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Research Article

DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD FOR THE SIMULTANEOUS DETERMINATION OF IMEGLIMIN HYDROCHLORIDE AND SITAGLIPTIN PHOSPHATE USING REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objectives: The aim of the study was to develop and validate a precise reverse-phase high-performance liquid chromatography (RP-HPLC) method for concurrent estimation of imeglimin hydrochloride (IMG) and Sitagliptin phosphate (SIT) in bulk and dosage forms as per International Council for Harmonization guidelines.

Methods: The RP-HPLC technique utilized a Fortis C18 column (100×4.6 mm, 2.5 µm) with a mobile phase consisting of methanol and water, along with 0.1% ortho phosphoric acid in a 43:57 ratio at pH 3.5 for the separation of IMG and SIT. The chromatographic parameters included a flow rate of 0.7 mL/min and an injection volume of 20 µL, with a brief run time under isocratic conditions. Detection was carried out using a diode array detector, and quantification was performed at 266 nm.

Results: The RP-HPLC method exhibited outstanding linearity, with R^2 values of 0.9995 for IMG and 0.9996 for SIT at 266 nm. The recovery rates were determined to be 99.61–101.23% for IMG and 99.27–102.48% for SIT. The limits of detection (LOD) and limit of quantification (LOQ) for IMG were established at 0.450 ppm and 1.365 ppm, respectively. For SIT, the LOD and LOQ were found to be 0.028 ppm and 0.086 ppm, respectively.

Conclusion: The method developed in this research is accurate, robust, and offers excellent separation and resolution of IMG and SIT. The findings support the potential use of this method for quality control of combination formulations.

Keywords: Imeglimin hydrochloride, Sitagliptin phosphate, Analytical method, Validation, Reverse phase high-performance liquid chromatography.

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INTRODUCTION

In diabetes treatment, controlling blood glucose levels through monotherapy is essential and difficult. Combination therapy utilizes medications with distinct and complementary mechanisms of action [1,2]. Fixed-dose combination (FDC) formulations represent an innovative therapeutic approach that improves patient adherence, reduces the daily pill burden, enhances efficacy, and simplifies treatment regimens [3]. The global prevalence of type 2 diabetes mellitus (T2DM) currently affects hundreds of millions of people and is projected to increase significantly over the coming decades [4]. Despite the availability of various anti-diabetic drugs, none effectively target all three aspects of diabetes pathophysiology: Excessive hepatic glucose production, increased insulin resistance, and insufficient insulin secretion due to pancreatic β-cell dysfunction [5].

Imeglimin hydrochloride (IMG) (Fig. 1a) is a novel glimins-class drug with a distinct mechanism of action. IMG enhances insulin signaling, boosts glucose uptake in muscle tissues, preserves β-cell mass, and improves mitochondrial function to enhance insulin sensitivity [6-8]. Sitagliptin phosphate (SIT) (Fig. 1b), an inhibitor of the dipeptidyl peptidase-4 enzyme, is indicated for improving glycemic control in T2DM by facilitating insulin secretion and reducing glucagon levels [9-11]. The FDC formulation of IMG and SIT is promising for the management of Type 2 diabetes, which presents benefits over monotherapy, thereby offering an improved treatment option [12,13]. Establishing a simple, accurate method for the simultaneously estimating of IMG and SIT is crucial for developing of FDC formulations.

The literature review indicated that no analytical method has been documented for the simultaneous estimation of IMG and SIT, underscoring a significant analytical gap and the need to develop an appropriate method to support the formulation of FDC products.

Thus, the present study aimed to develop and validate a simple, precise, sensitive, and reproducible reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of IMG and SIT in bulk drugs and pharmaceutical FDC formulations [14-18]. RP-HPLC is a widely accepted analytical technique. In the present research, the new method was developed and validated in accordance with the International Council for Harmonization (ICH) guidelines to confirm its suitability [19,20].

MATERIALS AND METHODS

Materials

IMG and SIT with purity of ≥98% were generously supplied by Ami Life Sciences Private Limited, located in Vadodara, India, and Lee Pharma Limited, based in Hyderabad, India, respectively. Methanol and water of HPLC grade were obtained from Merck, Mumbai, India. Imeglimin and Sitagliptin Tablets were acquired from a local pharmacy in Vadodara, India. All other reagents utilized were of HPLC grade obtained from Merck, Mumbai, India.

