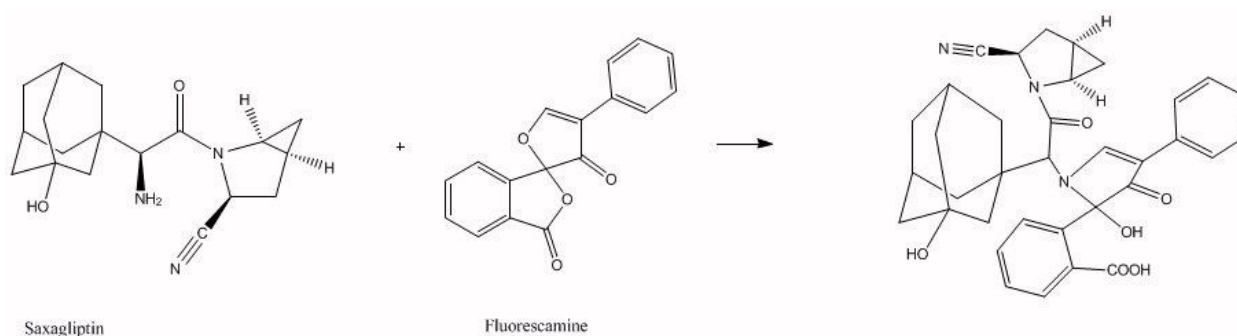

Figure 1. The derivatization reaction process between SGX and fluorescamine

MATERIALS AND METHODS

Chemicals and Reagents

SGX was supplied from Shanghai Yingxuan Pharmaceutical Science & Technology (China), Onglyza® tablets containing 5 mg SGX were purchased from local drug store. Acetonitrile, orthophosphoric acide (HPLC grade) and hexane, isopropanol (analytical grade) were supplied from Merck (Darmstadt, Germany). Fluorescamine was obtained from Sigma (MO, USA). Water was purified by Human (Japan) ultrawater purification system.

Solutions

A stock solution of SGX (0.1 mg/ml) was prepared in water and diluted with water to give standard solutions of 0.05, 0.5, and 1 µg/ml.

Borate buffer was prepared by dissolving 0.620 g boric acid and 0.750 g potassium chloride in 100 ml water. The pH level was adjusted to 8.5 with 0.1 M sodium hydroxide solution, and the volume was made up to 200 ml with water. All solutions except fluorescamine, were stored at 4ºC and were stable at least for 2 weeks. Fluorescamine solution was freshly prepared in acetone at 1 mg/ml concentration.

Instrumentation

The HPLC analyses were performed on a Shimadzu (Japan) LC 20 liquid chromatograph which consisted of a LC-20AT pump, SIL AT-HT autosampler part, a SPD-20A HT UV detector, which was set at 378 nm for excitation and 463 for emission nm and CTO 10 AC column oven were used. Chromatographic separation was achieved isocratically at 40ºC on a GL Sciences (Japan) C18 (ODS) column with the dimensions; 4.6 mm I.D, 150 mm length and 5 µm particle size. The mobile phase was acetonitrile-10 mM orthophosphoric acid (pH 2.4) containing with a flow rate of 1.3 ml/min.

Optimization Studies for Experimental Parameters

The different experimental parameters affecting the development of the reaction product were carefully studied and optimized. Such factors were changed individually while others were kept constant. These factors were: pH, concentration of the reagent, temperature and heating period, acetone-water ratio in the reaction medium. According to the results pre-column derivatization procedure was conducted.

