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DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHIC ENANTIOMER SEPARATION METHOD FOR THE ESTIMATION OF (S)-ENANTIOMER IN SITAGLIPTIN

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ABSTRACT: A direct chiral separation method development was carried out for sitagliptin and its (*S*)-enantiomer on ten diverse chiral stationary phases. Chiral stationary phases, which were tested include, R, R Whelk-O1 column, macrocyclic glycopeptides namely, Chirobiotic R (Ristocetin A), Chirobiotic V (Vancomycin), Chirobiotic T (Teicoplanin), Chirobiotic TAG (Teicoplanin aglycone), OJ-H, OJ-RH belonging to tris (4-methylbenzoate) of cellulose. Chiral selectivity was observed on polysaccharide based CSP columns namely Chiralpak IA-3, Chiralpak IC-3 belonging to tris-(3,5-dimethylphenyl carbamate) of amylose, tris-(3,5-dichlorophenyl carbamate) of cellulose respectively and OD-H (tris-3,5-dimethylphenylcarbamate) of cellulose. Better enantioselective separation has been achieved on cellulose tris-(3,5-dichlorophenyl carbamate) column (Chiralpak IC-3), using IPA and n-hexane as mobile phase, both containing 0.05% ethylene diamine and at 0.5 mL/min flow rate. Detection was carried out at 266nm using PDA detector and column maintained at 35ºC. The method was validated for precision, accuracy, linearity and robustness. The advantages of the method are rapid equilibration and less solvent consumption due to short column length. Efficient enantio separation (Resolution 3.38) is due to small particle size of 3 µm. Therefore this method is suitable for chiral purity determination of sitagliptin and its (*S*)-enantiomer.

INTRODUCTION: Sitagliptin belongs to dipeptidyl peptidase-4 (DPP-4) inhibitor class, which is in use as an oral antihyperglycemic drug [1]. DPP-4 enzyme breaks down the incretins GLP-1 and GIP, the gastrointestinal hormones released in response to a meal. Sitagliptin works to competitively inhibit the enzyme DPP-4, preventing GLP-1 and GIP inactivation; thereby they increase the secretion of insulin and suppress the release of glucagon by the pancreas.

This drives blood glucose levels towards normal [2, 3]. This drug is used either alone or in combination with other oral antihyperglycemic agents for the treatment of type 2 diabetes mellitus. **Figure 1** shows the structure of (*R*)-sitagliptin.

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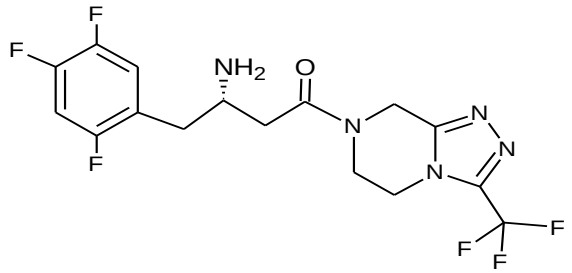

FIGURE 1: STRUCTURE OF (*R*)-SITAGLIPTIN

Sitagliptin, the active substance contains a chiral centre and is used as a single (*R*)-enantiomer for therapeutic purpose, therefore other (*S)*-enantiomer is regarded as an impurity. High performance liquid chromatography (HPLC) coupled with various detection techniques is widely used for the qualitative and quantitative analysis in separation science. There have been analytical and bio-analytical methods reported for the determination of sitagliptin alone using HPLC [4], LC-MS/MS [5], HTLC-MS/MS [6] and in combination with other antidiabetic drugs using HPLC/Q-TOF MS [7].

Sitagliptin analysis also has been reported with its degradation product and metformin using HPLC [8], with its N-acetyl impurity, metformin and metformin related impurities using UPLC [9]. So far there are only two published direct enantiomeric separation methods using AD-H column [10], an OD-RH column [11] and an indirect method [12].