Instruments

The study employed an Agilent Technologies (1100) HPLC system (Mumbai, India), which included a column temperature oven, an autosampler (G1313A), a pump (G1310A), and a diode array detector

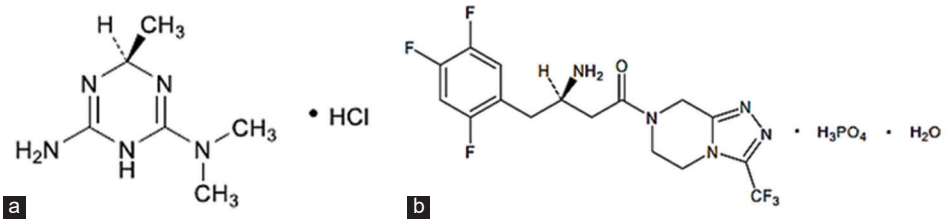

Fig. 1: Structure and IUPAC name of imeglimin hydrochloride (IMG) and Sitagliptin phosphate. (a) IMG; (4R)-6-N,6-N,4-trimethyl-1,4-dihydro-1,3,5-triazine-2,6-diamine;hydrochloride, (b) Sitagliptin phosphate 1,2,4-triazolo[4,3-a]pyrazine, 7-[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8-tetrahydro-3-(trifluoromethyl)-, phosphate (1:1) monohydrate

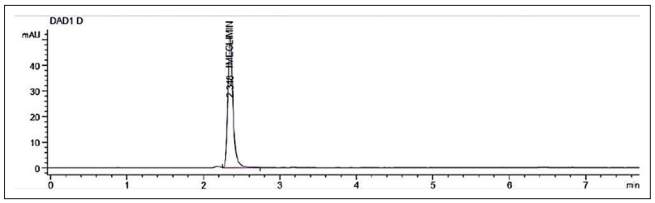

Fig. 2: Chromatogram of imeglimin hydrochloride

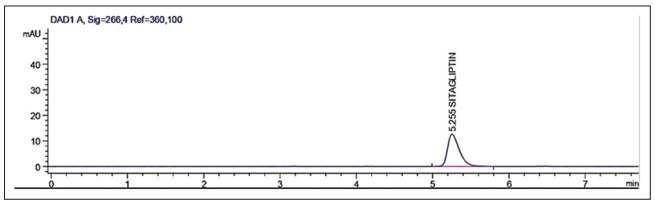

Fig. 3: Chromatogram of sitagliptin phosphate

(DAD) (G1314B) detector. Data acquisition was conducted using ChemStation 10.1 software. A Fortis C18 column with dimensions of 100×4.6 mm and a particle size of 2.5 µm was utilized (Fortis, Bangalore, India). The injection volume was set at 20 µL. A pH meter (Lab India), an analytical balance (Mettler Toledo; Sensitivity 0.01 mg), ultraviolet (UV)-visible spectrophotometer (Jasco V-630, double-beam, Japan), a Fourier Transform Infrared (FTIR) spectrometer (Thermo Nicolet, Mumbai, India), and an ultrasonicator (Sonics make) were employed for the study. Class A glassware was consistently used throughout the research.

Identification of IMG and SIT samples

IMG and SIT samples were identified using UV and FTIR spectroscopy [21,22].

By UV-visible spectroscopy

A 10 µg/mL solution of IMG and a 10 µg/mL solution of SIT were prepared separately in methanol, and their UV spectra were recorded.

By FTIR spectroscopy

IMG and SIT were scanned separately using an FTIR spectrometer over the range 4000–400 cm^{-1} characteristic functional group bands.

Selection of detection wavelength (λ_{max})

The suitable detection wavelength for HPLC analysis was determined by recording UV spectra of individual IMG and SIT solutions in the range of 200–400 nm, followed by spectral overlay [23-27].

Chromatographic conditions

The method was developed using a C18 column (100×4.6 mm, 2.5 µm) with a mobile phase consisting of methanol and water containing 0.1% ortho phosphoric acid (OPA) (43:57 v/v) adjusted to pH 3.5. The flow rate was set at 0.7 mL/min, the column temperature was maintained at 33°C, and the injection volume was 20 µL under isocratic conditions. Detection was performed with a DAD detector with quantification at wavelength 266 nm.