Sample Preparation and General Procedure

5 ml venous blood samples were collected from peripheral veins of a volunteer (informed consent form was obtained according to ethical commitee approval) into tubes containing disodium EDTA and centrifuged at 4500 × g for 10 min. The resultant plasma samples were stored at −20°C. To extract the drug from the plasma samples, 0.5 ml plasma was alkalinized with 250 ml 0.1 M NaOH, and the solution was then extracted into 3 ml of hexane-propanol (2:1, v/v). The contents were mixed with vortex mixer at moderate speed for 5 min and centrifuged at 4500 × g for 3 min. The aqueous layer was discarded. The organic layer was evaporated to dryness under a stream of nitrogen at 40ºC. To the residue, 1 ml water, 500 µl pH 8.5 borate buffer and 500 µl 1 mg/ml fluorescamine solution were added. The solution was vigorously mixed with a vortex mixer for 30 sec. and 20 µl of the derivatized sample was injected into the HPLC system.

RESULTS

Derivatization and Separation

Reaction conditions of SGX with fluorescamine were investigated. The optimum reaction time, temperature, pH, buffer type, proportions of acetone-water, and mole ratio of fluorescamine/SGX were determined.

Effect of pH

The reaction of fluorescamine with primary amines has been shown to be strongly pH dependent [9]. It was noticed that the fluorescence emission was developed only in alkaline medium by using borate buffer 5-8 and therefore, study of the pH was restricted to within the range 7-11 using borate buffer. Maximum absorbance was obtained at pH 8.5.

Effect of time and temperature

In order to determine the optimum temperature and time required for the reaction, the derivatization reaction was carried out at different temperatures and durations. It was found that the fluorophore was formed immediately at room temperature. It is an advantage that fluorescamine does not require any heating procedure or long reaction time.

Effect of fluorescamine concentration

The effect of fluorescamine concentration on the derivatization reaction was studied. It was found that 1.3×10^{-3} mmol (0.5 ml of 1% (w/v)) fluorescamine solution was sufficient to obtain maximum fluorescence. The effects of these parameters on fluorescence intensity of the derivatives are shown in Figure 2.
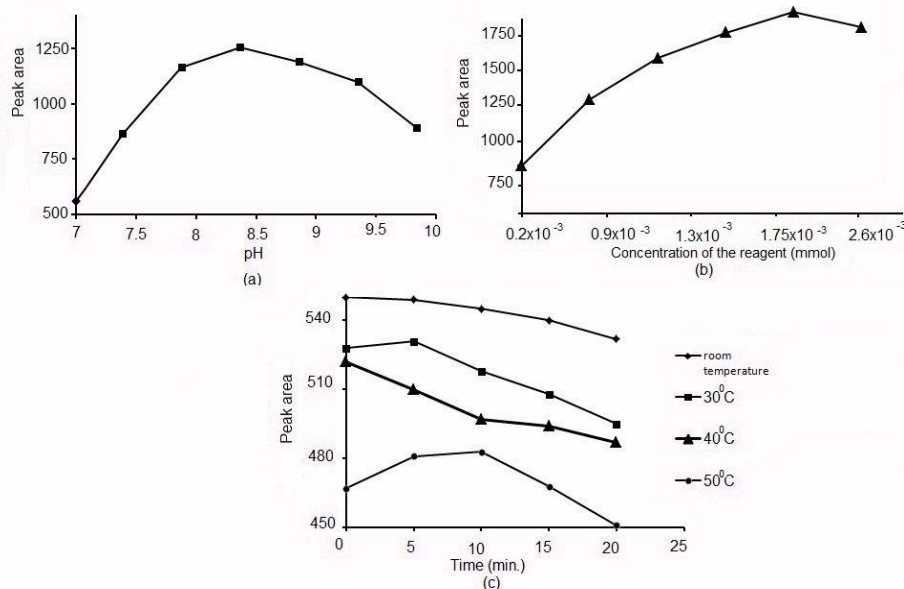

Figure 2. a. The effect of pH on the development of the reaction product of SGX with fluorescamine.

b. The effect of fluorescamine concentration on the development of the reaction product of SGX with fluorescamine.

c. The effect of temperature and time of heating on the reaction between SGX with fluorescamine.

Effect of acetone to water ratio in derivatization medium

Different volumes of acetone and water, were trialed where the concentrations of drug, buffer and fluorescamine solutions were kept constant. The maximum peak area was observed by using a ratio of acetone to water as 1:1.

