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Research Article

Enantioselective RP-UFLC Method for the Simultaneous Estimation of Sitagliptin (STG) Enantiomers (R and S) in the Racemic Mixture and their Pharmacokinetic Assessment in Male Wistar Rats

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ABSTRACT

The present work aims to develop and validate a simple, rapid, and reproducible reverse phase ultra-fast liquid chromatography method with a UV detector (RP-UFLC-UV) was developed for the separation and determination of sitagliptin (STG) enantiomers (S-(STG) and R-(STG)) simultaneously. Baseline separation was achieved on Lux cellulose-1 column, (cellulose tri-(3,5-dimethyl phenyl carbamate (Chiralcel OD-RH, 250 mm × 4.6 mm, 5 µm) column and mobile phase consisted of 20mM borate buffer solution (pH = 9±0.05) and ACN in the ratio of (60:40, v/v) at a flow rate of 0.8 mL/min was used. Detection of peaks was monitored at 262 nm. For analyzing the STG enantiomers in the rat serum and pure API, a method was developed using the chiral RP-UFLC-UV method was validated. The single oral dose of 2.5 mg/kg of STG racemate was administered to a group of 6 healthy rats for a comparative pharmacokinetic study of both the enantiomers. The linear range of the calibration curve for each enantiomer was 0.5–64 µg/mL. The precision of this method at concentrations between 0.5–48 µg/mL was within 8.65% and the % recovery (accuracy) of both sitagliptin (STG) enantiomers (S-(STG) and R-(STG) were 98.47–101.02% and 98.93-100.52%. The precision at LLOQ (0.5 µg/mL) was between 8.65%-7.09%, which was poor than that at QC levels, and the average extraction recovery was higher than 85% for both sitagliptin (SGT) enantiomers at QC levels, and its pharmacokinetics of enantiomers was found to be stereoselective.

Introduction

Food & Drug Administration (FDA) recommended the development of a highly sensitive, reliable, and specific method for chiral drugs.[1] Chiral drug's quantitative isomeric composition with a chiral center is important for pharmacological, toxicological, and clinical studies, and also it is needed for the analysis of pharmacokinetics of single/mixture of enantiomer. Chiral compounds require the derivatization of enantiomers into diastereomers as they cannot be separated by traditional High-pressure liquid chromatography (HPLC) stationary phases. In the initial stages of drug development itself, quantitative assays for enantiomers should be developed by manufacturers. Over the past fifteen years, powerful chiral stationary phases (CSPs) have become available (Pirkle-type, protein-based, cyclodextrin, cellulose/amylose, etc.) that allow direct separation and quantitation of enantiomers without the need to convert them to diastereomers. Each type of CSP has specific advantages, and no single CSP can claim a universal application.[2]

Type 2 diabetes mellitus (T2DM) is a concerning problem worldwide. Approximately 8% of the adult population is being affected. It is estimated that there will be 400 million cases by 2030.[3] Sitagliptin (STG) ((2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4] triazolo[4,3-a] pyrazin-7 (8H)-yl]- 1-(2,4,5- trifluorophenyl)butan-2-amine) (Fig. 1) is prepared synthetically by chiral Mannich

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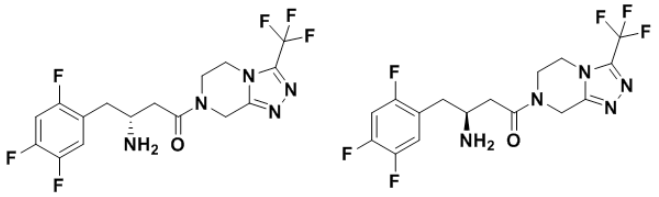

R-SITAGLIPTIN S-SITAGLIPTIN

Fig. 1: Chemical structure of R-Sitagliptin and S-enantiomer of sitagliptin

adducts obtained from primary alkyl substrates. It was highlighted by the first organ catalytic, coupling-reagent-free synthesis of the antidiabetic drug (-)-(R)-sitagliptin.