Preparation of analytical solutions

Preparation of diluent

The diluent used in this study was a mixture of methanol and Milli-Q water containing 0.1% OPA, in a 40:60 v/v ratio.

Preparation of mobile phase

The mobile phase was prepared by combining HPLC-grade methanol with milli-Q water in a 43:57 v/v ratio, with the addition of 0.1% OPA. The pH of this solvent system was modified to 3.5. The mobile phase was subjected to sonication for 15 min and then filtered using a 0.45 µm membrane filter. The mixed solvents were degassed and utilized as the mobile phase.

Preparation of standard solution

The FDC tablet formulation under study contains 50 mg SIT and 1000 mg of IMG. To accurately represent the actual dosage strength and ensure precise analytical quantification, a 1:20 drug ratio (SIT: IMG) was selected for the development and validation of the analytical method. This ratio was consistently maintained throughout the analysis to simulate the formulation composition and facilitate accurate quantification of both drugs. This selected ratio provided well-resolved peaks for both drugs without compromising linearity or analytical robustness. For standard solution preparation, 5 mg of SIT and 100 mg of IMG were accurately weighed and dissolved in 25 mL of methanol, yielding stock concentrations of 200 µg/mL for SIT and 4000 µg/mL for IMG.

The chromatograms of individual standard solutions were recorded during method development (Figs. 2 and 3).

Preparation of sample

Using a mortar and pestle, 20 tablets were ground into a fine powder. An accurately measured 154.3 mg portion of this powder was transferred into a 25 mL volumetric flask, to which 10 mL of methanol was added and mixed thoroughly to dissolve it completely. The mixture was then subjected to ultrasonication for 15 min to ensure the analyte was completely dissolved, resulting in a final concentration of 200 ppm for SIT and 4000 ppm for IMG. From this solution, 5 mL was taken and diluted to 100 mL with diluent.

Method validation

Analytical method validation is a process for ensuring a method operates consistently and meets the criteria for its intended analytical purpose. In the present study, the HPLC method was developed and validated in accordance with ICH guidelines. Method validation included testing parameters such as specificity, linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantification (LOQ).

RESULTS

Identification of IMG and SIT samples

By UV-visible spectroscopy

The UV spectra of the test samples showed absorption maxima at 245 nm for IMG (Fig. 4) and 274 nm for SIT (Fig. 5), confirming the identity of both drugs.

