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ICH GUIDELINE PRACTICE: A VALIDATED POLAR IONIC MODE CHIRAL HPLC METHOD DEVELOPMENT AND ITS APPLICATION FOR THE DETERMINATION OF SAXAGLIPTIN STEREOISOMERS IN BULK AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT

SAXAGLIPTIN (SAX) is a commonly used and orally active medicinal ingredient for the therapy ofnon-insulin-dependent diabetes. Three out of all the stereo isomers were generated in minor quantities as impurities during the synthesis of SAX, lowering the drug's purity. The primary goal of this work was to investigate several chiral stationary phases (CSPs) in order to identify the phase providing the most efficient and reliable separation of the stereoisomers. Subsequently, chromatographic requirements were adjusted, and the developed technique was comprehensively confirmed using multiple standard validation parameters. CH_3OH:CH_3COOH:$(C_2H_5)_3N$ (100:0.1:0.1, v/v/v/) . Compared to Chiralpak-IG, the Chirobiotic V2 column effectively separated the SAX stereoisomers with good resolution in Polar Ionic Mode. The validated chiral HPLC method showed excellent selectivity with resolution >1.95 and tailing factor <1.35 for all analytes. The validated analytical procedure showed outstanding sensitivity, with limits of detection ranging from 0.32 to 0.68 µg/mL and limits of quantification spanning 1.09 to 2.13 µg/mL for DIA-1, DIA-2, ENA, and SAX. The proposed analytical strategy demonstrated strong selectivity, precision, accuracy, linearity, and robustness for the reliable identification and quantification of SAX stereoisomers, according to ICH guidelines. The described approach has been used to determine the stereoisomers of SAX bulk pharmaceuticals and pharmaceutical formulations with great success.

Keywords: SAX, Chromatography, Stereoisomer, Chiral Stationary Phase, Pharmaceuticals, Polar Ionic Mode, Chirobiotic V2 Column

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INTRODUCTION

SAXAGLIPTIN (SAX) (I) (Fig.-1) is an orally active and widely used pharmacological ingredient for the therapy of non-insulin-dependent diabetes.[1] The drug exerts its antidiabetic action by inhibiting the DPP-4 enzyme, thereby prolonging the activity of incretin hormones and improving glycemic control. Due to its favourable pharmacokinetic profile and clinical efficacy, saxagliptin has gained significant importance in modern antidiabetic therapy.[2] It has four stereogenic sites chemically, resulting in 16 stereoisomers. Three stereoisomers are created in minute amounts of impurities during the synthesis of SAX, which influence the drug's quality. Chemical structures of SAX stereo isomers were displayed in Fig.-1. As a result, assuring the therapeutic efficiency and safety of SAX pharmaceutical compositions requires

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stereospecific isolation of SAX and its stereoisomers. Among the various chiral separation strategies, polar ionic mode chiral HPLC has garnered considerable attention for basic polar analytes due to enhanced enantio recognition. In polar ionic mode, chiral stationary phases such as macrocyclic glycopeptides (e.g., vancomycin-based Chirobiotic phases) are combined with polar organic mobile phases modified with acidic and basic additives. This approach enables multiple interaction mechanisms-including ionic interactions, hydrogen bonding, and π–π interactions-leading to improved selectivity and resolution of stereoisomers, particularly for DPP-4 inhibitors.[3] Although several analytical methods have been reported for saxagliptin, most focus on achiral reversed-phase HPLC for assay determination or stability-indicating methods without addressing stereoisomeric separation. These methods-validated for parameters such as specificity, linearity, accuracy, and precision-serve routine assay purposes but do not resolve individual stereoisomers. For example, reversed-phase HPLC approaches have been successfully established and validated, aimed at the simultaneous estimation of saxagliptin with other antidiabetic agents in bulk and formulation matrices, but they do not provide stereoisomeric resolution.[4] RP-HPLC techniques for quantifying SAX in combination with Metformin in pharmaceutical medicines have been disclosed in the literature.[5-7] There was also an HPTLC technique for assessing SAX in medicinal dose form.[8,9-12] LC-MS/MS approaches for determining SAX in biological fluids have been published. The development of analytical methods for chiral compounds and the assessment of enantiomeric purity remain challenging due to the identical physical and chemical characteristics of enantiomers, which hinder their effective separation. The present study aims to develop and validate a innovative polar ionic mode chiral HPLC technique for the effective separation of saxagliptin stereoisomers. Method development involves systematically optimising chromatographic conditions to achieve adequate enantiomeric resolution. Full validation is conducted in compliance with ICH Q2 (R1/R2) guidelines.