The phosphate salt of (-)-(R)-sitagliptin, which incorporates an aliphatic chiral amide backbone, is commercially available as a potent, orally active, and as selective Dipeptidyl peptidase-4 (DPP-4) inhibitor for the treatment of T2DM.[4-6] Therapeutic activity of STG is assigned specifically to the (*R*)-enantiomer while the (*S*)-enantiomer is an impurity.[7] STG increases glucose-dependent insulin secretion, decreases abnormal glucagon secretion, decelerates stomach motion, increases the β-cell number, and reduces appetite.[8-11]

The glycemic control and regulation of the growth of insulin-producing β-cells in pancreatic islets are improved by sitagliptin. Sitagliptin has a bioavailability of 87% and exhibits low reversible binding to plasma proteins. The pharmacological activity of sitagliptin is assigned specifically to R-enantiomer, and the plasma binding capacity of two enantiomers differs significantly, influencing its efficacy.

Thus, monitoring of undesired S-enantiomer of sitagliptin in biological matrices is of great importance because of different pharmacological/toxicological properties and the possibility of different metabolism.[12–14]

A thorough literature search has revealed that liquid chromatography-tandem mass spectroscopy (LC-MS/MS) has been widely used to determine sitagliptin (STG) in biological fluids such as plasma, urine, and hemodialysate of rats and humans.

LC-MS/MS method for determination of STG in human plasma, urine, and hemodialysate was reported indicating clinical pharmacokinetic studies.[15, 16] Different methods are reported using liquid-liquid extraction (LLE) followed by LC-MS/MS to determine sitagliptin enantiomer in human plasma.[17,18] HPLC determination of STG in rat plasma and urine was reported using molecularly imprinted polymer-based solid phase extraction [SPE] technique.

Liquid chromatography (LC), LC-MS, and Laser diode thermal desorption (LDTD-MS/MS) methods for simultaneous identification and quantification of oral hypoglycemic drugs, including metformin, in pharmaceutical preparations plasma and dried blood spots were also reported.[19–22]

In addition to LC-MS, spectrofluorimetry (SF) using fluorescamine as the derivatization reagent was also used to determine STG in tablets and serum samples. [23] Analysis of STG interaction with β-cyclodextrin derivative by capillary electrophoresis is also reported.[24] Another method involved pre-column derivatization of STG with *o*-phthalaldehyde (OPA) and *N*-acetyl-*L*-cysteine to form diastereomeric derivatives separated on a C18 column detected using (SF) detection with excitation at 330 nm and emission at 450 nm.[25]

However, all these methods were achiral in nature and do not address the separation of STG enantiomers from the racemic mixture. A selective pharmacokinetic study was almost nonexistent by the direct reverse-phase UFLC-UV method. Pharmacokinetics of enantiomers may be stereoselective,[26,27] and chiral inversion may occur[28,29] *in vivo* or *in vitro* hence the pharmacokinetic and chiral inversion studies of enantiomers become considerable.

The separation of enantiomers in SGT depends on interaction between solute and polar carbamate groups present in CSP. The interaction is through hydrogen bonding between C=O and NH group present in the CSP with C=O and OH group in the STG drug molecule.[30,31]

Hence, to the best of our knowledge, no chiral LC method by direct analysis of sitagliptin (STG) enantiomers in rat serum has been reported so far in the literature. Hence, this study was undertaken. The UFLC-UV method on the chiral stationary phase (CSPs) was established to separate and determine STG enantiomers. Based on the optimization of operation parameters, a simple, rapid, selective, and sensitive method was developed and validated. After oral administration, the method was used for pharmacokinetic studies of enantiomers in rat serum. According to International Council for Harmonization (ICH) guidelines, the developed method was validated. [32]

Materials and Methods

Chemicals and Reagents

Sitagliptin (STG) enantiomers (S and R form) were obtained as gift samples from Novartis, India. The pure compound of rosiglitazone (Internal standard, IS, purity: 99.9%) was obtained from JiGS Chemical (Gujarat, India). Disodium tetraborate decahydrate (BORAX) was purchased from Finar Limited (Ahmedabad, Gujarat, India). HPLC grade methanol, ethanol, acetonitrile, and triethylamine (TEA) were purchased from Merck. All analytical grade reagents were used.

Instrument

The research work was conducted using Shimadzu LC-20 AD Ultra-fast Liquid Chromatography (UFLC) system equipped with a binary pump and UV detector. The instrument was controlled using LC Lab solutions software. Trials were conducted using polysaccharide-

based CSPs (Lux cellulose 1, 2, 3, and 4) for method optimization using reverse phase mode. Ultrasonicator is used for mixing the drug in a suitable solvent. Separation of serum from blood samples was performed using a centrifuge. Micropipettes with a measuring capacity of 100-1000µL were used. Serum samples were filtered using 0.25-µm syringe filters and stored at -20 ˚C in a deep freezer until analysis. The pH was measured using a pH meter (LI 127, Elico, India).