We have developed and validated a chiral separation method which is superior in terms of efficiency and column ruggedness as the chiral selector is immobilized. The column we have used belongs to latest generation polysaccharide based chiral chemistry technology. As the particle size of this stationary phase is 3 µm, column efficiency has increased multiple folds leading to reduction in column length which in turn reduces the mobile phase consumption. In this case a resolution of 3.38 is obtained with 0.5 mL/min flow within a reasonable runtime.

EXPERIMENTAL:

Chemicals and Materials: Sitagliptin was isolated from Januvia™ tablets obtained from local pharmacy. Sitagliptin was racemized following a published procedure and has been characterized using NMR and mass spectroscopy, in-house.

Ultra high pure water was obtained from in-house Millipore Milli Q water system. Acetonitrile and methanol were purchased from JT Baker, USA. *n*-Hexane and Dichloromethane were procured from Merck, India. Ethyl alcohol was procured from Les alcohols, Canada. *n*-Heptane was procured from Qualigens, India. Ammonium acetate (Fluka, Germany), Trifluoroacetic acid (TFA), Ethylene Diamine (EDA) and Diethyl Amine (DEA) were procured from Sigma Aldrich, India. Methyl t-butyl ether was procured from Spectrochem, India. Isopropyl alcohol (IPA) was procured from Rankem, India.

Chiral Stationary Phases: Chiralcel OD-H, Chiralcel OJ-H with dimensions 250x4.6 mm, 5 µm, based on (tris-3,5-dimethylphenylcarbamate) of cellulose, tris (4-methylbenzoate) of cellulose respectively, Chiralcel OJ-RH with dimensions 150x4.6 mm, 5 µm, based on tris (4-methylbenzoate) of cellulose, Chiralpak IA-3, and Chiralpak IC-3 with dimensions 150x4.6 mm, 3 µm, based on tris-(3,5-dimethylphenyl carbamate) of amylose, tris-(3,5-dichlorophenyl carbamate) of cellulose respectively were procured from Daicel, Japan. R, R Whelk-O1 column with dimensions 250x4.6 mm, 5 µm, was procured from Regis Technologies, USA. Macrocyclic glycopeptides namely, Chirobiotic R (Ristocetin A), Chirobiotic V (Vancomycin), Chirobiotic T (Teicoplanin) and Chirobiotic TAG (Teicoplanin aglycone) with dimensions 100x4.6 mm and 250x4.6 mm, 5 µm, were procured from Astec Technologies, USA.

Instrumentation: HPLC separations were carried out using Agilent 1200 series HPLC systems equipped with diode array detector (G1315B), quaternary pump (G1311A), an on-line degasser (G1322A), auto-sampler (G1367B), with auto-sampler thermostat (G1330B) and a column thermostat (G1316A). Data acquisition and system suitability calculations were performed by Agilent chemstation software.

Chromatographic method: Final optimized normal phase separation method was developed on Chiralpak IC-3 column. The mobile phase was *n*-hexane, IPA, in the ratio of 40:60 respecively, containing 0.05% ethylene diamine.

The flow rate was adjusted to 0.5 mL/min, the UV detection wavelength was 266 nm and the column temperature was set to 35ºC. A sample volume of 10 µL was injected from each vial throughout the validation process. The enantiomeric separation chromatogram obtained with optimized conditions for racemic sitagliptin is given in **Figure 2**. Other chiral stationary phases mentioned in section 2.2 were used to explore chiral selectivity with various mobile phases compatible to the respective CSP.

Sample preparations: For method development samples were prepared in their respective mobile phase related solvents at a concentration of 1 mg/mL. For method validation the samples were prepared in IPA. Stock solutions of (*S*)-enantiomer were prepared at a concentration of 1 mg/mL. Precision, recovery and linearity samples were prepared from stock solution of (*S*)-enantiomer by serial dilution.