Stoichiometry of the reaction

The molar ratio of fluorescamine to SGX in the reaction mixture was studied according to Job's method of continuous variation [10]. Utilizing equimolar solutions of SGX and fluorescamine, the reaction stoichiometry was found to approximate to a 1:1 ratio (reagent/drug). According to peak areas, it is correct to say that all of the reagent is consumed, and there is no shortfall or excess of the reagent in this stoichiometric ratio.

Upon testing derivatization reactions, all solutions were injected into the HPLC system and peak areas were measured to find the optimal conditions. Derivatives, prepared under the above mentioned conditions, remained stable for at least 24 h.

A good separation of the derivatives and endogenous compounds of plasma was obtained using an isocratic elution system and RP-HPLC as described above. Representative chromatograms of the blank plasma and plasma samples spiked with SGX (50 ng/ml) are shown in Figure 3a and b, respectively. No interference was detected with the plasma constituents. The retantion time of SGX is about 3.5 min.
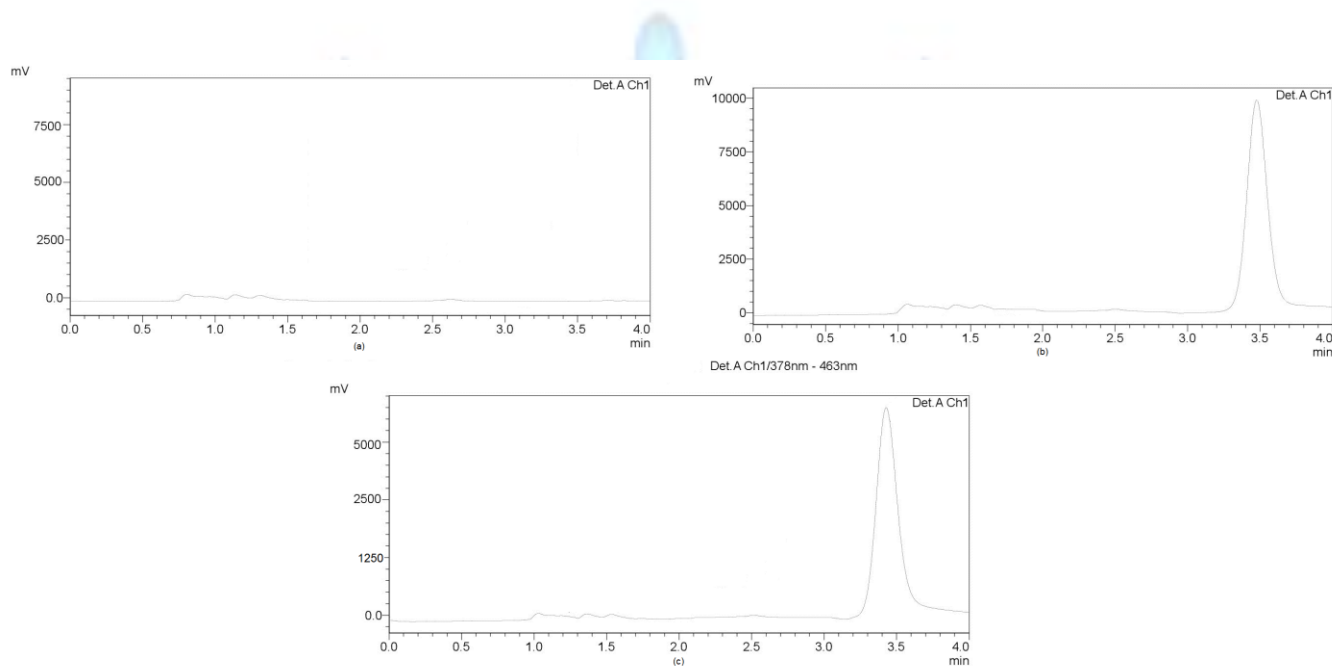

Figure 3. Chromatograms obtained from an extract of (a) human blank plasma (b) human blank plasma spiked with 50 ng/mL SGX (c) plasma samples from the volunteer after 2 h after a single oral dose of 5 mg drug containing 23.5 ng/mL SGX

Validation of the method

Validation of the method was carried out according to the following guidelines given by the International Conference on Harmonization (ICH) [11].