Chromatographic Conditions

Enantiomeric separation of sitagliptin (STG) was operated on Lux cellulose-1 (Chiralcel OD-RH (3,5-dimethyl phenyl carbamate) (250 mm × 4.6 mm, 5 µm) column using a mixture of a borate buffer (pH = 9±0.05) and ACN in the ratio of (60: 40, v/v) as mobile phase. The column temperature was maintained at 25°C; the flow rate was 0.8 mL/min. The wavelength for detection of the sitagliptin (STG) enantiomers peaks was set at 262 nm, and the injection volume was 20 µL.

Preparation of 20mM Borax buffer solution[33]

Accurately weighed 3.81grams of borax was taken and transferred into a 500 mL beaker. The content was dissolved with ultrapure water, and the pH (9 ±.005) of this solution was adjusted to (9 ± 0.005) with 0.1% triethylamine (TEA). The mobile phase was prepared by mixing acetonitrile and buffer solution in a specific ratio, followed by degassing on an ultrasonic bath.

Preparation of Standard Solutions

Standard stock solutions of enantiomers [1-mg/mL] and internal standard (IS) [1 mg/mL] were prepared. By diluting the stock solutions, concentrations of 100µg/ml were prepared for both enantiomers and internal standards (IS). The prepared solutions were preserved at 4°C and were brought to room temperature before analysis.

Sample Preparation

Sample preparation was performed by protein precipitation method. Aliquots of 100 µL serum and 10µL of working solution with 350 µL methanol were put into a centrifuge tube, then vortex-mixed for 5 minutes. After centrifugation at 16000 rpm under 4 °C for 10 minutes, the supernatant was transferred into another tube and evaporated in a vacuum oven. The residue was re-dissolved in 100 µL methanol, then vortexed for 2 minutes and centrifuged at 16000 rpm under 4 °C for 10 minutes. The supernatant was injected into the UFLC-UV system for analysis.

Calibration Curve and Quality Control (QC) samples

Calibration curves were prepared by spiking blank rat serum with the appropriate amount of enantiomers & internal standard. Then the prepared samples were extracted as per the procedure described above in sample preparation. The final serum concentration levels were 0.5, 1, 2, 4, 8, 16, 34 and 64 µg/mL. QC samples were prepared by mixing the blank serum with the known amount of concentration followed by extraction, which resulted in concentrations equivalent to 0.5, 1.5, 32, and 48 µg/mL.

Method Validation

The method was validated according to the bioanalytical method validation guidelines as per FDA. [34]

Specificity and Selectivity

This was assessed by injecting six different blank rat serum with *rac*-sitagliptin and IS at LLOQ level. Peak areas of coeluted peaks at LLOQ less than 20% of the peak area of the drug peak were obligatory.

Linearity

The sample processing was the same as described in sample preparation. Calibration curves were fitted to the equation y = mx + c, where x represents the plasma concentration of enantiomer, and y refers to the peak area ratio of enantiomer to IS dependent on the plasma concentration.

Sensitivity

It is universally received that LLOQ is defined as the lowest concentration in the linearity curve. The LLOQ concentration was injected 5 times in three replicates into the UFLC system. The analyte response 5 times more than the drug response of zero calibrators was compulsory.

Precision and Accuracy

The intra-batch precision and accuracy were measured by analyzing six serum samples spiked with enantiomers at each QC level (Lower limit of quality control (LLQC), limit of quality control (LQC), Middle-quality control (MQC), and Higher quality control (HQC)) in a batch and the inter-batch precision. For determination of accuracy, three replicate serum samples spiked with enantiomer were analyzed at each QC level in three consecutive batches on different days. The precision (relative standard deviation (RSD)) and accuracy (the percentage of the actual concentration over the theoretical concentration) should not exceed 15%.

Extraction Recovery

The extraction recovery of enantiomers was determined by comparing peak area ratios of enantiomer to IS obtained from extracted serum samples (n = 6) with working concentration solutions (n = 2). This was investigated with four different concentrations (0.5, 1.5, 32, and 48 µg/mL).