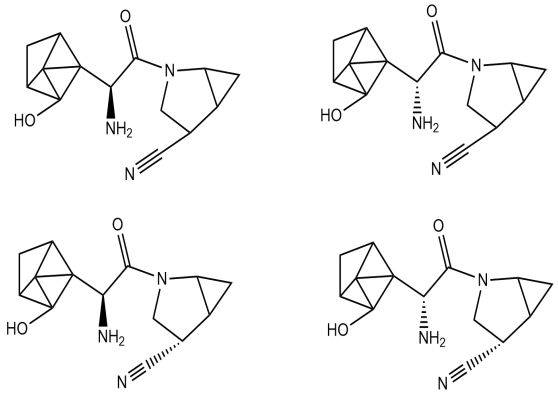

Fig.-1: Chemical Structures of SAX Stereo Isomers

EXPERIMENTAL

Chemicals and Reagents

Pharmaceutical grade stereoisomer contaminants and SAX standards procured from Daicel Chiral Technologies (India) Pvt. Ltd, Hyderabad. Deionised water was glass-distilled using a Nanopure purification system from Barnstead (IA, USA) Ethanoloicacid and N, N-Diethylethanamine (S.D. Fine Chem, Mumbai, India). After degassing, each solvent was filtered through a 0.22µm membrane.

Instrumentation and Analytical Conditions

The chromatographic analyses stayed approved out on a Shimadzu HPLC system from Kyoto (Japan) equipped with dual LC-20AD solvent delivery pumps. LC-Solution software (Shimadzu, Kyoto, Japan) installed on an HP-Vectra workstation (Hewlett-Packard, Waldron, Germany). Enantiomeric separation was achieved using CHIRALPAK IG-3 and CHIROBIOTIC V_2 columns (250 × 4.6 mm, i.e., 5 µm particle size; Supelco, PA, USA).

Preparation of Standard Working & Stock Solutions

Primary standard solutions of racemic saxagliptin and its individual stereoisomer stayed ready at a concentration of 1.0 mg mL^{-1} by precisely weighing each compound and dissolving it in a solvent mixture.

The resulting solutions were preserved at 4 °C under light-protected conditions to maintain their stability.

Method Development

Preliminary method optimisation was achieved on a CHIRALPAK IG column, which employs an immobilised polysaccharide chiral stationary phase containing amylose tris bonded to a silica support. Owing to its immobilised nature, the column offers enhanced solvent compatibility, improved durability, and extended column lifetime, allowing operation under both normal-phase and reverse-phase conditions with improved chiral recognition capability. The corresponding Key chromatographic variables and the conditions are presented in Table-1. Preliminary experiments were performed using normal-phase mobile systems consisting of n-hexane–methanol–triethylamine and n-hexane–ethanol–diethylamine mixtures in a 90:10:0.1 (v/v/v) ratio. However, these conditions did not result in satisfactory separation of saxagliptin stereoisomers. Further optimisation was attempted by varying the proportions of n-hexane and substituting n-heptane, but no improvement in resolution was observed. Subsequently, alternative organic solvents, including dichloromethane and methyl tert-butyl ether, and tetrahydrofuran, were evaluated in combination with different modifiers; however, these trials also failed to achieve stereoisomeric separation. Effective separation was achieved only under polar ionic mode conditions using a mobile phase containing ethanol, acetonitrile, and isopropanol in a 20:80:0.1 (v/v/v) ratio," which provided adequate resolution and reproducibility and was therefore selected for further method optimisation and validation. "The chromatogram of saxagliptin stereoisomers, obtained using a mobile phase of ethanol, acetonitrile, and isopropanol (20:80:0.1, v/v/v), is presented in Fig.-2."

Table-1: Optimised Chromatographic Conditions for Method Development Using the CHIRALPAK IG Chiral Column

Columns	CHIRAL PAK IG (250x4.6mm, 3µ particle size)
MobilePhase	EtOH: ACN: IPA(20:80:0.1v/v/v)
Flowrate	1mL/min
Wavelength	225 nm
Injection samplerVolume	10 µL
Columntemperature	25^{0}C
Detector	Photo diodearray

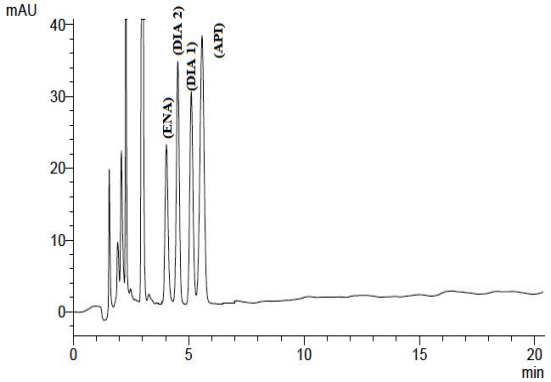

Fig.-2: Chromatographic Separation of Saxagliptin Stereoisomers was Achieved Using an Ethanol–Acetonitrile–Isopropanol Mobile Phase (20:80:0.1, v/v/v).