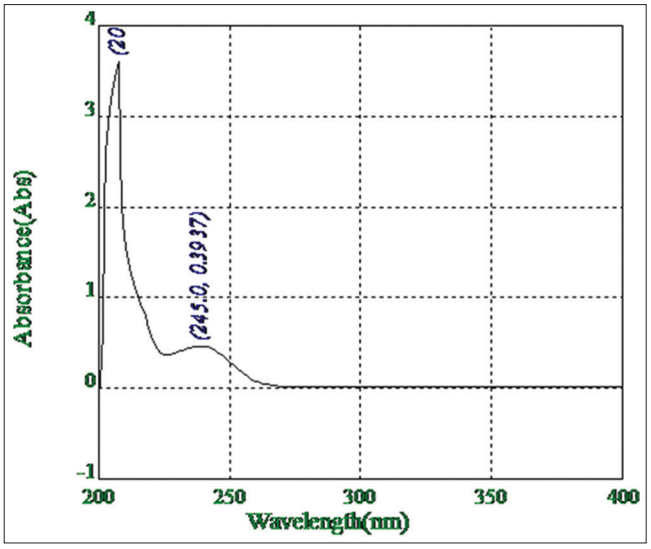

Fig. 4: Ultraviolet spectrum of imeglimin hydrochloride

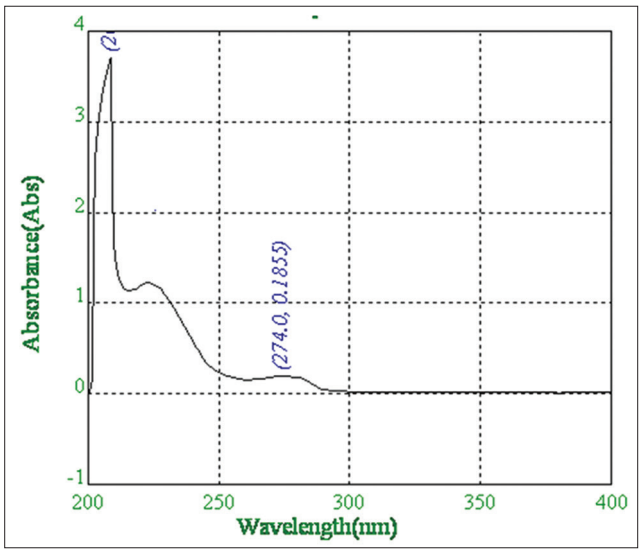

Fig. 5: Ultraviolet spectrum of sitagliptin phosphate

By FTIR spectroscopy

The distinct functional group peaks observed in the FTIR spectra confirmed the identity of IMG and SIT, as shown in Figs. 6 and 7.

Selection of detection wavelength (λ_{max})

The overlain UV spectra of IMG and SIT intersected at 266 nm, indicating an isosbestic point with stable absorbance. This wavelength was therefore selected for HPLC detection (Fig. 8).

Method development and optimization

In the initial phase of method development process, a relevant literature was reviewed and the information was gathered for physicochemical characteristics of IMG and SIT. The collected information was used to refine the preliminary chromatographic parameters, including the detection wavelength, stationary phase (column), and mobile phase composition.

A structured experimental approach was used to develop the RP-HPLC method to achieve optimal chromatographic separation of IMG and SIT. Each trial was assessed based on resolution, retention characteristics, and signal response to determine the conditions that

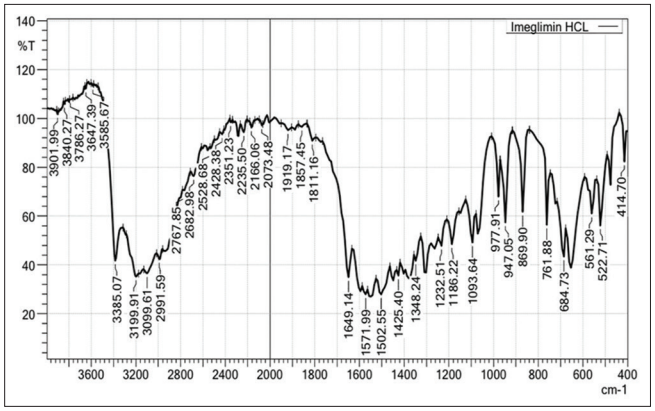

Fig. 6: Infrared spectra of imeglimin hydrochloride

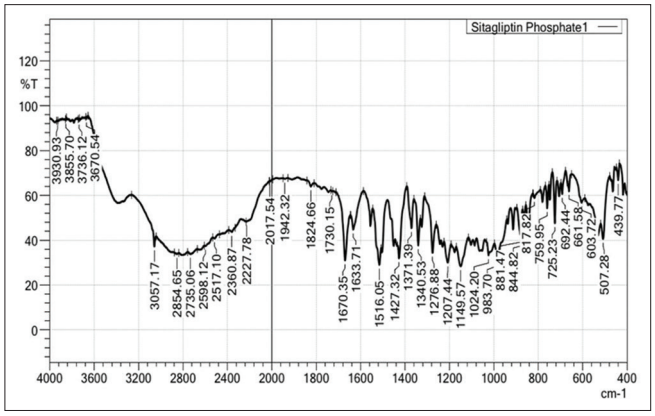

Fig. 7: Infrared spectra of sitagliptin phosphate

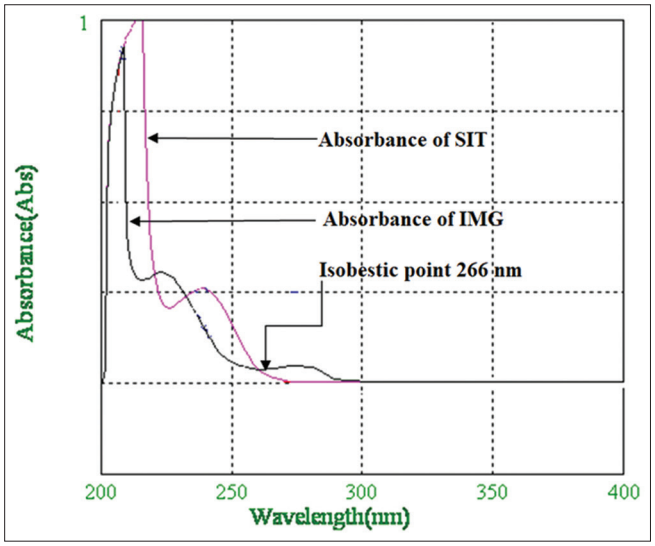

Fig. 8: Selection of isosbestic point for imeglimin hydrochloride and sitagliptin phosphate