Robustness

Robustness was determined by evaluating any small influence in the chromatographic conditions like changing flow rate, mobile phase composition, and column temperature. The retention and resolution between two

enantiomers were used to evaluate the separation under modified conditions.

Stability

The stability of enantiomers in rat serum for the duration of storage and sample processing was investigated through analyzing samples in six replicates at two QC levels. All analyte concentrations within the stability study samples have been measured by the use of freshly prepared stock solutions. A freshly prepared sample was measured immediately after sample extraction. The short time and residue stability were analyzed after 12 hours at room temperature and at 4 °C, respectively. The freeze-thaw stability was assessed by analyzing the samples frozen (-20 °C, 12 hours) and thawed (room temperature) in cycles for two times. The accuracy should be in the range of 80 to 120%, and the precision should be less than 15% for all samples at QC levels.

Method Applications (Pharmacokinetic Studies)

The pharmacokinetic study of enantiomers was evaluated using six healthy male Wistar rats (weighing 180–250 g) for *in vivo* bioavailability study. Animals were fasted overnight before the study and had free access to only drinking water. The studies were conducted with prior approval by the Institutional Animal Ethical Committee (File No: IAEC/10/UCPSc/KU/2020). After overnight fasting, each rat was orally administered 10 mg/kg body weight with the racemic mixture by diluting it with a normal saline solution (2.5 mg/mL) using an oral feeding tube. Blood samples (About 0.3 mL) were collected from the orbital venous plexus into Eppendorf tubes at 0 h (pre-dose) and 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 84, and 96 hr time points after drug administration. The serum was separated from blood by centrifugation process at 5000 rpm for 10 min and then stored at -20 °C until analysis.

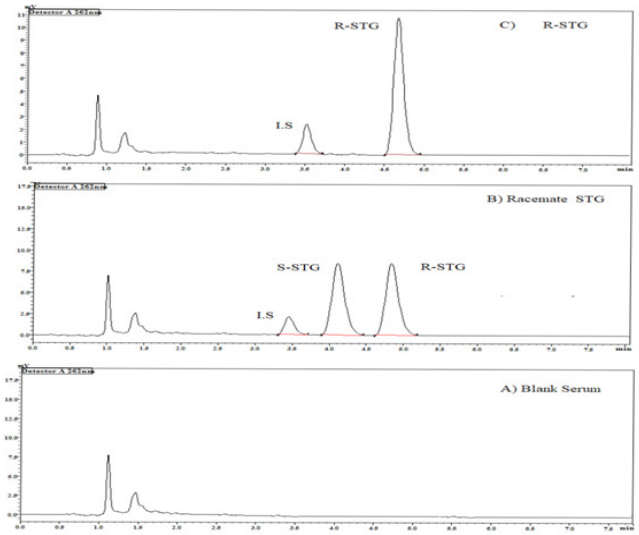

Fig. 2: Representative chromatograms of enantiomers and the IS resulting from analysis of A) a blank serum, B) rat serum spiked with racemic STG, and C) R-STG with internal standard (IS) at HLOQ.

Pharmacokinetic and Statistical Analysis

Drug and Statistics (DAS) 2.0 software was used for calculating the pharmacokinetic parameters based on mean serum concentration at respective time points. GraphPad Prism 6.0 was used to validate results statistically. A parametric model was used to estimate Pharmacokinetic parameters of enantiomers.

Results

Method Development

The mobile phase plays a major role in the separation of enantiomers in sitagliptin using polysaccharide-based chiral stationary phase (CSPs). The separation of sitagliptin enantiomers was conducted using reverse phase mode. The separation of enantiomers in SGT depends on an interaction between CSP's solute and polar carbamate groups. The interaction is through hydrogen bonding between C=O and NH group present in the CSP with C=O and OH group in the STG drug molecule.[30]

After trying with various combinations of Borax buffer and ACN at different concentration levels, the mobile phase consisting of a mixture of 20mM Borax buffer solution (pH 9±0.05) and ACN (60:40, v/v) was selected and determined in reverse phase mode at a flow rate of 0.8 mL/min at ambient temperature. (Fig. 2) shows the typical chromatogram A) a Blank serum, B) rat serum spiked with racemic STG, and C) R-SGT at HLOQ. The chiral impurity (S-SGT) was well resolved from drug substances (R-SGT) with a resolution factor greater than 4.0, and typical retention times of internal standard, S- and R-SGT enantiomers were 3.5, 4.06, and 4.66 min, respectively. The values of retention factor (k: 1-10) and resolution factor (Rs >1.5) were within specified limits.