Method Optimisation using the Chirobiotic V_2 Column

The results and discussion demonstrate that basic compounds exhibit enhanced chiral selectivity under polar ionic mode conditions.[13–17] In this mode, secondary and tertiary amines are effectively resolved on the CHIROBIOTIC V_2 column due to strong ionic and stereoselective interactions. Saxagliptin contains an ionizable secondary amine at its stereogenic centre (Fig.-1), which facilitates selective interaction with the chiral selector, making the CHIROBIOTIC V_2 column particularly suitable for the stereoisomeric separation of SAX. The optimised chromatographic settings for the chiral HPLC separation of saxagliptin stereoisomers using the CHIROBIOTIC V_2 column are summarised in Table-2. A typical chromatographic profile recorded under these optimised conditions is presented in Fig.-3.

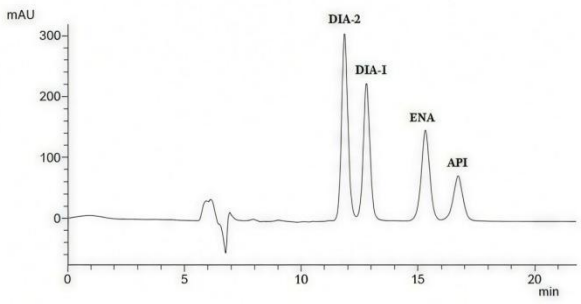

Fig.-3: The Chromatographic Separation of Saxagliptin Stereoisomers was Performed using a Methanol-Based Mobile Phase Modified with Acetic Acid and Triethylamine in a Ratio of 100:0.1:0.1 (v/v/v)

Table-2: Optimised Chromatographic Conditions for the Chiral HPLC Separation of Saxagliptin Stereoisomers Using the Chirobiotic V_2 Column

Columns	Chirobiotic V_2 (4.6mm i.d × 250mm)
MobilePhase	CH_3OH:CH_3COOH:$(C_2H_5)_3N$ (100:0.1:0.1, v/v/v)
Flowrate	1mL/min
Wavelength	225nm
Injection samplerVolume	0.5 µL
Columntemperature	30^0C
Detector	Photo diode array

Method Validation

The liquid chromatographic technique was authenticated in accordance with ICH rules.[18]

Accuracy

SAX levels in three concentrations (i.e., 50, 100, and 200 g/ml) were used to assess the assay's accuracy in triplicate, and recoveries were computed for each concentration. Considering a saxagliptin attentiveness of 800 µg mL^{-1} in the drug substance, recovery experiments were performed in triplicate for the related impurities at four concentration stages: LOQ: 50%, 100%, and 200%. These corresponded to impurity concentrations of LOQ, 0.5, 1.5, and 2.1 µg mL^{-1}, based on a nominal specification level of 0.15%, and impurity recoveries were calculated.

Precision

System precision was assessed by analysing six replicate injections of assay solutions containing saxagliptin at 100 µg mL^{-1} and stereoisomer solutions prepared from saxagliptin at 800 µg mL^{-1} pointed with 0.15% of each stereoisomer. To assess the intermediate precision of the method, six different preparations of SAX (800 g/ml) spiked with 0.15 per cent stereoisomer were injected. Intermediate accuracy was assessed by different analysts on separate days using different column batches and independent chromatographic systems within the same laboratory. Six independently prepared samples containing saxagliptin at 800 µg mL^{-1}, spiked with the enantiomer and two diastereomers at their respective LOQ levels, were analysed for precision at LOQ levels for each associated drug, and the per cent RSD of peak area was computed. Six separate assays of SAX samples at a concentration of 100g/ml against an appropriate reference standard were used to assess the precision of the assay procedure. It also had a different analyst examine the test method's intermediate precision, using a similar process as in the case of linked chemicals. The %RSDs of the SAX examine was computed.

Linearity

Linearity was assessed over five concentration levels corresponding to 50–150% of the assay concentration (50, 75, 100, 125, and 200 µg mL^{-1}), which were used to create linearity test solutions for SAX stock solutions. The stock solutions were mixed and diluted to the appropriate concentrations to create the linearity test solutions for the linked compounds.

Robustness

Method robustness reflects its capacity to produce consistent results despite minor, deliberate variations in chromatographic parameters. Accordingly, the influence of small changes in column temperature (±2 °C) and flow rate (±0.1 mL min^{-1}) was investigated. The composition of the mobile phase did not change when the temperature or flow rates did. The SAX assay recovery and system suitability parameters were examined under various conditions.