Calibration and sensitivity

Calibration curves were obtained using linear least-squares regression analysis by plotting of peak areas of the derivatives versus the corresponding SGX concentrations. The mean equation of the calibration curve (n = 6) obtained from five points was: $y = 0.123x + 0.3472$ (correlation coefficient = 0.9996) where y represents peak area of SGX-fluorescamine derivative and x represents the concentration of SGX.

The limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined using the formula: LOD or LOQ= kSDa/b, where k=3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The parameters for the analytical performance of the proposed method are summarized in Table 1.

Table 1. Analytical parameters of the method

Parameters	Method
Concentration range [a] (ng/ml)	3-100
Regression equation[b]	
Intercept± SD	0.3472±0.097
Slope± SD	0.123±0.024
Correlation coefficient (r^2)	0.9996
LOD (ng/ml)	0.15
LOQ (ng/ml)	0.5

[a] Average of six determinations

[b] $y=xC+b$ where C is the concentration in ng/ml and y is the peak area

Accuracy, precision and recovery

Accuracy and precision were assessed by determination of the QC samples at three concentration levels, (10.0, 50.0, and 75.0 ng/ml) that can be classified as low, medium and high concentrations were prepared. The accuracy was expressed by recovery values and RME and the precision by RSD. The absolute recovery of SGX from plasma was examined by extraction and derivatization of spiked plasma samples and comparison with peak areas obtained after derivatization of the same amounts of aqueous unextracted SGX solutions. The mean absolute recovery of SGX were of 89.17%. The relative recovery was calculated as 97.37% by the comparison of the amounts that is added on to plasma and measured by the calibration curve.

Six replicates of samples at each concentration were assayed on the same day for intraday and on six different days for interday precision and accuracy. The RSD values of both intraday and interday assays were all less than 2.92%. According to all these results summarized in Table 2 good precision and accuracy were observed.

Table 2. Accuracy and precision of the method

Added concentration (ng/ml)	Found concentration (ng/ml) (Mean±SD[1])	Recovery (%)	RSD of recovery	RSD of intraday variation	RSD of interday variation
10.00	9.53 ± 0,09	95.30	0.66	2.80	2.92
50.00	48.86 ± 0,42	97.72	1.25	2.25	2.76
75.00	74.33 ± 0,35	99.10	1.45	1.67	1.72

Mean relative recovery = 97.37

Robustness

Robustness was assessed by changing the flow-rate, column oven temperature and acetonitrile and water phase contents of the mobile phase. The mobile phase proportions were changed from 30:70 (acetonitrile-acidic solution) to 35:65 and 25:75; column temperature was changed from 40ºC to 35ºC and 40ºC; and the flow rate was changed from 1.3 to 0.8 and 1.8 ml/min. These changes had no significant effect on peak areas.

Stability

The effects of freezing and thawing on SGX concentrations were studied using spiked SGX plasma standards at 10, 50, 75 ng/ml, which were subjected to four freeze–thaw cycles before analysis. The stability of derivatives in spiked plasma stored at room temperature for 24 h and –20°C for 2 weeks was evaluated, as well. Stock solutions of SGX were stable at least for 30 days when stored at −20ºC. After 30 days no decrease was observed in the concentration of SGX in plasma.

Derivatized solutions were found to be stable for 4 days, if the samples were kept at 4°C using a sample cooler, 1 day at room temperature.