Method Validation

Specificity and Selectivity

The specificity of the method was investigated by analyzing blank rat serum samples and the serum spiked with enantiomers and IS. The endogenous substances present in the blank serum sample did not show any interference with the analyte of interest. A better separation was achieved within 7.0 min; the retention time of internal standard, S-STG, and R-STG were 3.5, 4.06, and 4.66 min, respectively. (Fig. 2)

Linearity and Sensitivity

The calibration curves were prepared by assaying standard rat serum samples at eight concentration levels of 0.5, 1, 2, 4, 8, 16, 34, and 64µg/mL. The calibration curves for enantiomers were described by the equation: $y = 0.758x + 0.175$ ($r^2 = 0.999$, R-STG) and $y = 0.744 x + 0.309$ ($r^2 = 0.999$, S-STG), where y corresponds to the peak area ratio of enantiomer to IS, and x refers to the concentration of enantiomer in serum (µg/mL). LLOQ

value for both enantiomers was shown to be 0.5 µg/mL. The precision was observed to be within 20% on injecting five standard injections (0.5 µg/mL). The results proved the sensitivity of the developed method can be applied to pharmacokinetic studies in the rat. (Fig. 3)

Precision and Accuracy

The % RSD values of within-run precision ranged from 1.07% to 8.65% for (*S*)-sitagliptin (STG) and 0.15 % to 5.60% for (*R*)-sitagliptin (STG). The between-run precision values ranged from 1.19 % to 7.09% for (*S*)-sitagliptin, S-(STG) and 1.34% to 4.98% for (*R*)- sitagliptin (STG). The consistency of this data indicated the reliability of the developed method. The results for accuracy and precision in terms of LLOQ, LQC, MQC, and HQC are summarized in Tables 1 and 2.

Recovery

The extraction recoveries of (*S*)-sitagliptin (STG) from rat serum were 98.47, 101.02, and 100.53% at LQC, MQC, and HQC, respectively. For (*R*) - sitagliptin (STG), the extraction recoveries at the same QC levels were 98.93, 100.07, and 100.52 %, respectively (Table 2). The reported results confirmed that the protein precipitation method (using methanol) as a successful method with reliable recovery results for the analysis of both sitagliptin enantiomers.

Robustness

Robustness was checked to evaluate any small influence in the chromatographic conditions by changing flow rate, mobile phase composition, and column temperature.

Table 1: The inter- and intra-batch accuracy and precision for the determination of Sitagliptin enantiomers.

Concentration (µg/mL)	*(S)- (STG) - enantiomer*	*(R)- (STG) - enantiomer*
% RSD (within run)		
LLQC(0.5)	8.65	5.60
LQC(1.5)	2.67	3.64
MQC(32)	1.07	0.15
HQC(48)	1.12	1.23
% RSD (between run)		
LLQC (0.5)	7.09	4.98
LQC (1.5)	3.43	2.62
MQC (32)	1.26	1.341
HQC (48)	1.19	1.67

Table 2: Extraction recoveries of sitagliptin (STG) enantiomers in rat serum

Quality control sample (µg/mL)	*%Recovery*	
	(S)- (STG)	*(R)- (STG)*
LQC (1.5)	98.47%	100.52%
MQC (32)	101.02%	100.07%
HQC (48)	100.53%	98.93%

The resolution and retention between two enantiomers were used to evaluate for the separation under modified conditions. The resolution of enantiomers was greater than 4.0 under all conditions Table 3.

Stability

The % RSD values for stability testing of sitagliptin enantiomers were reported to be within the acceptance criteria as per the bioanalytical guidelines, as shown in Table 4. The results proved the good stability of sitagliptin enantiomers during the study period without racemization or conversion. The method is thus confirmed to be helpful in routine analysis.