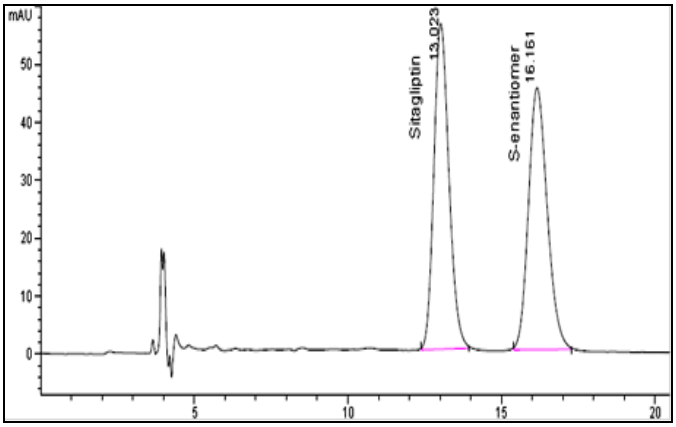

FIGURE 2: CHROMATOGRAM OF SITAGLIPTIN RACEMATE

Validation of the method: HPLC method was validated according to ICH guideline [13] for linearity, precision, accuracy and robustness. LOD and LOQ were determined at signal to noise ratio of 3 and 10, respectively for (*S*)-enantiomer. Further, LOQ concentration was confirmed by six replicate injections and measuring their area %RSD. Linearity of detector response was evaluated for six different concentrations starting with LOQ (1, 2, 4, 6, 8 and 10 µg/mL). These solutions were prepared by serial dilution of stock solution of (*S*)-enantiomer. Solution of each concentration was prepared in triplicate by serial dilution from stock solution and analyzed in six replicates for three consecutive days.

Linearity plot was established from concentrations and their respective mean peak areas. Correlation coefficient and *y*-intercept were calculated from the linearity plot.

The precision was determined by calculating %RSD of area and retention time of six replicates of 0.5% spiked (*S*)-enantiomer in 1 mg/mL solution of sitagliptin analyzed for three consecutive days.

Recovery study of (*S*)-enantiomer in presence of sitagliptin was performed to establish accuracy of the analytical method and evaluated at three concentrations i.e. 0.25%, 0.5% and 0.75% of (*S*)-enantiomer spiked with 0.5 mg/mL concentration of sitagliptin in triplicate.

The percent recoveries of (*S*)-enantiomer were calculated from the slope and *y*-intercept of the linearity plot. The method robustness was evaluated by deliberately varying experimental condition one at each time and monitored the resolution between sitagliptin and (*S*)-enantiomer.

RESULTS AND DISCUSSIONS:

Chiral separation method development: Several trials on various CSPs with different mobile phase combinations in reverse phase, polar organic and normal phase modes, with and without additives were carried out. However, glycopeptide based chirobiotic columns and pirkle columns did not show considerable signs of separation during screening. Better separations were observed with immobilized polysaccharide chiral stationary phases (Chiralpak-IC3, Chiralpak-IA3) compared to coated polysaccharide chiral stationary phases (Chiralcel OJ-H, Chiralcel OD-H) used in this study.

Based on the trials it was observed that normal phase mode with basic additives was more favorable for separation compared to polar organic mode. Though reverse phase mode could provide chiral selectivity, these separations could not be optimized further. Among normal phase solvents, good selectivity was observed with *n*-hexane or heptane in combination with alcohol (ethanol, IPA). Differential selectivity was observed for basic additives (DEA, EDA) and also for alcohols (ethanol, IPA) on Chiralpak-IA3 and IC3 columns.

Better separation was observed with *n*-hexane and ethanol compared to IPA combination, using basic additives on IA-3 column. Basic additives did play major role in achieving selectivity on Chiralpak-IA3 column, where better separation was observed with 0.05% DEA in *n*-hexane and ethanol (Resolution 2) compared to 0.05% EDA in *n*-hexane and ethanol (Resolution 1.3). Albeit moderate change in the resolution observed with *n*-hexane or heptane and alcohols (ethanol, IPA) with different basic additives (DEA, EDA), finally resolution >3 was achieved with 0.05% EDA in *n*-hexane and 0.05% EDA in IPA at 40:60 ratio respectively on Chiralpak-IC3 column. Results of some selected method development trials are given in **Table 1**.