RESULTS AND DISCUSSION

The developed polar ionic mode chiral HPLC method was systematically evaluated and used for the separation and quantification of DIA-1, DIA-2, ENA, and saxagliptin (SAX) in accordance with the chromatographic conditions established during method development. The method demonstrated excellent chromatographic performance, with resolution values greater than 1.95 between all adjacent analytes and tailing factors below 1.35, confirming effective stereoisomeric separation and high method selectivity. System suitability outcomes, abridged in Table-1, validate the adequacy of the chromatographic system for routine analysis. The LOD values for DIA-1, DIA-2, ENA, and SAX were found to lie between 0.32 and 0.68 µg mL^{-1}, while the corresponding LOQ values ranged from 1.09 to 2.13 µg mL^{-1}. These results demonstrate the capability of the method to detect and quantify trace-level stereoisomeric impurities, a critical requirement for pharmaceutical quality control and regulatory compliance. Precision studies further confirmed method reliability, with system precision %RSD values below 2.3%. Method precision and intermediate precision studies showed %RSD values of 0.5% for SAX and within 2.5–4.2% for stereoisomers, including LOQ-level precision values below 3%, indicating consistent and repeatable performance. Linearity was established over the validated concentration varieties of 50–200 µg mL^{-1} for SAX and LOQ–250% for related stereoisomers. Excellent linear relationships were observed between peak areas and corresponding concentrations, with correlation coefficients (r^2) greater than 0.99 for all analytes. The linearity parameters, including slopes, intercepts, and coefficients of determination, are presented in Table-3. Representative chromatograms illustrating the baseline separation of saxagliptin stereoisomers under optimised conditions are shown in Fig.-2 and 3, enhancing visual clarity and supporting the quantitative findings. Collectively, the findings demonstrate that the proposed method exhibits excellent selectivity, sensitivity, precision, accuracy, and linearity, making it appropriate for routine stereoisomeric evaluation of saxagliptin in bulk materials and finished dosage forms. The reliable identification and quantification of chiral impurities achieved with this approach contribute significantly to pharmaceutical quality control and ensure the consistent manufacture of safe and effective products.

Table-3: Validation Characteristics of the Developed HPLC Method for DIA-2, DIA-1, ENA, and SAX

Parameter	DIA-2	DIA-1	ENA	SAX
System				
Suitability[a]				
RT (min)	12.85	13.60	15.56	16.90
Rs	7.32	6.17	2.25	1.98
k′-	1.01	1.52	2.46	3.01
As	1.19	1.21	1.36	1.42
N	3412	2394	4645	4683
Sensitivity				
LOD (µg/mL)	0.32	0.41	0.57	0.68
LOQ (µg/mL)	1.09	1.25	1.84	2.13
Linearity[b]				
Range (µg/mL)	0.29-3.3	0.41-3.1	0.32-2.9	50-200
r^2	0.9979	0.9985	0.9996	0.9998
Slope	11769.59	14832.85	16068.51	9732.61
Intercept	-153.72	-97.35	-258.26	19788.18
Precision (% RSD)[a]				
System	1.87	2.01	2.33	0.59

Method	0.52	1.58	1.89	2.48
Intermediate	4.19	2.98	2.18	0.54
LOQ	2.91	2.21	1.83	NA
Accuracy				
50%	97.02	101.11	99.96	102.86
100%	98.34	101.61	101.00	99.26
200%	101.37	102.45	98.68	99.92
LOQ	103.00	101.29	97.86	NA

Retention time (RT); resolution (Rs); N is the number of theoretical plates; where s represents the tailing factor, k denotes the retention factor, and r^2 indicates the correlation coefficient; a corresponds to the mean of six replicate measurements, while b represents the mean of three replicate measurements.

CONCLUSION

A fast and reliable polar ionic mode chiral HPLC method was established and validated for stereoselective separation of saxagliptin stereoisomers using a Chirobiotic V_2 column and a methanol–acetic acid–triethylamine mobile phase. The method met all ICH validation requirements and demonstrated excellent selectivity, precision, linearity, and robustness in the analysis of bulk drug substances and pharmaceutical formulations. Future studies may extend this approach to LC–MS compatibility, stability-indicating studies, and chiral impurity profiling of other DPP-4 inhibitors. By ensuring the quality and safety of antidiabetic medicines.

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CONFLICT OF INTEREST

The authors report that they have no conflicts of interest related to this work.

AUTHOR CONTRIBUTIONS

This study aligns with *SDG-3: Good Health and Well-Being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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