provided reproducible, well-resolved peaks suitable for simultaneous estimation. Multiple trials were conducted using a Fortis C18 column (100 ×4.6 mm, 2.5 µm), and the outcomes of method optimization were assessed (Table 1). To refine the chromatographic conditions, various methanol-to-water ratios were examined in isocratic mode to achieve satisfactory resolution, peak shape, efficiency, and retention time. The examination of the performed trials indicated that compositions high in methanol did not provide acceptable separation, often

Table 1: Method development and optimization results

Trial no.	Mobile phase composition (methanol: water with 0.1% OPA)	Flow rate (mL/min)	Wavelength (nm)	Observation	Result
1	90:10	0.7	235	No detectable peaks	Rejected
2	70:30	0.7	235	Single sharp peak	Rejected
3	90:10	0.7	235	Two peaks	Rejected
4	95:5	1.0	235	Three peaks	Rejected
5	50:50	0.7	235	Single sharp peak	Rejected
6	60:40	0.7	235	Single prominent peak	Rejected
7	50:50	0.7	234	Single sharp peak	Rejected
8	43:67	0.7	266	Good resolution	Accepted

OPA: Ortho phosphoric acid

resulting in undetectable, merged, or additional peaks; in contrast, balanced or aqueous-rich compositions produced sharper peaks with reduced retention times. Among all the investigated mobile phase compositions, the mobile phase consisting of methanol and 0.1% OPA in water (43:57 v/v) at pH 3.5, with a flow rate of 0.7 mL/min, column temperature of 33°C, and detection wavelength of 266 nm produced sharp, symmetrical, and well-resolved peaks. Under the optimized conditions, the retention times for IMG and SIT were observed at 2.349 and 5.264 min, respectively (Fig. 9).

System suitability testing (SST)

At the beginning of the injection sequence, a blank sample was injected to assess any potential interference from the solutions. A second blank was injected at the conclusion of the sequence to ensure that no carryover effect occurred. Bracketing standards were intermittently included throughout the run to evaluate the system's performance.

For the SST [28], a reference standard solution of SIT and IMG was utilized. The system was deemed acceptable if the % relative standard deviation (RSD) of six replicate injections for peak area remained within 2%, the average theoretical plates were not <2000, the tailing factor not more than 2.0, and the resolution between the peaks was at least 2.0.

Method validation

Specificity

Specificity evaluation is the essential requirement for analytical method validation. Specificity was assessed by injecting blank samples, standard solutions, and sample solutions. The method exhibited total separation of both analytes without any interference. The results confirmed that the developed method successfully achieved complete separation of both analytes without any interference (Figs. 10 and 11).

Linearity

The linearity of the method was determined by diluting the standard stock solutions of IMG and SIT to generate concentration ranges of 40–200 µg/mL and 2–10 µg/mL, respectively. A calibration curve was constructed with the concentrations of the dilutions plotted on the X-axis and the corresponding peak area on the Y-axis (Figs. 12 and 13). The linearity data are presented in Table 2.

Accuracy

The precision of the established method was assessed by examining solutions with concentrations of 80%, 100%, and 120% of the target sample. The results obtained were compared against the anticipated values to evaluate accuracy, which indicates the method's capability to measure the analyte consistently. The percentage recovery at each concentration level was determined to be within the acceptable range (Table 3).

Precision

Precision refers to the consistency with which a method can provide the same results when applied under the same conditions. This is quantified as the % RSD of repeated measurements. To evaluate the method's

Table 2: Results of linearity study

Analyte	Concentration (µg/mL)	Area	Slope	Intercept	R^2
IMG	40	274.5801	6.577	9.935	0.9995
	80	538.9124			
	120	812.7271			
	160	1070.1755			
	200	1309.4600			
SIT	2	15.1072	8.038	0.744	0.9996
	4	30.9126			
	6	46.8434			
	8	63.4992			
	10	80.3122			