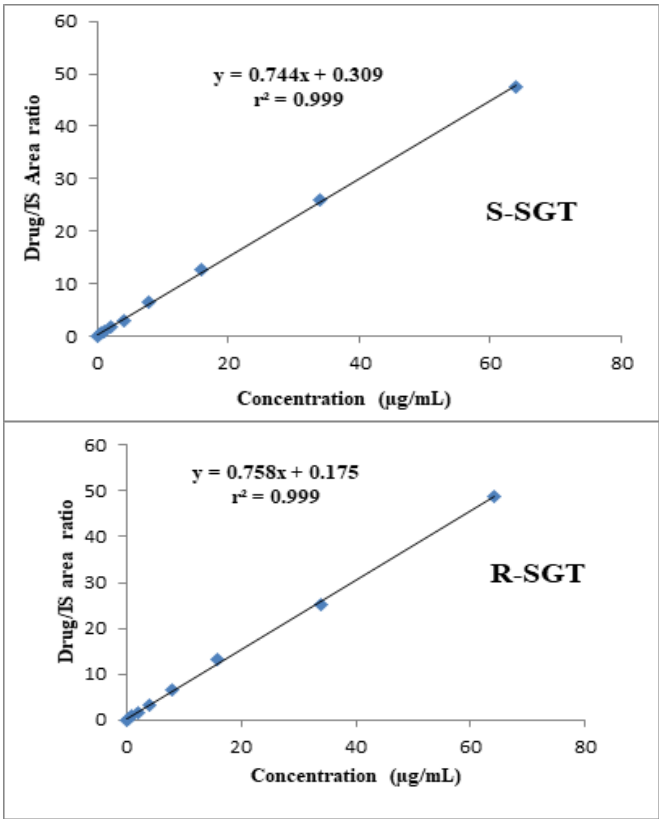

Fig. 3: Linearity plot of R-STG and S-STG enantiomers in rat serum

Table 3: Robustness of the method under different conditions

Flow rate (mL/min)	*ACN (%)*	*Column (temperature)°C*	*Resolution (R_S)*
0.6	15	25	4.45
0.8	15	25	4.28
1	15	25	5.22
1.2	15	25	3.65
1	10	25	5.12
1	15	25	4.35
1	15	25	3.56
1	15	25	4.15
1	15	20	4.75
1	15	30	5.25

Table 4: Stability testing of sitagliptin (STG) enantiomers

	% Accuracy		*% RSD*	
Concentration (µg/mL)	*(S)- (STG)*	*(R)-(STG)*	*(S)-(STG)*	*(R)-(STG)*
Bench-top stability				
LQC (1.5)	98.47	100.07	2.61	3.43
HQC (48)	100.053	98.93	1.67	1.18
Long-term Analyte stability (after 30 days)				
LQC (1.5)	99.58	99.9	2.67	3.64
HQC (48)	99.83	98.56	1.12	1.23

Table 5: Pharmacokinetic parameters for sitagliptin (STG) enantiomers

Parameters	*(S)- (STG)*	*(R)- (STG)*
T½ (h)	15.29 ± 0.16	11.82 ± 1.56
C-max (µg*/mL)	2.58 ± 0.02	3.97 ± 0.12
T-max (h)	4.0	4.0
AUC_{0-t} (µg*h/mL)	61.07 ± 0.17	102.45 ± 0.401
MRT	25.97 ± 0.06	25.65 ± 0.44

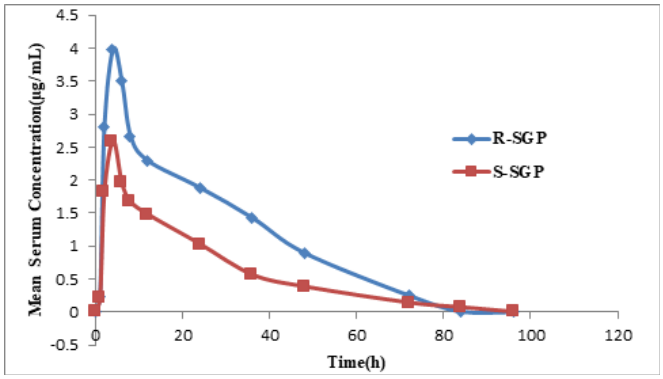

Fig. 4: Serum concentration-time curves of enantiomers in rat serum.

Stereoselective Pharmacokinetic Study

The validated UFLC method was applied for stereoselective pharmacokinetic study of sitagliptin (STG) enantiomers in rats after oral administration of 2.5 mg/kg of the racemic drug. The mean serum concentration versus time profiles of sitagliptin (STG) enantiomers over 96 hours (Fig. 4) and the pharmacokinetic parameters are summarized in Table 5. Statistical significance of different pharmacokinetic parameters between sitagliptin (STG) enantiomers was assessed by Student's unpaired *t-test* using Kinetica 2000 software (version 5.0, Innaphase Corporation, Philadelphia).