TABLE 1: Selected chiral selectivity results using basic additives and different CSPs

Chiral Column	Mobile phase	Retention time (min)	Resolution
IC3	0.05% DEA in IPA, 0.5 mL/min	09.8, 11.3	1.28
IC3	0.05% DEA in ethanol, 0.5 mL/min	5.9	-
IC3	0.05% DEA in IPA-Hexane(80:20), 0.5 mL/min	10.4, 12.5	1.98
IC3	0.05% EDA in IPA-Hexane(80:20), 0.5 mL/min	11.7, 14.4	2.26
IA3	0.05% DEA in IPA, 0.5 mL/min	06.7, 07.0	0.5
IA3	0.05% DEA in ethanol, 0.5 mL/min	08.5, 09.1	1.02
IA3	0.05% DEA in ethanol-hexane(90:10), 0.5 mL/min	09.7, 10.7	1.97
IA3	0.05% EDA in ethanol-hexane(90:10), 0.5 mL/min	09.0, 10.0	1.28
IA3	0.05% DEA in IPA-hexane(90:10), 0.5 mL/min	06.1, 06.2	0.49
OD-H	0.05% DEA in IPA, 0.5 mL/min	18.4, 20.6	1.01

TABLE 2: VALIDATION RESULTS OF CHIRAL HPLC METHOD

Validation Parameters	Results
Precision %RSD (n=6)	
Peak area, Sitagliptin	0.13
Peak area, (*S*)-enantiomer	0.99
Retention time, Sitagliptin	0.12
Retention time, (*S*)-enantiomer	0.16
Intermediate Precision %RSD (n=6)	
Peak area, Sitagliptin	0.38
Peak area, (*S*)-enantiomer	1.15
Retention time, Sitagliptin	0.43
Retention time, (*S*)-enantiomer	0.55
LOD-LOQ, (S)-enantiomer	
LOD (µg/mL)	0.1
LOQ (µg/mL)	1
Precision at LOQ, area %RSD (n=6)	1.25
Linearity, (S)-enantiomer	
Calibration range (µg/mL)	1-10
Calibration points	6
Correlation coefficient	0.9999
Regression equation	y=26.734x-1.1039

Validation results of the method: The Validation results are summarized in **Table 2**. Limit of detection and limit of quantification for (*S*)-enantiomer were found to be 0.1 µg/mL and 1 µg/mL, respectively. In the linearity study, excellent correlation (r^2=0.9999) observed between peak area and corresponding analyte concentration ranging from 1 µg/mL to 10 µg/mL.

Method precision percent RSD for area and RT were below 1.2 indicating good repeatability of results. Recovery study results are given in **Table 3**. The results from robustness study found to be within the satisfactory limits (**Table 4**).

TABLE 3: RECOVERY OF SPIKED (*S*)-ENANTIOMER IN SITAGLIPTIN BULK

Spiked conc. (µg/mL)	Calculated spiked conc.(µg/mL), %RSD	Recovery (%)
1.25	1.22, 0.32	97.5
2.5	2.38, 0.18	95.1
3.75	3.57, 0.19	95.3

TABLE 4: ROBUSTNESS RESULTS OF CHIRAL SEPARATION METHOD

Parameters	Resolution
Column temperature	
30ºC	3.14
35ºC	3.38
40ºC	3.12
Flow rate (mL/min)	
0.45	3.22
0.55	3.09
EDA conc. (%)	
0.0475	3.25
0.0525	3.08
Mobile phase composition	
54% IPA	2.39
66% IPA	2.83

CONCLUSIONS: Simple, precise, selective and rugged chiral separation method was developed and thoroughly validated on immobilized polysaccharide column. The diversity of mobile phases that can be used on immobilized chiral columns offered flexibility during method development. Based on our experience on extensive chiral separation method developments, we could conclude that immobilized chiral stationary phase columns with 3 µm particles offer excellent column life apart from being rugged in usage and result in saving of HPLC solvents compared to coated columns. This method is suitable for the quantitative estimation of (*S*)-enantiomer as an impurity in sitagliptin drug substance or formulation.

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