Table 3: Results of accuracy (recovery) study

Analyte	Level (%)	Recovery (%)	*Assay (%±SD)	% RSD
IMG	80	101.23	100.86±1.11	1.10
	100	101.74		
	120	99.61		
SIT	80	102.48	101.03±1.63	1.61
	100	101.34		
	120	99.27		

*Average of three determinations, SD: Standard deviation, RSD: Relative standard deviation

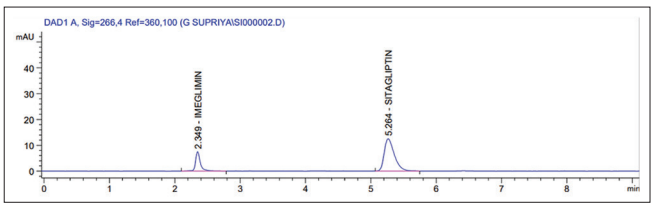

Fig. 9: Chromatogram of imeglimin hydrochloride and sitagliptin phosphate

precision (repeatability), six replicate injections (n=6) were performed for both analytes at concentrations of 120 µg/mL for IMG and 6 µg/mL for SIT. The findings are compiled in Table 4. The method demonstrated outstanding precision, with % RSD values of 0.83% for IMG and 1.76% for SIT, both of which fall well within acceptable thresholds.

Intermediate precision

Intermediate precision assesses the reliability of an analytical method under varying conditions within the same laboratory. This evaluation was conducted by performing analyses on different days, involving different analysts, and using various instruments. The method is considered acceptable if the % RSD lies within established limits. Precision was assessed as inter-day (intermediate) precision and

Table 4: Intra-day precision (method repeatability)

Analyte	Concentration (µg/mL)	Replicate	Area	*Mean (µg/mL)	*% Amount found	% RSD
IMG	120	1	120.9378	121.01	100.85	0.83
		2	120.9252			
		3	121.2947			
		4	121.0926			
		5	120.9349			
		6	120.9047			
SIT	6	1	6.0068	6.00	100.01	1.76
		2	5.9839			
		3	6.0020			
		4	6.0045			
		5	5.9873			
		6	6.0195			

*Average of three determinations, RSD: Relative standard deviation

intra-day (repeatability) by using three distinct concentrations with three replicate injections per concentration. The RSD of the peak areas was calculated and found to be below the specified thresholds (Tables 5 and 6).

Robustness

The robustness of the method was tested by making small, deliberate changes to the optimized chromatographic parameters, like flow rate (±0.1 mL/min), mobile phase composition (methanol: Water ratios ±1%), mobile phase pH (±0.2), detection wavelength (±2 nm), and column temperature (±3°C). These changes were made to assess whether the method could still maintain system suitability and consistent performance under slight variations. No significant differences were observed in peak area or retention time, and the % RSD and % recovery values remained within acceptable limits, confirming that the developed method is robust (Table 7).

Ruggedness

Both IMG and SIT were subjected to replicate injections at an identical concentration, which were subsequently analyzed under different conditions of wavelength and mobile phase. The outcomes were evaluated by computing the % RSD of peak areas and retention times. The determined % RSD was found to be below 2.0 %, thereby confirming that the method is robust and suitable for routine analysis across various conditions.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated using the formulas LOD=3.3 σ/s and LOQ=10 σ/s, where "σ" represents the standard deviation of the response and "s" denotes the slope of the calibration. The LOD and LOQ for IMG were established at 0.450 ppm and 1.365 ppm, respectively. In the case of SIT, the LOD and LOQ were determined to be 0.028 ppm and 0.086 ppm, respectively.

Analysis of tablet formulation

The assay determination for both drugs was performed on IMG 1000 mg and SIT 50 mg tablets, utilizing the developed and validated RP-HPLC method. The percentage content of IMG and SIT in the tablet formulation was found to be 99.85%±0.153 and 101.34%±0.594, respectively (Table 8).

DISCUSSION

Several analytical techniques have been reported for the quantification of IMG and SIT individually. For IMG, reported methods include UV spectrophotometry [29], RP-HPLC [30,31], and HPTLC [32]. Similarly, SIT has been analyzed using UV spectrophotometry [33-36], RP-HPLC [37-40], and HPTLC [41] methods. Although these techniques are specific and robust, they are limited to single-drug estimation.