Application of the Method to Pharmacokinetic Analysis

The proposed method was applied to the determination of the drug substance in plasma for the pharmacokinetic studies. A healthy 29 year-old male volunteer was administered a single oral dose of SGX (5 mg). Approximately, 5 ml venous blood samples were collected prior to dosage and 1, 2, 3, 4, 6, 8, and 10 hours afterwards on the first day. For the following days, blood samples were collected once a day for 10 days. The blood samples were processed to plasma as described above. Figure 3c shows a chromatogram of the plasma sample obtained 2 h after the single oral dose of 5 mg SGX from the volunteer. The samples were stored at –20°C until analysis.

Pharmacokinetic analysis was carried out using standard methods. Area under the plasma concentration-time curves (AUC_{0-96}, $AUC_{0-\infty}$) were calculated using the TOPFIT 2.0 pharmacokinetic and pharmacodynamic data analysis system [12]. A plasma concentration-time curve of SGX after an oral administration of a single dose of 5 mg of drug is shown in Figure 4. Pharmacokinetic parameters, which are given in Table 3, are identical to those previously found [2],[13].
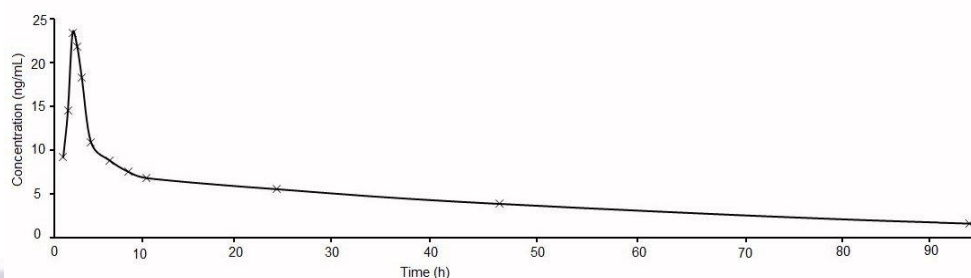

Figure 4. Pharmacokinetic curve of SGX between 0 and 96 hours after administration of 5 mg oral dose

Table 3. Pharmacokinetic parameters of sgx after administration of single oral dose of 5 mg

Parameter	Found value
$T_{max}{}^{a}$ (h)	2
$C_{max}{}^{b}$ (ng/ml)	23.5
$t_{1/2}{}^{c}$ (h)	2.5
$AUC^{d}{}_{0-96}$ (ng.h/ml)	71.6
$AUC^{d}{}_{0-\infty}$ (ng.h/ml)	1075

[a]Time to maximum concentration

[b]Maximum concentration,

[c]Elimination half life,

[d]Area under the concentration-time curve

DISCUSSION

The main advantage of the proposed method is the ability to determine SGX in human plasma with a lower cost than other techniques [3],[4]. It is difficult to apply those MS methods for routine analysis, because they require high cost equipment and sophisticated operator which are not generally available in routine laboratories. The proposed method is very sensitive with a 0.5 ng/ml LOQ value. This is very important for SGX because the drug has very low plasma concentrations in the pharmacokinetic studies. The other advantages are related to simplicity of the derivatization process and extraction procedure from plasma with high recovery values. Generally, fluorogenic derivatization require heat and longer time. In this method there is not any time consuming procedures. The retantion time of the drug substance is about 3.5 min, which shows the duration of the analyse is also very short. The presented method can certainly be used for bioequivalence and bioavailability investigations and routine analysis of the drug in plasma.

ACKNOWLEDGMENTS

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Projects

A New Spectrophotometric Method for the Determination of Maprotiline- Istanbul University Research Foundation Department- 2003

Development of a new HPLC Method for the Determination of Memantine in Human Plasma and Investigating the Pharmacokinetics of the Drug - Istanbul University Research Foundation Department- 2007

Development of an HPLC-UV method for the analysis of drugs used for combined hypertension therapy in pharmaceutical preparations and human plasma - Bezmialem Vakif University Research Foundation Department -2011

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