It was found that the pharmacokinetic parameters of R-STG were higher than S-STG in the rat; this indicates prominent variation in the stereo-specific behavior of enantiomers. The C-max and T-max values for (*R*)-sitagliptin (STG) are 3.97 µg/ml and 4 hours, respectively, whereas C-max and T-max values for (*S*)-sitagliptin (STG) were 2.58 µg/mL and 4.0 hours, respectively. The AUC_0 –*t* values were 102.45 and 61.07 µg*h/mL for (*R*) - sitagliptin (STG) and (*S*)-sitagliptin (STG), respectively.

Discussion

Authors aimed at the development of sensitive bioanalytical enantioselective RP-UFLC method in rat serum after oral administration of measured racemic STG. Most of the previous literature reported the analysis of STG by using indirect achiral methods. *Rao et al.*[18] analyzed STG enantiomers in rat plasma by an indirect, achiral method using C18 column connected to a fluorescence detector. Another method by *Rao et al.*[25] employed the use of molecularly imprinted polymer for selective extraction followed by LC determination in rat plasma and urine. In comparison, the present optimized method was simple, direct, and chiral (carried on chiral cell OD-RH column) without using any derivatization reagent. The % recovery was comparable with the present method (>98%). The reported methods also include analysis by capillary electrophoresis to study the interaction of STG with β-cyclodextrin derivative.[24] LC-MS/MS LDTD MS/MS techniques were also used to analyze STG in the biological matrices.[15,16,19,21]

Hess et al. [19] reported % recovery of STG to be 79.90-85.25% in human plasma by LCMS/MS, whereas the present method found % recovery to be from 98.93 to 100.52%. *Zeng et al.* [15] and *Nirogi et al.* [16] carried out quantification of STG in human urine &hemodialysate using turbulent flow online extraction with tandem MS and LLE with LC/Tandem MS, respectively. The % recovery of both the LC-MS methods was similar to the recovery rate by the present method. In addition to the aforementioned observation, the chiral RP-UFLC method is cost-effective compared to LC/MS techniques.

The analytical RP-UFLC method was reported along with its validation.[35] Compared to all the reported works on sitagliptin, the proposed optimized method could be applied for bioanalytical analysis, i.e., pharmacokinetics studies. The impurity of S-SGT was resolved from the active enantiomer R-SGT. This kind of profiling is the important desideratum for chiral drug bioanalysis. The above-stated objectives formed the basis of the work. The retention times of the optimized method on chiral cell OD-RH column were found to be 4.06 & 4.66 min., which is better in comparison to the work carried out with amylose-based chiral stationary phase.[31,35]

The method separated sitagliptin's (S) and (R) enantiomers in rat serum. The concentration of organic modifier (ACN) and buffer strength (Borax) played an important role in achieving better retention and resolution between the enantiomers of sitagliptin.

The selectivity of enantiomers on the chiral cell OD-RH column was good, and no endogenous peaks from serum interfered with the enantiomer peaks. Sitagliptin enantiomers were extracted efficiently from rat serum by a simple protein precipitating process using methanol as a precipitating protein agent. The system suitability parameters like separation factor (α) and capacity factor (k) were improved than the reported methods. The method's sensitivity was found to be extremely low compared with reported methods. The technique was developed in reverse phase mode. The results will be more reproducible than the existing chiral indirect mode and reverse-phase mode on the achiral stationary phase.[17, 25]

The developed RP UFLC method established its sensitivity, precision & accuracy as the result of LLOQC, LQC, MQC, and HQC were by ICH guidelines. The resolution between the enantiomers in serum was exemplary, as it was above the accepted limit of >1.5 (i, e 4.0). Stability testing indicated the sturdy nature of the enantiomer. Student unpaired *t*-test proved the significance of the application of a developed bioanalytical method for stereoselective pharmacokinetic study in rat serum. The pharmacokinetic parameters were assessed to be better and comparable to an indirect achiral method.

A present developed and validated method is sensitive, specific, and reproducible to quantitate sitagliptin enantiomers in rat serum. This method can be further used to investigate the quantification of sitagliptin enantiomers in clinical drug monitoring and in studying the pharmacokinetic profiles.

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