Table 5: Inter-day (intermediate) precision

Analyte	Concentration (µg/mL)	Amount found (µg/mL)	% amount Found	% RSD
IMG	80	79.95	99.94	0.488
	120	122.39	101.99	0.265
	160	161.73	101.08	0.493
SIT	4	4.15	100.79	0.52
	6	6.07	101.11	0.13
	8	7.86	98.28	1.85

RSD: Relative standard deviation

Table 6: Intra-day (repeatability) precision

Analyte	Concentration (µg/mL)	Amount found (µg/mL)	% Amount found	% RSD
IMG	80	80.06	100.08	0.26
	120	122.48	102.07	0.23
	160	161.68	101.05	0.32
SIT	4	4.05	101.17	0.88
	6	5.98	99.65	1.05
	8	8.09	101.09	0.03

RSD: Relative standard deviation

Table 7: Results of robustness

Parameters	Deviation	% RSD for peak area	
		IMG	SIT
Flow rate (mL/min)	0.6 mL/min	0.42	0.45
	0.8 mL/min	0.27	0.13
Mobile phase ratio	44:56	0.11	0.57
(Methanol: Water)	42:58	0.32	0.81
Mobile phase pH	3.3	0.63	0.36
	3.7	0.74	0.48
Wavelength (nm)	264 nm	0.14	0.05
	268 nm	0.32	0.98
Column temperature (°C)	30°C	0.70	0.34
	36°C	0.74	0.62

RSD: Relative standard deviation, IMG: Imeglimin hydrochloride, SIT: Sitagliptin phosphate

Table 8: % assay of tablet formulation

Drug	Label claim	% Assay*±%RSD
Imeglimin hydrochloride	1000 mg	99.85±0.153
Sitagliptin phosphate	50 mg	101.34±0.594

*Average of three determinations, RSD: Relative standard deviation

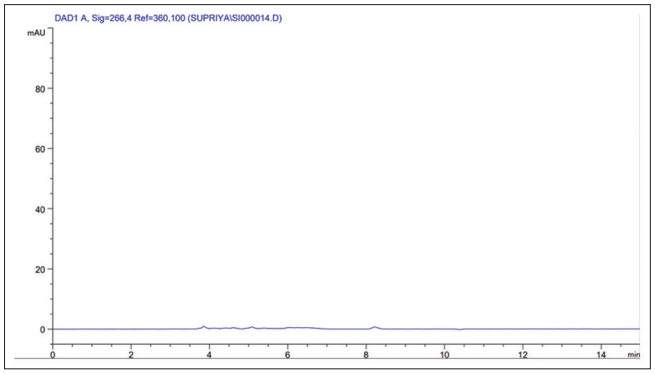

Fig. 10: Chromatogram of blank

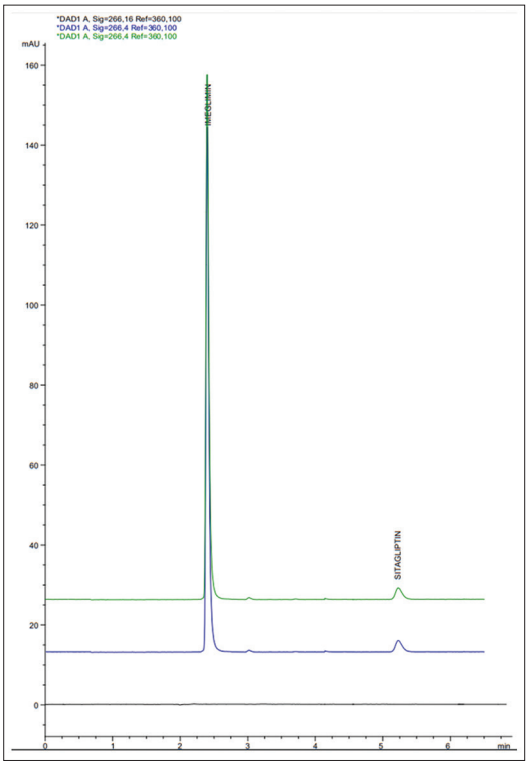

Fig. 11: Chromatogram of blank (black), mixed standard (blue) and formulation (green)

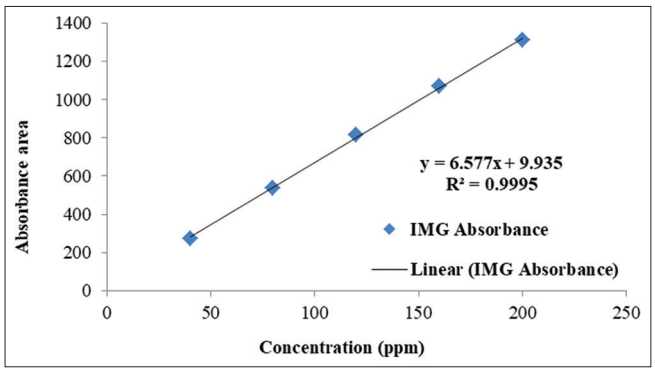

Fig. 12: Calibration curve of imeglimin hydrochloride

Despite several analytical methods being available for individual estimation of IMG and SIT, no method has been reported for their simultaneous analysis in a FDC. Existing approaches require separate runs, increasing time and resource consumption. This study presents the first simple, efficient, and economical RP-HPLC method for simultaneous quantification of IMG and SIT in a single run, making it suitable for routine quality control. The isosbestic point for IMG and SIT was determined using UV-visible spectroscopy and was found to be 266 nm, which was selected as a detection wavelength for the entire study. The drug samples were identified by UV and FTIR methods. Based on the literature search, the method development was initiated using a trial-and-error approach. One of the key challenges during method development was achieving a suitable mobile phase and wavelength that could provide sharp, well-resolved peaks for both analytes, particularly given the high-dose IMG and low-dose SIT combination in the FDC formulation. The chromatographic behavior of IMG and SIT under the optimized conditions can be attributed to their differential physicochemical characteristics and interactions with the C18 stationary phase.

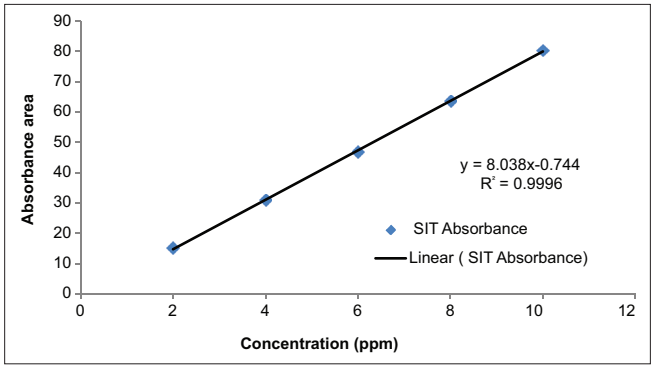

Fig. 13: Calibration curve of sitagliptin phosphate

Minor peak fronting was observed, likely due to secondary interactions with residual silanol groups on the column. Although this did not affect peak integration or validation outcomes, it is important to acknowledge this limitation for future method enhancements. The peak symmetry values for IMG and SIT were found to be 0.97 and 0.95, respectively, indicating acceptable peak shape and good chromatographic performance. Mechanistically, IMG eluted earlier due to its higher polarity and weaker hydrophobic interactions with the stationary phase, while SIT, with comparatively lower polarity, displayed stronger retention and eluted later.

The optimized mobile phase consisted of methanol and water with 0.1% OPA (43:57, v/v) at pH 3.5, flow rate 0.7 mL/min, column temperature 33°C (C18 column,100×4.6 mm, 2.5 µm) with DAD detector set at 266 nm. The method was found to be selective for simultaneous estimation of IMG and SIT. Linear correlation was observed between the peak areas with correlation coefficients exceeding 0.99 for both IMG and SIT. The method was specific and sensitive for assay of FDC tablets. Accuracy and precision were confirmed, with recovery values well within the limits. Robustness was evaluated for a limited set of parameters, with % RSD values remaining below 2%. Although the present study has certain limitations, the results indicate potential for further investigation and further method development.

CONCLUSION

The developed method was found to be practical and suitable for the determination of IMG and SIT in FDC products. Although the study involved a limited set of validation parameters, it addresses an analytical gap in the literature by providing an RP-HPLC method applicable to such formulations. Further comprehensive investigations are recommended to fully establish the method's suitability for routine quality control applications.

ETHICAL APPROVALS

This research does not involve any participation of animals or humans in any capacity.

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AUTHORS' CONTRIBUTIONS

Vijay Agrawal conducted research, experiments, and analysis. Komal Patel and Rajesh Varade supervised, guided the study, reviewed, and interpreted data. Both approved the manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

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